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BEHAVIOURAL AND PHYSIOLOGICAL MEASUREMENTS OF VISUAL PERFORMANCE IN THE CUTTLEFISH, SEPIA OFFICINALIS

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BEHAVIOURAL AND PHYSIOLOGICAL MEASUREMENTS OF VISUAL PERFORMANCE IN THE CUTTLEFISH, *SEPIA OFFICINALIS.*

By

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Behavioural and physiological measurements of visual performance in the cuttlefish, *Sepia officinalis*

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Abstract

This thesis investigated the behaviour and physiology of the visual system of *Sepia officinalis* by studying systematically its visual sensitivity and its spatial resolution. The cephalopod retina is composed of only photoreceptors and supporting cells, thereby providing a unique opportunity to study the interactions between photoreceptors, without the influence of other neurons, such as those typically found in the vertebrate retina.

The minimum separable angle (MSA), a measure of spatial resolution, of *S. officinalis* was determined from behavioural experiments to be 42' for animals of 8 cm mantle length at 15 $\mu W/cm^2$ light intensity. As the animals grew in size and as the ambient light intensity was increased, *S. officinalis* showed improved visual acuity. Through these experiments, it was revealed that each tested size of animal adapted to light with similar efficiencies, and that factors other than retinal growth were involved in the improvement of behavioural MSA with increasing size.

The minimum light intensity to which the retina of *S. officinalis* responded was 0.1 $\mu W/cm^2$, which was slightly higher than that to which individual photoreceptor cells responded. Retinal sensitivity decreased with increasing animal size. This was unexpected, as previous theoretical and behavioural studies in other species have shown sensitivity to increase with increasing animal size. Possible reasons for the decrease in sensitivity were a reciprocal decrease in cell resistance or an increase in dark noise. The visual sensitivity of *S. officinalis* was also affected by the stimulus flash wavelength and duration. Its retina adapted to background light in a way similar to vertebrate photoreceptors and the extracellular calcium concentration of the solution perfusing the retina affected this process. Finally, two series of experiments provided some evidence that functioning gap junctions exist in the retina of *S. officinalis*.

By completing a study of the visual sensitivity of *S. officinalis* at the three levels of single cell, retina and whole animal, the visual processing that occurs between these physiological levels was investigated. From the work presented in this thesis, it is concluded that, although *S. officinalis* did not prove comparable in every aspect to other species on an intracellular level, it would be a useful model of behavioural and extracellular visual processes for both invertebrate and vertebrate species.

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List of Abbreviations

Abbreviation	
ANOVA	Analysis of Variance
ASW	Artificial sea water
BL	Background light
BSA	Bovine serum albumin
$[Ca^{2+}]$	Concentration of calcium ions
CSF	Contrast Sensitivity Function
df	Degrees of freedom
DiI	Octadecyl indocarbocyanine
ERG	Electroretinogram
F	Test statistic for a parametric ANOVA
FITC-dextran	Fluoroscein 5(6) isothiocyanate dextran
H	Test statistic for a non-parametric ANOVA
LED	Light emitting diode
LI	Light intensity
ML	Mantle Length
MSA	Minimum Separable Angle
NaMBS	Sodium metabisulphite
ND	Neutral density
neurobiotin	N-(2-aminoethyl) biotinamide hydrochloride
n	Number of replicates
p	Probability
PBS	Phosphate buffered saline
PBS-ASW	Artificial sea water based on phosphate buffered saline
PFA	Paraformaldehyde
r^2	Square of the correlation coefficient of a best fit line to data
rpm	Rotations per minute
sd	Standard deviation
se	Standard error
sig.	Significance
TTX	Tetrodotoxin
V/log I curve	Graph of response amplitude against stimulus intensity

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Two quotes that struck a chord with me, and helped me to keep pursuing my PhD were:

"The journey itself and not only the great conquest is a fulfilment of human life." (Stern, 1965)

"Research often consists of groping." (Hubel, 1995)

Author's Declaration

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award.

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A programme of advanced study was undertaken, which included training in several electrophysiological techniques through participating in the Microelectrode Techniques for Cell Physiology workshop in September 2001 and 2002, training in histological and behavioural techniques, and the completion of two postgraduate courses, one in research methods (EAR5101) and the other in demonstrating (ENV5101). A course in health and safety was attended at Plymouth Marine Laboratories.

Relevant scientific seminars and conferences were regularly attended at which work was often presented.

Publications:

1. Groeger, G. and Williamson R. (2002) Intracellular recordings from the photoreceptors of the cuttlefish, *Sepia officinalis*. *J. Physiol. Lond.* **543**S:5P-6P
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3. Groeger, G. and Williamson, R. (2003) Some factors affecting the electroretinogram of the cuttlefish. *Proc. 29th Goettingen Neurobiol. Conf.* (Eds. N. Elsner and H. Zimmermann) Pg. 542

Presentations and Conferences Attended (in chronological order):

1. *University of Plymouth*: First year talks, February, 2001. Chair: Dr. Murray Brown. Oral presentation: Visual acuity of cuttlefish.
2. *MBA Council, Plymouth*: April, 2001
   Poster presentation: A cephalopod model for visual studies
3. *Physiological Society, Lancaster*: May, 2002

Poster presentation: Intracellular recordings from the photoreceptors of the cuttlefish, *Sepia officinalis*. (abstract published)

This poster was also presented at the MBA Council, April 2002, and the inaugural meeting of the Plymouth Institute of Neuroscience, April, 2002.

4. *Graduate Research Forum, Plymouth*: August, 2002. Chair: Dr. Declan Schroeder

   Oral presentation: The visual acuity of *Sepia officinalis*

5. *Society for Experimental Biology Meeting, Southampton*: April, 2003

   Poster presentation: Changes in cuttlefish visual performance with growth (abstract published)

   This poster was also presented at the MBA Council, April 2003.

6. *Goettingen Neurobiology Conference, Germany*: June, 2003

   Poster presentation: Some factors affecting the electroretinogram of the cuttlefish (abstract published)

7. *MBA seminar series, Plymouth*: June, 2003. Chairs: Dr. Helen Goddard, and Dr. Andrew Pemberton

   Oral presentation: Where did a poster on cuttlefish visual sensitivity fit into a neuroscience meeting?

Signed .....

Date..07/02/04.....

Chapter 1 General Introduction

1.1 Introduction

Cephalopods are highly evolved molluscs that live almost exclusively in marine environments (Barnes *et al.*, 1993). They first appeared in the fossil history approximately 500 million years ago (Packard, 1972). Since then, they have evolved to internalise and reduce their shell, develop neutral buoyancy and jet propulsion, and to increase the size of their central nervous system (Hanlon & Messenger, 1996). Packard (1972) called cephalopods "functional fish" and as such they compete with fish in the oceans. As with most active predators, cephalopods rely heavily on vision to catch their food (Wells, 1958) and are widely accepted as having one of the most sophisticated invertebrate eyes (Barnes *et al.*, 1993).

Vision is extremely important to most cephalopod species in many aspects of their lives. Not only do many cephalopods move their eyes to track their prey (Messenger, 1968), but the majority of cephalopods are also capable of colour change through the use of chromatophores in their skin. The ability to change colour and display different patterns plays an important role in social behaviour and in predator avoidance strategies (Hanlon & Messenger, 1996). Social signals involving different colour patterns, e.g. the zebra pattern, would be useless without a good visual system. While cephalopods can sense other stimuli, e.g. the presence of different chemicals (Boal & Golden, 1999) and vibrations through the lateral line (Bleckmann *et al.*, 1991), they are believed to be less important to their survival. A debate continues amongst cephalopod researchers as to whether cephalopod species can hear (Hanlon & Messenger, 1996). Although they are highly visual animals, all cephalopods have been shown to be colour blind (Messenger, 1981), apart from one species, *Watasenia scintillans* (Seidou *et al.*, 1990). There is general agreement that cephalopods rely heavily on vision (Hanlon & Messenger, 1996) and this means that they are ideal subjects to study visual processes.

The principal aim of this thesis is to characterise the cephalopod visual system, using *Sepia officinalis* (the European cuttlefish, Figure 1.1) as the study species. In doing so, two things can be accomplished. Firstly, the similarities and differences between it and other invertebrate species can be determined, and secondly, and perhaps more importantly, the usefulness of *S. officinalis* as a model for visual studies can be assessed. Land (1981) stated

> "The important properties of an eye, as far as its ability to collect and transmit information about the visual world is concerned, are very largely specified by its spatial resolving power and by its sensitivity."

Using this quote as a guideline, both the spatial resolution and the visual sensitivity of *S. officinalis* will be studied using behavioural and physiological techniques, so as to characterise its visual system. This chapter will introduce the cephalopod visual system, define spatial resolution and visual sensitivity and will finally outline the aims of each of the individual results chapter in this thesis.

Figure 1.1: A photograph of a juvenile *Sepia officinalis*, whose mantle length was approximately 10 cm (courtesy of John Rundle).

1.2 The basics of vision

Vision is the process whereby light from the outside world is transformed, via a chemical cascade, into electrical signals, which are then passed from one neuron type to

another and eventually interpreted by the brain. Light, itself, is often described as having a dual nature. On one hand, it is packaged in discrete units called photons, but on the other, it is regarded as a waveform. These two theories about the nature of light are meant to complement our understanding of light and should not be thought of as separate models (Loew, 1999). Both are referred to in this thesis, particularly when measuring the intensity of light, i.e. the amplitude of the wave, or the number of photons.

Photoreceptors are the basic unit of an eye, and within them rhodopsin is the chemical that allows the transformation of photons of light into electrical signals. Rhodopsin is composed of two parts, 11-*cis* retinal and an opsin, which is a membrane bound protein. The interaction between the opsin and the retinal determines the wavelength of light to which the rhodopsin will respond (Archer, 1999). Although the structure of rhodopsin is similar in both vertebrates and invertebrates, the chemical cascade they use to convert light to electricity is quite different. In invertebrates, rhodopsin converts to a stable *meta*-rhodopsin when activated by light, which in turn activates the phototransduction cascade (Rayer *et al.*, 1990 and Figure 1.2). When vertebrate rhodopsin is stimulated by light, the 11-*cis* retinal changes configuration to the all *trans* form of retinal, and breaks away from the opsin, initiating the vertebrate cascade (McIlwain, 1996). Both cascades employ several different enzymes and at least one second messenger that eventually lead to either opening or closing of ion channels in the photoreceptor's membrane (Figure 1.2). In vertebrates, the channels are closed, resulting in a hyperpolarisation of the cell, but in invertebrates, the channels are generally opened, resulting in a depolarisation of the cell's membrane (McIlwain, 1996 and Figure 1.2). This electrical signal is then passed to, and processed by other neural cells, which can eventually result in the animal reacting to what it sees in its environment. It is on these phototransduction cascades that the visual performances of animals are based.

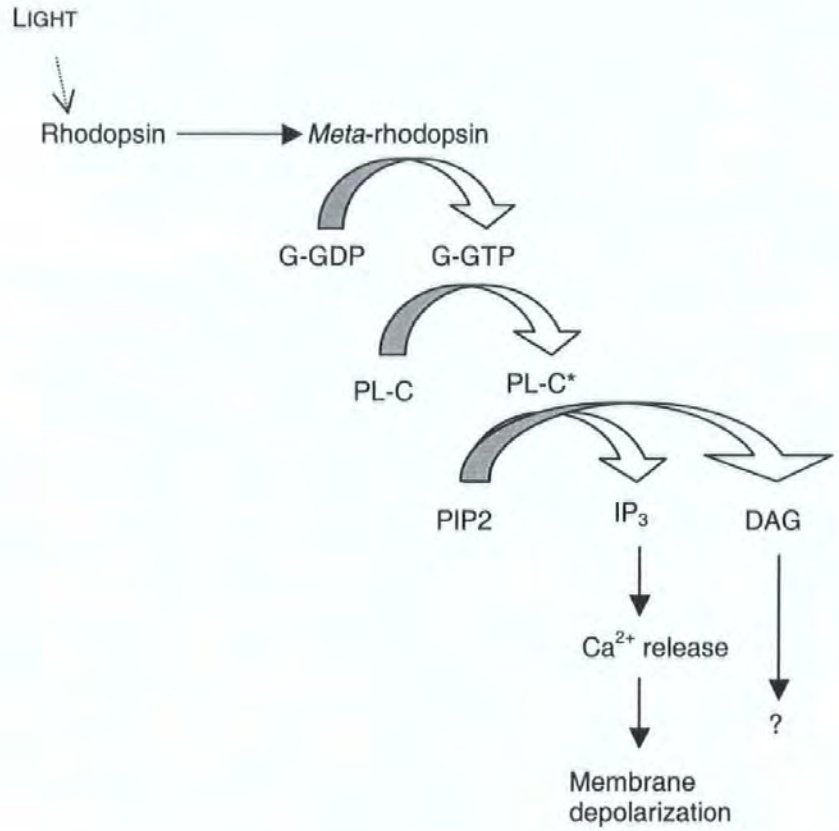


Figure 1.2: The invertebrate phototransduction cascade (adapted from Frank & Fein, 1991 and Rayer *et al.*, 1990). G = GTP binding protein, GDP = Guanosine Di-phosphate, GTP = Guanosine Tri-phosphate, PL-C = Phospholipase C, PL-C* = active form of PL-C, PIP_2 = phosphatidyl-inositol 4, 5-bisphosphate, IP_3 = Inositol 1, 4, 5,-trisphosphate, DAG = diacylglycerol. The function of DAG remains unknown.

1.3 Cephalopod visual system

The cephalopod visual system, consisting of the eyes and optic lobes (Figure 1.3a), comprises a large part of the animal's brain, with both optic lobes approximately three times the weight of the rest of the brain (*Sepia elegans*: Bullock, 1965). When the cephalopod eye is examined on a gross morphological level, it shows close similarity with the vertebrate eye (Wells, 1978; Messenger, 1981, 1991). On a cellular and molecular level, however, it remains closer to the eyes of other species within the molluscan phylum (Figure 1.3).

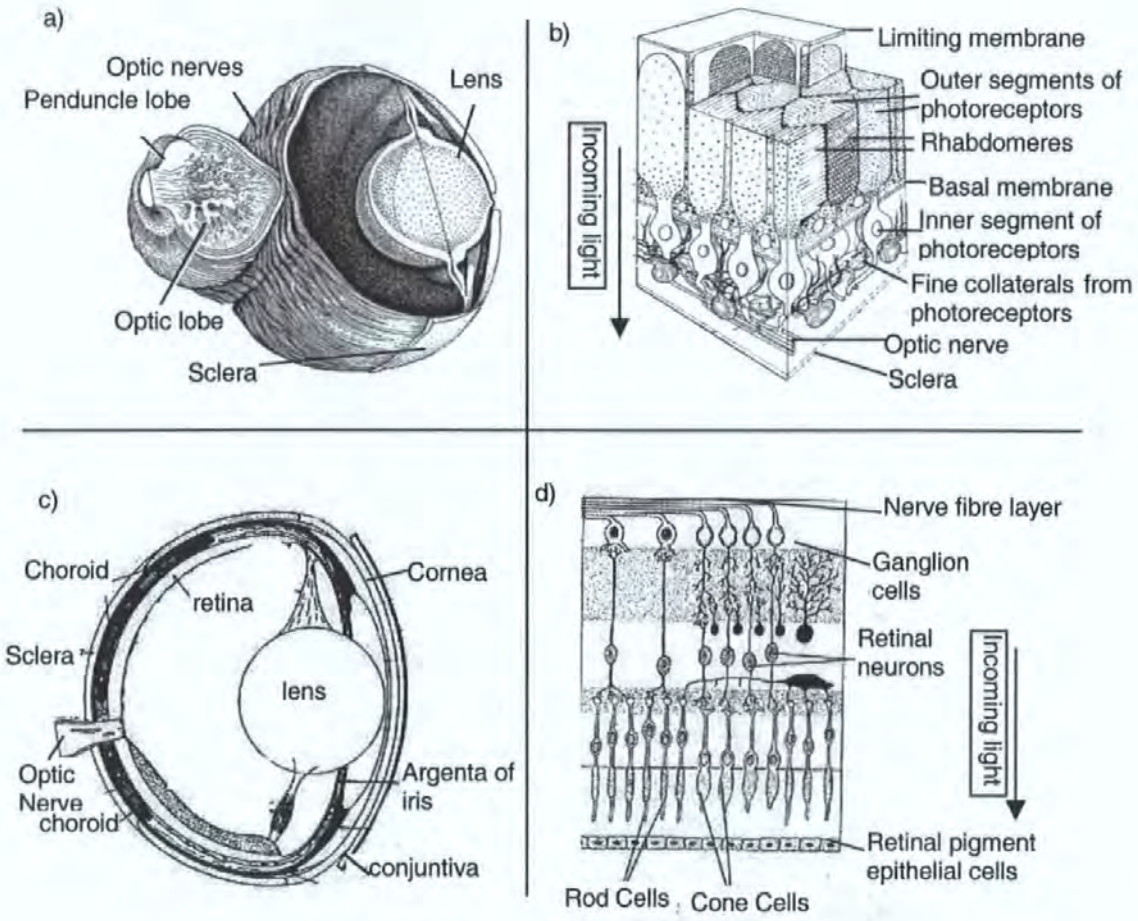


Figure 1.3: Diagrams of the a) cephalopod eye (Young, 1971) (no scale given in original diagram, but eye size as measured from lens to back of eye ball ranges from 0.5 cm to 5 cm), b) cephalopod retina (Wells, 1978) (the maximum width of an *Octopus* retina is 400 μm, c) vertebrate eye (Detwiler, 1943) (size range similar to cephalopod eye ball), and d) vertebrate retina (Campbell, 1993) (approximately 0.5 mm thick).

One of the main differences between the cephalopod eye and the vertebrate eye is the number of nerve cell types found in the retina (Figure 1.3b and d). Vertebrate eyes contain six major types of neurons: a) photoreceptors, b) horizontal cells, c) bipolar cells, d) amacrine cells, e) ganglion cells and f) interplexiform cells (McIlwain, 1996 and Figure 1.3d). Since photoreceptors are the only nervous cell type present in large numbers in the cephalopod retina (Nasi & Gomez, 1992 and Figure 1.3b), it should be easier to study

certain phenomena using it as a model, rather than the more complex vertebrate retina. Analogues of these other neuronal types occur in the cephalopod visual system but they are in the optic lobes of the brain rather than within the retina itself (Young, 1971).

Unlike the morphology of most vertebrate eyes where there are rods and cones, the cephalopod eye has only one photoreceptor type and it is of the invertebrate, rhabdomeric type. The cephalopod photoreceptor is long and thin, with thousands of microvilli (Cohen, 1973a). The dimensions of the photoreceptors change depending on the location within the retina, with the thinnest and longest cells at the centre of the eye (Young, 1962, 1963), thus some cephalopod species have a fovea-like area. The photoreceptor is composed of three main sections: the outer segment, the inner segment and the axon (Figure 1.3b). The outer segment is the long, thin part covered in microvilli and is where the phototransduction cascade takes place (Figure 1.2). The inner segment comprises of the cell body, with the nucleus and other cellular organelles. Finally there is the cell's axon, which leads from the eye to the optic lobe. These axons cross a dorsoventral chiasm, which rectifies the image, but there is no horizontal chiasm as found in vertebrates (Messenger, 1981). The outer segments of cephalopod photoreceptors point towards the incoming light, so the nerves do not need to pass through the retina, as they do in vertebrates (Figure 1.3b and d), and therefore there is no blind spot in the cephalopod eye. The microvilli are orientated in a specific manner, perpendicular to the long axis of the photoreceptor and this allows cephalopods to see polarised light (Williamson, 1995).

Photoreceptor cells, within the retinas of both cephalopods and vertebrates, are linked by gap junctions. Gap junctions are channels between cells that connect their cytoplasms (Spray & Bennett, 1985) and allow the passage of charged molecules and metabolites directly from one cell to another (Hardie, 1991a; Vaney *et al.*, 1998). One gap junction is composed of two hemi-channels (or connexons), one from each cell involved in the junction. Each hemi-channel is composed of six proteins (Saez *et al.*, 1993). Structurally, these six proteins can be in an opened or closed formation, between which

they can flip quickly (Saez *et al.*, 1993). When opened, they have a channel, approximately 1.5 nm in diameter, which will allow any molecule smaller than this to pass through (Saez *et al.*, 1993). In the closed configuration, the proteins are orientated differently so as to block the gap junction. Much more work in this area has been completed on the vertebrate retina than on the cephalopod retina, and many different gap junctions have been found (see Vaney, 1997 for review). For example, they have been shown to link amacrine and bipolar cells in mammals (McIlwain, 1996), horizontal cells and cones in fish (Kamermans *et al.*, 2001), rods and cones, and cones and cones in mammals (Raviola & Gilula, 1973). These intercellular channels are now recognised to play an important part in vertebrate visual processing (Vaney, 1997). The advantage of such connections, is that they decrease the noise in each rod, as they allow a population of rods to share the random noise, but it also has a disadvantage in that it blurs the target (Werblin, 1991). In cephalopods, electron microscopy studies have proven that gap junctions exist between photoreceptor collaterals, in the plexus layer of the retina (Cohen, 1973b; Yamamoto, 1984). The physiological techniques that have provided so much information about the functioning of gap junctions in vertebrate retina have never been applied to the retina of cephalopod species.

1.3.1 *The visual environment of* Sepia officinalis

Sepia officinalis hatch from eggs in the summer months in shallow coastal waters (maximum depth of 40 m) (Boletzky, 1983). They usually feed from their yolk sacs for the first 24 h, after which they will catch live prey (Wells, 1958). This shallow water environment corresponds to light intensities ranging from 10^{-2} (or even darker during moonless nights) – 10^{5} $\mu W/m^2$ (Clarke & Denton, 1962). As *S. officinalis* successfully catch prey 24 h after hatching in these light intensities, it is assumed that their visual systems operate successfully under these conditions. As the animal grows, it migrates to deeper waters (maximum depth of 150 m), where it spends the winter, and so spends most

of its time in light intensities ranging from 10^{-6} $\mu W/m^2$ on bright sun lit days, to much darker at night times. *Sepia officinalis* eat many different types of prey items ranging from shrimp to fish to crustaceans (Wells, 1958). They are ambush predators, hiding half buried in the substrate, until an opportunity presents itself and they pounce on their prey (Hanlon & Messenger, 1996). The visual environment of *S. officinalis* can be quite dark, and has a limited variation in light intensities, particularly when they spend the winter in deeper waters. They eat animals that are generally quite large, but can camouflage themselves, so *S. officinalis* need good spatial resolution at times to detect their prey. Due to evolutionary pressures, it is likely that the visual performance of *S. officinalis* will be well suited to its visual environment.

1.4 Visual acuity

Spatial resolution, or visual acuity, is a characteristic of a visual system that is important in determining its ability to amass information about the outside world (Land, 1981). It is defined as the ability to see fine detail in the visual scene (McIlwain, 1996) and is determined mainly by the spacing of the photoreceptor cells and the magnification of the eye. This thesis concentrated on one of the more commonly used measurements of visual acuity, that of minimum separable angle (MSA). MSA is defined as the critical angular spacing between two stimuli when they are just resolved (McIlwain, 1996). It gives an indication as to how grainy a picture the retina being studied produces. If the MSA is small, then the picture that the retina produces contains considerable detail. But as the MSA increases, the picture becomes grainier and detail is lost to the animal. MSA can be measured using a variety of techniques, most of which are based on the two very different methods of histology and behaviour.

The histological measurement of MSA is based on the fact that to distinguish between two stimuli, they need to be separated by at least one unstimulated photoreceptor (McIlwain, 1996). To calculate the MSA, the retina must be fixed, stained, and then the density of photoreceptors counted. A formula, which also needs the magnification of the eye, is then used to convert the photoreceptor density into the MSA. There are, however, different formulae in the literature, but these are all based on two typified by those published by Northmore & Dvorak (1979) and Tamura (1957). This difference has arisen as some people maintain that the distance required to distinguish between two objects is that of two unstimulated photoreceptors (Land, 1981). So care must be taken when comparing the MSA published in different papers. Watanuki *et al.* (2000) in an examination on the retina of adult *Sepia esculenta* found that the histological MSA was 1.12', using a formula close to that of (Tamura, 1957). This study gives an estimate of the predicted MSA of *S. officinalis*.

All behavioural tests used to measure visual acuity examine the reactions of animals to different sized stimuli, in particular to stimuli varying in width. Three different experimental designs have been used to measure MSA of cephalopods, but none is appropriate for use with every species. All three tests are described here, as they have been used in the past to measure the MSA of cephalopod species.

The first is a visual training experiment, where animals are taught to differentiate between gratings and uniform panels. The training is accomplished through associative learning with the use of rewards and deterrents. Three octopus species have been trained using this technique to discriminate between gratings and uniform control grey panels of the same luminance. In Sutherland's (1963) experiments, a panel (either the grating or the control) was associated with a reward (food), and the second panel with a punishment (an electric shock), but recently Muntz & Gwyther (1988, 1989) found the deterrent unnecessary. Once training is complete, the stripe width of the grating is reduced until the animal attacks the panel it was trained to attack at a chance rate. It is then determined to

have just passed its MSA. Most *Octopus* spp. tend to be territorial and so attack almost anything that moves within their home range. *Sepia officinalis* are not territorial and so only attack food-like items (Wells, 1958). Therefore this technique would not be ideal to use with them, it has however been used to measure the MSA of closely related species.

A second behavioural technique used to measure MSA is to record how far away a predator is from its prey when it first reacts to it. This parameter is called the reactive distance, and if the size of the prey is also known, then the angle subtended by the prey on the predator's eye can be calculated, i.e. the MSA. Any angle calculated by this method would probably be an over estimation, as most animals can probably see smaller items or more distant items, which they ignore. Reactive distances were used by Watanuki *et al.* (2000) to measure the MSA of *Sepia esculenta* (a Japanese cuttlefish) to be 2.75'. This technique would not be ideal to use with *S. officinalis*, as they tend to eat large prey items, and so a very large tank would be necessary to measure the reactive distance accurately.

The final behavioural method used to measure MSA is the optomotor apparatus and it should prove successful with *S. officinalis*. This method is based on the fact that to see an object clearly, its image must be held on the retina for a long enough time and with sufficiently slow slip velocity[1] to allow the brain to analyse the image (Dieringer, 1991). So for an animal to see a moving object clearly, it must at least turn its eyes (sometimes its head and body too) at the same speed and in the same direction as the movement of that object. This reaction is maximised in the optomotor apparatus, which consists of a drum that can be lined with stripes of various widths. The animal is placed at the centre of the drum, usually in a stationary container. The drum is rotated and the animal's reaction recorded. If it follows the speed and direction of the drum in a consistent manner, it must be able to see the stripes. But if it makes no attempt to do so, it is assumed that it cannot resolve the stripes, and probably sees a grey background (Rahmann *et al.*, 1978). By

[1] Slip velocity refers to the difference in velocities of the moving object and the animal (Dieringer, 1991). If it is too fast the image of the object is not held on the retina for sufficient time to allow it to be analysed, i.e. seen properly.

discovering the minimum stripe width to which an animal will respond, its MSA can be determined.

These three different behavioural methods do not measure precisely the same type of visual acuity, although all are termed MSA, so it remains unclear how closely related are the results obtained from them (Davson, 1990). Although some authors have said that the angle calculated by reactive distance is equivalent to MSA (e.g. Breck & Gitter, 1983), in reality it measures the minimum angle, which is the angle that an individual object must subtend on the retina to be distinguished from the background (Lit, 1968). All three methods also use slightly different triggers to elicit the required behaviour. The training method measures the reaction of an animal to a stationary object, while the optomotor apparatus measures an animal's reaction to moving stripes. It might be that the optomotor apparatus records the animal's reaction to the movement rather than to stripes of the apparatus (Douglas & Hawryshyn, 1990). The reactive distance technique needs the specific movement and form of the object to resemble a prey item to enable an animal to identify it as possible prey. With these different triggers, care must be taken when comparing the results from these methods. The results obtained using these methods in various cephalopod species give an estimation of the likely MSA of *S. officinalis*, for this value has not yet been determined. By measuring the MSA of *S. officinalis*, one aspect of its visual system will be characterised, and so the visual performance of this species, in relation to its spatial resolution, can be compared to that of other species.

1.5 Visual sensitivity

Visual sensitivity, the second characteristic important to the basic visual performance of an eye (Land, 1981), is the amount of light needed to activate a sufficient amount of rhodopsin in an eye to produce a measurable response (Autrum, 1981). This

parameter determines in how dark a habitat an animal can operate. It is influenced by many characteristics of the visual system, e.g. pupil size, screening pigment, pooling of information from individual photoreceptors, all of which determine how much light enters the eye and actually reaches the rhodopsin in the photoreceptors (Meyer-Rochow, 2001). Two measurements of visual sensitivity are used in this thesis. The first is absolute sensitivity, which is defined as the reciprocal of the light intensity that causes a certain response amplitude (Autrum, 1981). The second is absolute threshold, and is defined as the minimum amount of light needed to elicit a detectable response either in a cell, a retina, or from an animal (Autrum, 1981).

All definitions of sensitivity rely on measuring a response of the visual system to light, and how it varies with different intensities of light, although Land (1981) published a formula that used histological and morphological measurements alone. While any figures for sensitivity calculated or measured using these two disparate methods cannot be compared directly, they can be used to examine if measured physiological trends matched those predicted from theoretical calculations. This thesis is mainly concerned with the physiological measurement of sensitivity, but it also uses Land's formula to examine if sensitivity changes with certain histological variables in a predicted way. Sensitivity has been previously measured in various cephalopod species using electrophysiological methods (e.g. Tasaki *et al.*, 1963c; Hamasaki, 1968; Weeks & Duncan, 1974; Clark & Duncan, 1978; Lange *et al.*, 1979), but no such data are available for *S. officinalis*.

Behavioural measurements are at least one step further along the visual pathway from physiological measurements, as they generally encompass some additional processing in the central nervous systems. Behavioural measurements of visual sensitivity are relatively easy to make, and usually take the form of testing the minimum light intensity at which an animal will still perform a given task, e.g. feeding (Douglas & Hawryshyn, 1990). This technique has been used to measure the sensitivity of some fish species, for example Blaxter (1968, 1969) and Van der Meer (1994) found that the absolute sensitivity

of fish improves with age and with dark-adaptation. There are no published papers of the behavioural measurement of visual sensitivity in cephalopods (Muntz, 1999), and by doing so in this thesis, the visual performance of *S. officinalis* was characterised further than has been done so before.

1.5.1 Adaptation of the visual system

Adaptation is a change in sensitivity due to a constant background stimulus. It is the process whereby organisms adjust their sensitivities to improve their visual performances (Autrum, 1981). Most invertebrates adapt to different light intensities using some (or all) of the following mechanisms: pigment granule migrations, cell movements, optical adjustments (Meyer-Rochow, 2001) and intracellular changes (Laughlin, 1989). Each of these mechanisms combines to alter the sensitivity, so that the eye operates at maximum efficiency in as many different background light intensities as the animal needs. There are two separate forms of adaptation: light and dark adaptation. Light adaptation occurs more quickly, within minutes, but dark adaptation can take several hours (Meyer-Rochow, 2001). These are believed to be two different processes with different controls and together these two forms of adaptation allow an animal to see items under different light regimes. Only light adaptation is examined in this thesis.

The morphological changes that accompany light adaptation in cephalopods have been well studied. Young (1963) performed many histological experiments on the nature of light and dark adaptation in four cephalopod species. He found that cephalopod photoreceptors shorten and that screening pigment migrates to the photoreceptor tips in response to light. Young concluded that, as these movements occur in the isolated eye, they must not be controlled by efferent input from the brain. He did not consider that in the intact animal, the brain might have further control over these mechanisms, as Gleadall *et al.* (1993) later found to be true. Daw & Pearlman (1974) performed a chemical-based analysis of the pigment migration, and found that it was dependent on the amount of

rhodopsin that was isomerised. These changes in screening pigment migration and cell length alone would allow the retina of a cephalopod to cope with approximately 1.7 log units change in the light intensity of their environment. Muntz (1977) examined the changes of the cephalopod pupil with different amounts of light, and concluded that these changes could reduce the light reaching the retina by 0.6 log units. All of these morphological changes reduce the light intensity at the retina by a maximum of 2.5 log units, but most coastal cephalopods live in an environment where they can encounter changes of at least 10 log units in ambient light intensity, probably up to a maximum of 13 log units (Clarke & Denton, 1962). It is likely that other mechanisms, within cells and on a sub-cellular level, e.g. numbers of functioning gap junctions and intracellular biochemical changes, are important to allow cephalopods to adapt to the entire light intensity range they encounter on a daily basis (Muntz, 1999). Some of these mechanisms were investigated as part of this thesis, i.e. the adaptation capabilities of the retina alone and the presence of functioning gap junctions.

Adaptation, in all species, is still a cause of some debate, as the precise way in which it occurs within a cell is undetermined (Jindrova, 1998). It is known that intracellular calcium levels play an important role in adaptation, but the degree of their importance, and the presence of another chemical involved in the shutting down mechanism is debated (Fain *et al.*, 2001). One way in which calcium participates in adaptation is by controlling, to a certain degree, the amount of *meta*-rhodopsin in a cell through affecting guanosine di-phosphate (GDP in Figure 1.2) (Rayer *et al.*, 1990). As this in turn, influences the levels of rhodopsin, it can have some bearing on the cell's light reactivity (Autrum, 1981). Bader *et al.* (1976) injected calcium and sodium ions into an insect's photoreceptor and found that they had the same effect as light adaptation. Investigations into dark adaptation and the influence of calcium concentration on sensitivity in cephalopods (Pinto & Brown, 1977; Clark & Duncan, 1978; Duncan & Pynsent, 1979b) have had similar results to Bader *et al.* (1976). The light adaptation

capabilities of the excised retina and the influence of its extracellular calcium concentration are investigated in this thesis for *S. officinalis*. In doing so, some of the factors controlling the visual performance of *S. officinalis* will be quantified.

1.5.2 Physiological determination of visual sensitivity

To investigate adaptation and sensitivity of an animal's visual system, its responses to different light intensities must be measured. This can be done using physiological or behavioural methods and in Chapters 3, 4 and 5, the physiological measurement of the electroretinogram (ERG) was used. The ERG is a recording of the retina's field potential. It can be easily picked up by placing a low resistance electrode in the retina of an eye (e.g. Clark, 1975). Depending on the number of cell types in the retina, there can be several components of an ERG, each representing the different reactions in the various cell types within the eye (McIlwain, 1996). The cephalopod retina has only one neuronal cell type, so the ERG is relatively simple (Messenger, 1981). A second way in which it is possible to record from the cephalopod retina is to use intracellular recordings. As the name suggests, intracellular recordings involve placing an electrode within a cell and recording the difference in activity between it and that occurring outside the cell. Although intracellular recordings are technically more difficult to achieve than the ERG, as they require an electrode to remain within a cell, they allow greater access to investigate the events occurring within individual cells. Physiological measurements of the sensitivity and adaptation of the retina of *S. officinalis* can then be compared to some of the behavioural measurements and measurements from individual photoreceptors to gain a better understanding of the visual processing that occurs in cephalopods. This new information on the visual performance of *S. officinalis* will then be compared to that of other animals.

1.6 The aims of this thesis

The overall aim of this thesis was, as previously stated, to characterise the cephalopod visual system, using *S. officinalis* as the study species. Although there have been several papers published on various aspects of cephalopod vision (reviewed by Messenger, 1981), very few actually relate to the same species. By using some well established techniques on *S. officinalis* to measure some visual parameters, and by applying others that have not been previously used on this species, it is intended that a picture of the initial stages of visual processing in *S. officinalis* will be assembled. This will permit better characterisation of the cephalopod visual system and comparisons to be drawn with other such sophisticated visual systems

The two invertebrate species whose visual systems are most studied are *Limulus polyphemus* (the horseshoe crab) and *Drosophila melanogaster* (the fruitfly) (see Barlow *et al.*, 2001 and Zuker, 1996 reviews on respective species). Although the information available on vision in both these species is extensive, it is not complete. The genome sequence of *D. melanogaster* is known, so the research using this species has concentrated on the genes associated with vision. For *L. polyphemus*, the focus of the research is on the ecology of vision and how the animal interprets what it sees. The reviews by Barlow *et al.* (2001) on *L. polyphemus* and by Zuker (1996) on *D. melanogaster* show that the knowledge about vision in these two species far exceeds that known about any single cephalopod species, but there is a substantial amount known about cephalopod vision in general (Messenger, 1981). This thesis examines various aspects of vision in just one cephalopod species. By measuring the basic capabilities of the *S. officinalis* eye to collect and transmit visual data, it is anticipated that a foundation would be established on which to develop this species as a model for visual studies in the future. *Sepia officinalis* is particularly suited to this purpose as it has the overall structure of a vertebrate eye, but without the complexity of different neuronal cell types within its retina.

This thesis has five main results chapters, each of which had a separate objective, that complement each other to form a systematic study of the visual system of *S. officinalis*, in order to characterise its visual performance. The individual objectives were as follows:

- Chapter 2: To investigate changes in the spatial resolution of *S. officinalis* with increases in animal size and increases in ambient light intensity, using the optomotor apparatus;
- Chapter 3: To study the sensitivity of *S. officinalis*, using the ERG, and to explore how it varied with animal size, stimulus wavelength and stimulus duration;
- Chapter 4: To examine how the retina of *S. officinalis* adapts to light, including the dependency of this process on the extracellular calcium concentration of the retina;
- Chapter 5: To determine if functioning gap junctions are present in the retina of *S. officinalis*, using physiological techniques;
- Chapter 6: To characterise the intracellular response of the retinal photoreceptors of *S. officinalis*, including ascertaining the sensitivity of individual photoreceptors.

In achieving these aims, a complete picture of the visual sensitivity of *S. officinalis* will be acquired, from the level of an individual cell to that of the behaviour of the animal. Whenever possible, the work is carried out on a range of animal sizes, thus testing if there are any developmental change in the ability of *S. officinalis* to respond to its visual environment. Some factors either affecting sensitivity, or somehow related to it, are also investigated to complete the picture of the events that might control visual performance in *S. offinicalis*. The experimental results will be compared to those from other species to determine the usefulness of *S. officinalis* as a model for visual studies.

Chapter 2 Measuring the Visual Acuity of *Sepia officinalis* Using the Optomotor Apparatus

2.1 Introduction

Previous studies on cephalopod vision have focussed on shape discrimination, rather than on the measurement of basic visual functions, such as sensitivity and acuity (Muntz, 1999). Visual acuity (or spatial resolution) is the ability to discriminate fine detail (Tansley, 1965). It plays an extremely important role in the lives of animals, as it allows them to navigate in space, evade predators, catch prey, and in some species differentiate between males and females. There are several measures of visual acuity defined in scientific literature, but a commonly used one is the minimum separable angle (MSA). The formal definition of the MSA is the critical angular spacing of two stimuli when they are just resolved (McIlwain, 1996). At a fundamental level, the MSA is related to the spacing of the photoreceptors in the eye. For an animal to determine that two objects are separate, the images of the objects must fall on photoreceptors that are separated by at least one unstimulated photoreceptor. The smallest MSA therefore corresponds to the diameter of one photoreceptor. Other types of acuity measure slightly different angles, and do not rely necessarily on an unstimulated photoreceptor, e.g. vernier acuity, that measures the relative positions of objects (McIlwain, 1996), and minimum angle, the angle that a single object must subtend on the eye to be resolved (Neave, 1984).

In the past, the MSA of animals has been measured using the two very disparate methods of histology and behaviour. All behavioural tests used to measure MSA involve testing the reactions of animals either to stimuli of various widths at one distance, or to stimuli of similar widths at various distances. There are three main experimental designs used to measure behavioural acuity, a) training to discriminate between plain panels and gratings, b) measuring the reactive distance between predator and its prey items and c) the optomotor apparatus (see Chapter 1 for further details), and all have been used with

cephalopod species. Three octopus species have been trained to attack panels with different grating patterns in order to measure their MSA (Table 2.1).

Species	MSA	Reference
Octopus vulgaris	17'	Sutherland (1963)
O. pallidus	7.1'-9.7'	Muntz & Gwyther (1988, 1989)
O. australis	7.1'-9.7'	Muntz & Gwyther (1988, 1989)

Table 2.1: The minimum separable angle (MSA) of *Octopus* spp. determined using the training method.

The reactive distance technique was used by Watanuki *et al.* (2000) to measure the visual acuity of *Sepia esculenta* (a Japanese cuttlefish) to be 2.78'. Packard (1969) used the optomotor apparatus to measure the MSA of juvenile *Octopus vulgaris* to be 27'. The MSA of *S. officinalis* has yet to be determined using any behavioural method. These three different behavioural methods do not measure precisely the same type of MSA, so it remains unclear if results obtained from them are comparable (Davson, 1990). Also the three rely on different behavioural triggers, so angles measured using them must be compared with caution (see Section 1.4).

The optomotor apparatus was selected to measure the MSA of *S. officinalis* in this study instead of the other two behavioural techniques for the following reasons. The training technique has worked well with *Octopus* spp. as they tend to be territorial and so attack anything that moves within their home range. *Sepia officinalis* are not territorial and usually attack only food-like items (Wells, 1958), a complication which would increase the time needed for training. A second reason for not choosing to train *S. officinalis*, was that younger animals learn slowly (Wells, 1958), which would add to the training time necessary, and so limit the number and size, of animals that could be tested. The reactive distance method would be quite difficult to use as *S. officinalis* eat large prey items (mysid,

prawns and crabs; Hanlon & Messenger, 1996), which means that there would have to be a large distance between the prey and *S. officinalis* to calculate the reactive distance. These distances would be larger than the dimensions of normal aquarium tanks. Also any angle calculated using the reactive distance would probably be an overestimation, as most animals can probably see smaller or more distant items, which they ignore (Hairston and Li, 1982). The reasons for choosing the optomotor apparatus, rather than just rejecting the other two methods, were as follows. Two separate studies (Collewijn, 1969; Messenger, 1970) showed that *S. officinalis* responded positively when tested with the optomotor apparatus, but the MSA was not measured in these papers. This positive reaction was important, as various animals such as frogs and some fish species do not exhibit an optomotor response (Dieringer, 1991). Other advantages of the optomotor apparatus are that it allows animals to be tested individually and relatively quickly, thereby allowing adequate replication of experiments.

Although there have been histological measurements of MSA made on *S. officinalis*, and behavioural measurements done on other cephalopod species (e.g. Table 2.1), the behavioural MSA of this species has never been studied. In non-cephalopod species, investigations using the optomotor apparatus have been taken a step further, and the influence of other factors on the MSA has been considered. Only once has such a study been completed in a cephalopod species. Packard (1969) found that as *Octopus vulgaris* increased in size, their MSA decreased. Many authors have used the optomotor apparatus to show that this decrease occurred in other species also (e.g. Vestal, 1973; Rahmann *et al.*, 1979; Neave, 1984; Pankhurst, 1994). Similar trends were found when the histological MSA was examined in different sized animals (Shand, 1997). Behavioural studies have also shown that light intensity influences acuity (Shlaer, 1937; Pirenne & Denton, 1952), but not in all species (Rahmann *et al.*, 1968; Vestal, 1973). A report of the influence of different light intensities on the MSA of a cephalopod species has never been published. These studies in various non-cephalopod species illustrated that not only can

the optomotor apparatus be used to measure visual acuity, but can also be used to examine some of the factors that influence it.

The aims of this chapter were:

- to measure the behavioural MSA of *S. officinalis*, using the optomotor apparatus, and compare it with that of other cephalopods,
- to examine how the MSA was affected by size of the animal,
- to determine the influence of light intensity on the MSA of *S. officinalis*, and
- to ascertain if there was any interaction between the effects of size and light intensity on the MSA of *S. officinalis*.

2.2 Materials and Methods

2.2.1 *Animals*

Sepia officinalis in four size groups (1, 2, 4, and 8 cm in mantle length, ML) were used for these experiments. For the three smaller groups, cultured animals reared in captivity from hatching were used, but for the largest group, both cultured animals and animals caught in the wild were tested. The length of each animal's mantle was measured at the beginning of each experiment. Usually the individuals of each group were held in isolation, using fish breeding traps (3-Way Breeder Multi-Purpose, Lee's Aquarium and Pet Products, USA) for the duration of an experiment, but sometimes the largest animals were kept in groups and identified by their individual markings (damage to their skin usually obtained from trawling methods or fighting). All animals were fed daily and kept on a 12 h light:dark cycle. The 12 h light lasted from 6.30 am to 6.30 pm and varied between 30 and 100 $\mu W/cm^2$.

Twenty individual animals of the three smaller sizes were tested at each of four light intensities. Every effort was made to use animals that seemed healthy, by choosing those that were sitting on the bottom of the tank and showing a normal colouration. Whenever possible an animal was only tested once to avoid any possibility of habituation to the stripes and apparatus affecting the results (highlighted as a possibility by Vestal, 1973), but due to death of cultured animals, and to the small numbers caught in the wild this proved impossible for the 8 cm animals. Every one of the eleven 8 cm animals was therefore tested at each of the four light intensities. In the statistical analysis, the 8 cm group was treated differently from the other three size groups (see Section 2.2.5).

2.2.2 *Apparatus*

The optomotor apparatus consisted of a drum (152 cm diameter) that was rotated by a reversible motor. The bottom of the drum was lined with black material to mask any visible defects the animal might follow. A central platform, supported by transparent nylon thread from an overhead beam, carried the experimental container (23 cm diameter) (Figure 2.1). A video camera was also connected to the beam, directly over the container, and this allowed the movements of the animal to be viewed on a monitor in a screened area of the room. A light, whose output was varied using neutral density filters (grey plastic purchased from Stage Electrics, UK), illuminated the drum. The interior wall of the drum was lined with stripes. Nine different widths of stripes (ranging from 0.07 to 7.0 cm) were used to assess the MSA of *S. officinalis*. Larger stripe widths were printed on A4 sheets, which were then glued together. The three finest lines used were printed by professional printers on continuous sheets of paper. The lack of an optomotor response to a control of plain grey cardboard implied that the animal could not see any possible defects in the drum. The speed of rotation of the drum was 3 rotations per minute (rpm). Light intensity was measured using an optical power meter (TQ8210, Advantest, Japan). The four light intensities used were 4, 6, 10, and 15 $\mu W/cm^2$.

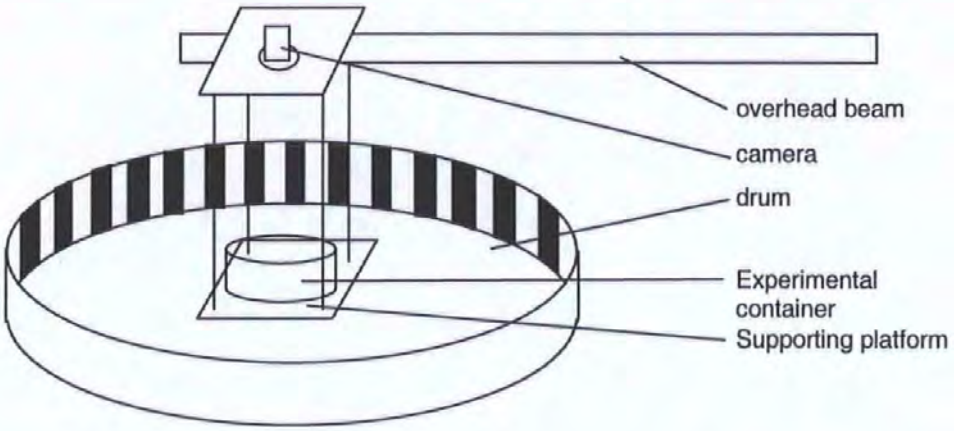


Figure 2.1: A diagram of the optomotor apparatus used for these experiments (not drawn to scale).

The stripe widths used were chosen by using the previously published papers on cephalopod acuity as guidelines (e.g. Sutherland, 1963; Muntz & Gwyther, 1988, 1989; and Watanuki *et al.*, 2000). First, the stripe width necessary to measure the MSA of *Octopus* spp. at the distance of 152 cm was calculated. Then, other stripe widths, above and below this width were printed to give a range of stripes to use. The speed of the drum (3 rpm) was chosen to reduce the chance of the animal responding to the movement of the drum rather than to the stripes, and because other papers tended to use values that were in the range of 1 to 6 rpm (e.g. Packard, 1969; Vestal, 1973). The critical flicker fusion frequency of *S. officinalis* is 40 rpm (Nelson, 2003), so theoretically they should be able to follow any speed slower than this. The light intensities were chosen to equate to the light intensities *S. officinalis* would experience in their natural habitat, using ranges given in Clarke and Denton (1962) and Boletzky (1983). The lower end of their natural range was tested here to reduce the visibility of any defects in the optomotor apparatus.

2.2.3 *Preliminary experiments*

Two preliminary experiments were completed using groups of 10 animals, of 1 and 2 cm ML (as the larger animals were not as numerous as the smaller ones). Each animal was used for one experiment only. Firstly, the time taken for *S. officinalis* to acclimate to the new surroundings of the experimental container was investigated. Animals were put into the experimental container, brought to the optomotor apparatus and the time they took to settle down was measured. Settling down was defined as spending at least 10 s without showing behaviours such as squirting, inking, jetting, or moving quickly. The maximum time allowed for this to occur was 5 minutes. To determine when *S. officinalis* first reacted to moving stripes, the 4.5 cm (equivalent to 4.7° MSA) stripe pattern was used. The animal was placed in the experimental container, in the optomotor apparatus, and allowed to settle down for 1 min. The apparatus was then rotated for five minutes. The time at which the animal first responded to the stripes was noted. The results from these experiments were used to determine the timings of the main experiment.

2.2.4 *Measuring the minimum separable angle of* Sepia officinalis *using the optomotor apparatus*

To measure the MSA of *S. officinalis*, each animal was placed alone in the experimental container, and brought to the optomotor apparatus. The container was placed on the central platform, and the animal given one minute to settle. The drum was then rotated for one minute, followed by a rest period of 30 s. This was repeated another three times, with the direction of rotation randomly chosen. In total, the animal was in the apparatus for 6.5 min after which it was returned to its holding tank. If the animal maintained the direction and speed of the rotating drum for at least one quarter of a rotation, it was deemed to have followed the stripes. If this occurred in at least two of the four rotations, the animal was considered to have exhibited a positive optomotor response.

If the animal only followed the drum once, this might have been coincidence, and therefore was not considered a true optomotor response.

Ten animals were tested in each run using the same stripe pattern and light intensity. Two or three runs were completed each day, holding the light intensity constant, but changing the stripe pattern. The order of stripe width and light intensity used was randomised. The one exception to this was that the control was always tested first with the brightest light to ensure the animals did not respond to visual cues, other than the stripes. Each group of animals was tested using various stripe widths until its MSA was measured. This usually involved 4 stripe widths and the control. Experiments were always carried out at the same times each day. The first run was completed between 08:30 and 10:30, the second between 11:00 and 13:00 and the third between 14:00 and 16:00.

2.2.5 *Statistical analysis*

The computer packages used to analyse the data from all experiments were SigmaPlot 2001 for Windows (version 7.0; SPSS Inc, USA), Microsoft Excel 2000 (Version 9.0.2720, Microsoft, USA) and SPSS (Version 11.0.1; SPSS Inc, USA). Microsoft Excel and SigmaPlot were used to manipulate the data and to draw graphs. SPSS was used to perform the statistical tests. Levene's test for homogeneity of variance was done before a parametric Analysis of Variance (ANOVA) was completed on the data. If the data were non-homogenous, after standard transformations, a non-parametric equivalent was used to investigate the significance of the observed trends.

Due to the differences in sample sizes of the largest groups with all others, an all-encompassing statistical test could not be performed. Instead, the MSA of the 1, 2 and 4 cm animals were examined in an expanded Kruskal-Wallis test, using the two factors of size and light intensity. The results measured at one light intensity were then selected, and the MSA of all size groups at this light intensity compared. This was to eliminate the problem of the repeated measures obtained from the 8 cm group, as the MSA of each 8 cm

animal was measured at the four different light intensities. To ensure equal sample sizes, 11 animals were randomly chosen from the three smaller size groups (using www.randomizer.org/index.htm).

2.3 Results

2.3.1 *Preliminary experiments*

2.3.1.1 The time taken by *S. officinalis* to settle down in the optomotor apparatus

All animals tested in this preliminary experiment settled down within one minute, with 60% of them settled within the first 10 s, as shown by the histograms in Figure 2.2. Therefore the animals in the main experiment to measure their MSA were allowed one minute to acclimate to the experimental container and apparatus before rotation of the apparatus began. When doing the main MSA experiments, a note was always taken of whether the animal had settled or not within the first minute. In the vast majority of cases (>99%), animals had settled within this time. This included the larger animals for which the preliminary studies had not been carried out, thereby indicating that this one minute interval to acclimate to the apparatus was appropriate for all sizes.

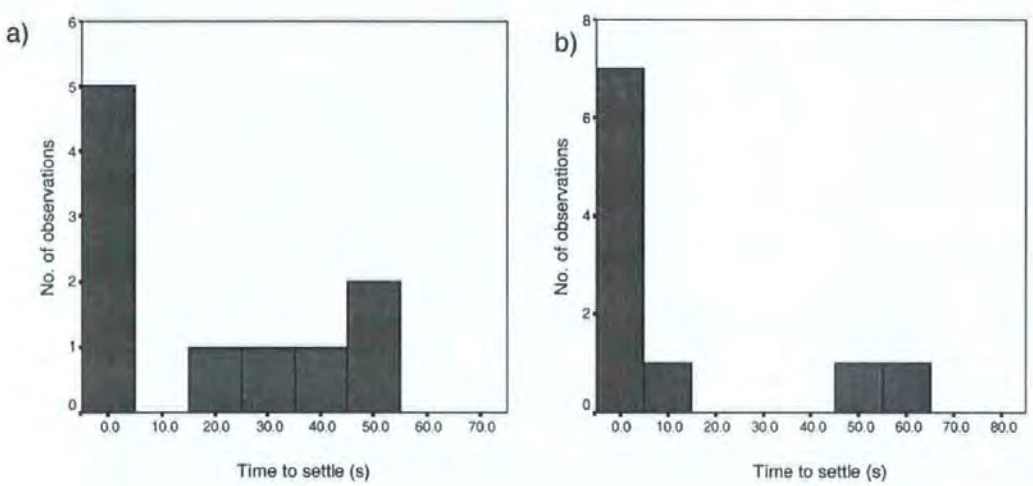


Figure 2.2: Histograms showing the length of time taken by two sizes of *S. officinalis* to settle down in the optomotor apparatus; a) animals 1 cm in mantle length (ML); b) animals 2 cm in ML.

2.3.1.2 The time taken for *S. officinalis* to react to moving stripes in the optomotor apparatus

Twenty different animals were used to determine when *S. officinalis* first responded to moving stripes. Within the first minute of rotation, 85% of the animals began to follow the movement of the stripe pattern. Three of the twenty animals did not respond in the first minute, while two animals (one from each size group) did not respond at all (Table 2.2). There was no significant difference between the two groups, of 1 and 2 cm ML, in the time taken to respond to moving stripes (Mann-Whitney U test, U=32.5, df=16, p>0.05). In experimental trials, a one minute rotation period was used to determine the MSA, as this was considered to be most likely to obtain a response from the animals. It also minimised the time the animals spent in the experimental container.

Mantle Length	Animal Number			Median time to respond (s) (95% Confidence limits)
	Responded in < 1 min	Responded in > 1 min	No response	
1 cm	7	2	1	10 (7-23)
2 cm	8	1	1	17 (11-30)

Table 2.2: The time animals took to react to the rotation of a 4.5 cm stripe in the optomotor apparatus.

2.3.2 *The minimum separable angle of* Sepia officinalis

2.3.2.1 The effect of light intensity and mantle length on the minimum separable angle of *Sepia officinalis*

The mantle length of *S. officinalis* and the light intensity at which it was tested, significantly affected its MSA (Table 2.3). This applied to the 1, 2 and 4 cm animals only, tested at four light intensities, as these had the same number of animals in each group.

Source	Sum of Squares	df	H	Sig.
Sample	369079	11	81.9	0.000
ML	130694	2	29.0	0.000
LI	149972	3	33.29	0.000
SIZE * LI	88413	6	19.6	0.025

Table 2.3: The results of an extended Kruskal-Wallis test with minimum separable angle (MSA) as the dependent variable, and mantle length (ML) of the animals and light intensity (LI) in the optomotor apparatus as the fixed factors.

The largest animals at the higher light intensities could differentiate more detail than the smallest animals at lower intensities, as was indicated by the separation of the letters assigned to these groups using post-hoc pairwise tests (Table 2.4). As there was a significant interaction between light intensity and size (Table 2.3), the comparison of the rank means (Table 2.4) had to be interpreted with caution (Underwood, 1997). The four cells in Table 2.4 with the same letters of bcd indicated that at different light intensities these three size groups had indistinguishable MSAs under those conditions (see the appendix for data on the number of animals that responded to each stripe width, including the control, with each of the light intensities, Table A.1).

ML (cm)	Light Intensity ($\mu W/cm^2$)			
	4	6	10	15
1	197.9 (e)	137.2 (cd)	120.8 (cd)	115.2 (bcd)
2	163.9 (de)	116.8 (bcd)	118.8 (bcd)	63.4 (ab)
4	108.3 (bcd)	88.2 (abc)	63.1 (ab)	41.8 (a)

Table 2.4: A comparison of the rank means of the MSA of three mantle length (ML) groups of animals, tested under four ambient light intensities, using a post-hoc pairwise test. Any two samples sharing a common letter were not significantly different at the p=0.05 level.

Mantle length had a significant effect on the MSA of *S. officinalis* (Table 2.5), when the results from the 8 cm animals were included with those from the smaller ML groups in a parametric ANOVA, that examined the results obtained at one light intensity only (6 $\mu W/cm^2$).

Source	Sum of Squares	df	Mean Square	F	Sig.
ML	168.701	4	42.175	45.766	0.000
Error	34.097	37	0.922		
Total	202.798	41			

Table 2.5 : The results of a parametric ANOVA testing the effect of mantle length (ML) on the minimum separable angle of *S. officinalis*.

The data from the 8 cm group were then included in a graph of MSA and light intensity to see how the different sizes compared under these conditions (Figure 2.3). At lower light intensities the MSA of each group was very different, with 8 cm animals able to discriminate the finest lines. At higher light intensities, the pattern was not as orderly as there was a degree of overlap between some of the sizes. Overall as light intensity decreased, the MSA increased considerably, faster for the 1 and 2 cm animals than for the 4 and 8 cm animals (Figure 2.3).

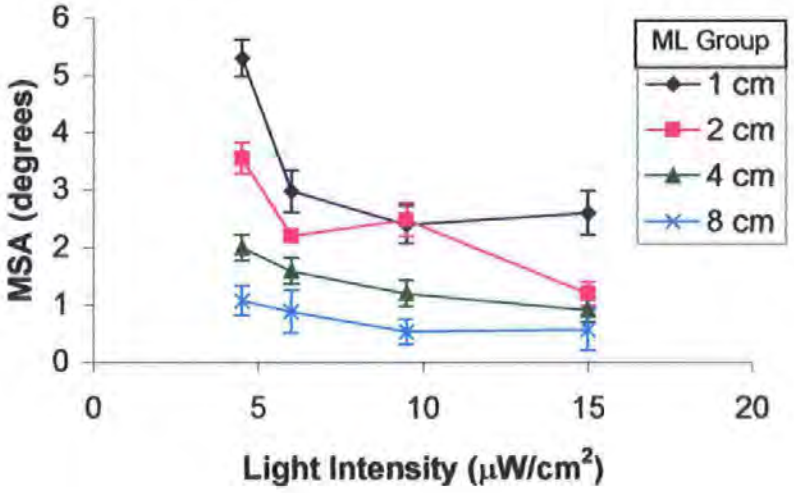


Figure 2.3: The effect of light intensity on the minimum separable angle (MSA) of four mantle length (ML) groups of *S. officinalis* (Error bars represent +/- one standard error (se), n=20 for the 1, 2 and 4 cm ML groups, n=11 for the 8 cm ML group).

2.3.2.2 The influence of mantle length increase on minimum separable angle

To assess the way in which MSA changed with ML, various curves were fitted to the mean MSA of different sized animals measured at 15 μW/cm^2 (Figure 2.4, and Table 2.6). Overall, as *S. officinalis* grew, their MSA decreased. A logarithmic curve was fitted to the data as logarithmic growth commonly occurs in these animals (Boletzky, 1983), but the fit was not significant (Table 2.6). When the SPSS curve estimation function was used to fit several different equations, three functions were found to fit the data in Figure 2.4 significantly (Table 2.6). These were an inverse function, an S- function and a power function. The other eight equations that SPSS fitted to these data were not significant (results not shown).

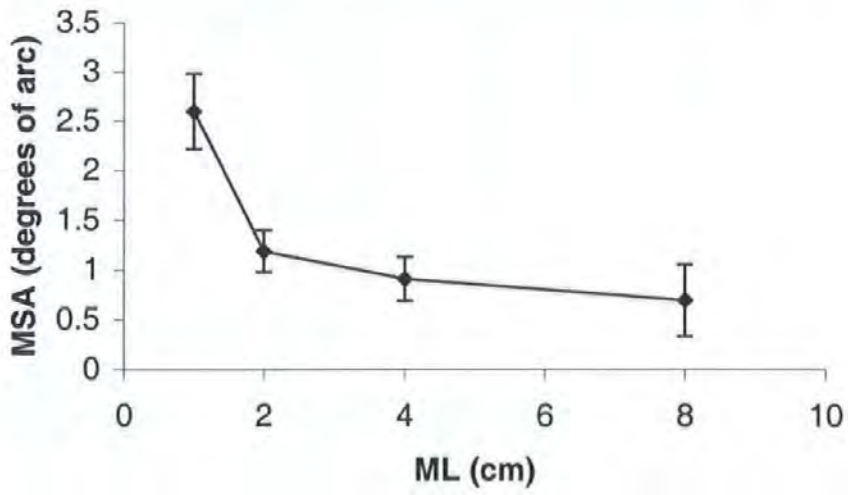


Figure 2.4: The decrease in the minimum separable angle (MSA) of *S. officinalis* with increasing mantle length (ML) at the highest light intensity used (15 μW/cm^2) (error bars = +/- 1 se; n=20 for each ML except 8 cm animals where n=11).

Type of line	Equation of best fit line	r^2 value	Significance level
Logarithmic	y = -0.87Ln(x) + 2.25	0.813, df=2	F=8.7; p=0.099
S-curve	$y = e^{(-0.52+1.47/x)}$	0.994, df=2	F=334.2; p=0.003
Inverse	y=0.33+(2.18/x)	0.968, df=2	F=60.3; p=0.016
Power	$y =2.23(x^{-0.61})$	0.919, df=2	F=22.54; p=0.042

Table 2.6: The equations of the best fit lines for the data presented in Figure 2.4.

2.3.2.3 The interaction of mantle length and light intensity

On initial inspection of Figure 2.3, it appeared that the MSA of larger animals did not change with light intensity to the same extent as the smaller ones, i.e. there was a much larger difference between the MSA measured at 4 μW/cm^2 and at 15 μW/cm^2 for the smaller animals than for the larger animals. When this was examined in absolute terms, the change in MSA for the 8 cm animals was 0.5° between the two extremes of light

intensity used, compared to a 2.7 ° change for the 1 cm animals (Table 2.7). This implied that a reduction in light intensity did not affect the larger animals to the same extent as the smaller animals. But when the proportional change was considered, this difference disappeared, and all animals resolved approximately 50% less detail at 4 $\mu W/cm^2$ than they did at 15 $\mu W/cm^2$ (Table 2.7).

ML Group (cm)	Absolute Decrease (º)	Proportional Decrease
1	2.70	51%
2	2.36	66%
4	1.09	54%
8	0.50	47%

Table 2.7: The change in the mean minimum separable angle (MSA) between the lowest (4 $\mu W/cm^2$) and highest (15 $\mu W/cm^2$) light intensity used in these experiments for each of the ML groups of *S. officinalis* tested.

2.4 Discussion

Using the optomotor apparatus to measure the reaction of *Sepia officinalis* to different widths of stripes revealed its best behavioural MSA to be 0.7° of arc (42') (Figure 2.4). This was the mean MSA of 8 cm ML tested at 15 $\mu W/cm^2$. At almost every light intensity, larger *S. officinalis* had a significantly lower MSA than smaller ones (Table 2.3 and Figure 2.3). Increasing the light intensity in which the experiments were run decreased the MSA of all sizes. An inverse function, a power function and an S-function all fitted the decrease in MSA with increasing ML better than a normal logarithmic growth function (Table 2.6). Each ML of *S. officinalis* tested responded to changes in the light intensity with a decrease in their MSA of approximately 50% (Table 2.7).

2.4.1 *A comparison of the MSA of* Sepia officinalis *with other cephalopod species*

The smallest MSA measured here for *S. officinalis* was 42', but *Octopus* spp. have MSA's under 30' (Sutherland, 1963; Packard, 1969; Muntz & Gwyther, 1988, 1989); see Section 2.1). Sutherland (1963) and Muntz and Gywther (1988, 1989) used adult animals of 50 to 500 g (different species have different weights at maturation), not hatchlings and juveniles, which were used here. As MSA was shown here to rely on size (Figure 2.4), this difference in the sizes of animals is the likely reason for the difference in MSA. It is important to remember that the optomotor apparatus gives a range within which the MSA lies, i.e. between the smallest stripe to which the animals responded and the largest to which they did not. Taking this into consideration, the MSA of *S. officinalis* could be closer to 10', than to 42'. All other papers that reported results utilising the optomotor apparatus used the narrowest stripe to which the animals responded as the MSA, which was the protocol followed here. The circadian rhythms of an animal are known to influence its visual processes (Barlow *et al.*, 1984; Wang & Mangel, 1996), but as these experiments were always completed within the light phase of the diurnal cycle of *S. officinalis*, it is highly unlikely that they influenced the results presented here.

Packard (1969), however, also used the optomotor apparatus, and only tested small animals up to 17 g, and still obtained results in agreement with other studies on *Octopus* spp., but slightly lower than those obtained here. It is, therefore, a possibility that *O. vulgaris* has better spatial resolution capabilities than *S. officinalis*. There was one large methodological difference between the two studies that could account for this difference in MSA. Packard noted eye, head and body movements when monitoring the animals he used for a positive optomotor response, but this study only used body movements. This meant that his technique was more sensitive than the one used here. This was highlighted by the fact that the control he used sometimes elicited a positive response. He factored this into his analysis, by subtracting any positive reactions to his control from the number of

reactions to the stripes. Packard did not measure the light intensity at which he tested *O. vulgaris*, so it could not be compared with those used here, but if a difference existed between the two lighting regimes, it would have added to the disparity in the measured MSA of *S. officinalis* and *O. vulgaris*.

The smallest MSA of *S. officinalis* (42') estimated from this study was much larger than that of *S. esculenta* (3'; Watanuki *et al.*, 2000). Even if the true MSA of *S. officinalis* lay closer to the largest stripe to which it did not respond, rather than to the narrowest one to which it did, i.e. closer to 10' than 42', *S. esculenta* still had three times better MSA than *S. officinalis*. Watanuki *et al.* tested *S. esculenta* using the reactive distance method, so a slightly different type of visual acuity was measured (see Section 2.1). The main reason for this discrepancy, however, was likely to be due to the different experimental methods used. Watanuki *et al.* monitored animals that were in a tank together. The distance they used to calculate the MSA was between the prey item and the furthest animal to react to it. They did not control for cuttlefish reacting to other cuttlefish. Therefore the furthest away animal might have reacted in the direction of the prey, not because it saw the prey, but because it saw another cuttlefish moving in that direction. The reactive distance they measured was therefore probably larger than the actual one, so the MSA they calculated from this would be an underestimate. A second reason for the difference between the two studies is that Watanuki *et al.* used adults, but hatchlings and juveniles were tested here, and as could be seen from Figure 2.4, MSA depends on size. Finally, the light intensities varied between the two studies. Watanuki *et al.* used between 500 and 4,500 lux, which is equivalent to 70 - 659 $\mu W/cm^2$. In this study, the maximum light intensity used was 15 $\mu W/cm^2$. This difference in light intensity would also contribute to the dissimilar MSA's reported in the two studies. Although Watanuki *et al.* reported a much smaller MSA for *S. esculenta* than that measured in this chapter for *S. officinalis*, it is unlikely that this represents a true difference in these species discriminating capabilities. To investigate the

differences in acuity between these species, both would have to be tested using the same method.

2.4.2 *The change in minimum separable angle of* Sepia officinalis *and other species with an increase in body size*

The results in this chapter agreed with those from other studies listed in Table 2.8, in that the MSA of an animal was influenced significantly by its size (Table 2.5). The data from different studies were difficult to compare in detail because of the different variables used to measure the size or age of the animals, but all species showed a reduction in MSA with increasing size/age. The final MSA of *S. officinalis* was within the ranges of the other species in Table 2.8. There was much greater variation in the first MSA measured in each species, than the last. The reason for the extremely high values in some species was that their eyes had not opened fully at the time of testing. Although the best MSA for *S. officinalis* was somewhat larger than other cephalopod species (section 2.4.1), the pattern it followed during development was very similar to other species.

Species	Method	MSA range	Size/age range	Reference
Pagrus auratus (f)	Optomotor	2280' - 488'	3.1 - 4.9 mm	Shand (1997)
Lepomis macrochirus (f)	Reactive distance	40' - 19'	27 – 152 mm	Breck & Gitter (1983)
Lepomis macrochirus (f)	Reactive distance	27' - 15'	37 - 58 mm	Hairston & Li (1982)
Alosa pseudoharengus (f)	Reactive distance	370' - 100'	9.8 – 40 mm	Miller *et al.* (1993)
Perca flavencens (f)	Reactive distance	250' - 90'	9.8 – 40 mm	
Coregonus hoyi (f)	Reactive distance	100' - 80'	11 – 40 mm	
Pleuronectes platessa (f)	Optomotor	2520' - 11'	0 – 70 days	Neave (1984)
Scophthalmus maximus (f)	Optomotor	1260' - 20'	2 – 42 days	
Salmo gairdneri (f)	Optomotor	1800' - 14'	52 d post fert-adult	Rahmann *et al.* (1979)
Peromyscus maniculatus (m)	Optomotor	163' – 18.2'	0 – 8 days	Vestal (1973)
Peromyscus leucopus (m)	Optomotor	221.8' – 18.2'	0 – 8 days	
Octopus vulgaris (c)	Optomotor	49' – 27'	0.47 – 17 g	Packard (1969)
***Sepia officinalis* (c)**	**Optomotor**	**156' – 42'**	**10 – 80 mm**	**This study**

Table 2.8: The reduction in MSA with size/age in several species (f= fish, c=cephalopod, m=mammal).

This was the second time that a size range of cephalopods was tested with an optomotor apparatus. Both Packard's (1969) study and this one show similar results, but with younger *O. vulgaris* having much better visual acuity than young *S. officinalis*. One of the differences between Packard's apparatus, and the one used here was the diameter of the drum. Packard's drum was 63 cm, but the one used here was 152 cm. Vestal (1973) showed that *Peromyscus* spp. (deermice) responded optimally to stripes when they were 10 cm away (he also tested distances of 5 and 20 cm). It is a possibility that the narrower drum used by Packard better suited smaller cephalopods, thereby decreasing the measured MSA.

Changes in the behavioural MSA were likely to be determined by changes in the parameters used to calculate the histological MSA and other changes in the visual system that affect the processing of the visual signal. Histological MSA decreases with increasing size for two possible reasons, a) the density of photoreceptors increases, and b) the magnification power of the lens increases (Shand, 1997). The change in behavioural acuity measured here could not be due to a change in photoreceptor density, as this does not change in cephalopods with growth (Packard, 1969), but must have been due to a change in lens/eye size and possibly other factors of the visual system. Research on other classes of animals has found that the increase in acuity is due to the increase in lens size, rather than to an increase in photoreceptor density. In fact, in some fish species the number of cones actually declines as the fish grows, but the acuity still improves (Shand, 1997). Other factors are also known to change with age, but as yet none of these has been investigated in relation to visual acuity. Rahmann *et al.* (1979) and Neave (1984) suggested that the improvement in MSA of young fish was due more to the growth of the optic tectum, than to growth of the eye. This cannot be measured by examining the histology of the retina, but investigations into the behavioural MSA might shed some light on the issue.

This study examined the decrease in behavioural MSA with an increase in ML, by fitting different functions to the data collected. Functions other than a logarithmic curve significantly fitted the decrease in behavioural MSA with an increase in ML (Table 2.6). A logarithmic curve best described the improvement in histological MSA with animal growth (S. Farley, unpublished results). As other curves better fit the improvement in behavioural MSA, it was likely that development in the brain was an important factor in determining behavioural MSA. The most likely reason for the inverse curve fitting better was that as the ML of the animal increased, the area of the retina increased by the square of the increase in length. The area of the retina is a parameter that is not considered directly in histological calculations of MSA. This study did not produce enough data to distinguish between the two possible options of development in the brain, or an increase in area of the retina to be the major cause of improvement in behavioural MSA with increasing ML.

2.4.3 *The effect of measuring the minimum separable angle of* Sepia officinalis, *and other species, under different ambient light intensities*

The light intensities used in these experiments resemble those *S. officinalis* would encounter in their natural habitat (Clarke & Denton, 1962). The animals could see more detail at higher intensities due to the adaptive mechanisms in their visual systems. Cephalopods adapt to different light intensities using pupil size changes (Muntz, 1977), migration of the screening pigment within the retina (Daw & Pearlman, 1974), changes in length of the photoreceptors (Young, 1963) and biochemical changes within the cells (Muntz, 1999). From Table 2.7, it was possible that these mechanisms operate to the same degree within all sizes of *S. officinalis*, to decrease MSA by 50% with an increase in light intensity from 4 – 15 $\mu W/cm^2$. Even though the animals were only given one minute to adapt to the different light intensities used in these experiments, which was probably not enough time to allow full adaptation (Meyer-Rochow, 2001), it allowed comparisons to be made between the different sized animals, as each one underwent the same treatment.

Larger *S. officinalis* have smaller MSA's at lower light intensities than smaller *S. officinalis* (Table 2.4). *Sepia officinalis* eggs are laid in shallow coastal waters, and so this is where hatchlings of 1-2 cm spend most of their time (Boletzky, 1983). As *S. officinalis* grow, they migrate to deeper waters, so the change in MSA observed here probably relates to this change in habitat. Larger animals need to distinguish more detail at lower light intensities, as they live in darker waters than smaller ones.

The decrease in visual acuity with an increase in light intensity (Figure 2.3) has been found in other studies (Shlaer, 1937; Pirenne & Denton, 1952). Two studies that used various species of *Peromyscus* (Rahmann *et al.*, 1968; Vestal, 1973) found that the light intensity at which experiments were carried out did not affect the visual acuity of all species tested. Both studies used a wide range of intensities, so a difference would have been expected. Not enough detail is given in the Rahmann *et al.* (1968) paper to comment on the methodology used for the light intensity experiments. Vestal (1973), however, states that fixed inter-trial intervals were not used. This implies that different animals could have had different lengths of time to adapt to the surrounding light intensity, which would result in differences in visual acuity at each light intensity, decreasing the chance of finding a pattern in MSA with decreasing light intensity. The photoreceptors of *S. officinalis* increase in length, as the animals increase in ML (S. Farley, unpublished results). This means that larger animals should have a greater sensitivity (Land, 1981), and so be able to distinguish more detail at lower light intensities, which was found to be so with *S. officinalis* in this study. Another factor that influences sensitivity is the aperture of the eye, i.e. pupil size (Land, 1981). Although no study has been published on the rate of increase in pupil size with the increase in the principal focal length of the eye of *S. officinalis*, it is assumed that they increase in such a way as to decrease the F-number of the eye, and so increase the amount of light reaching the photoreceptors. This would also enable larger animals to see more detail at lower light intensities than the smaller animals, as was found here.

2.4.4 *Conclusions*

The MSA of four different ML groups of *S. officinalis* under four different light intensities were measured (Figure 2.3) using the optomotor apparatus. Although the absolute values measured here were slightly larger than those found in similar species, the trends with changing size and light intensity were similar to those previously published. Larger animals had smaller MSA's while all sizes could not see as much detail at lower light intensities. Changes in histological parameters, that are usually used to calculate MSA, were unlikely to explain all the improvement in the behavioural MSA. Other parameters, not investigated in this study e.g. changes in retinal area or visual processing, would appear to offer a more accurate explanation for the decrease in behavioural MSA with an increase in ML. Increases in light intensity affected the four sizes of animals tested equally by reducing their MSA by 50%, implying that all sizes of *S. officinalis* adapt to changes in light intensity with the same efficiency.

Chapter 3 The Absolute Visual Sensitivity of *Sepia officinalis* as Determined by the Electroretinogram

3.1 Introduction

To investigate further the visual capabilities of *Sepia officinalis*, the reaction of its retina to different intensities of light, i.e. its sensitivity, was investigated. A report on the visual sensitivity of *S. officinalis* has yet to be published, and overall, there have been very few studies on the sensitivity of cephalopod species. Duncan & Pynsent (1979a) studied the sensitivity of *Sepiola atlantica* and Tomita (1968) did the same for some *Octopus* spp. In this chapter, and throughout the rest of this thesis, sensitivity refers to the amount of light necessary to elicit a response in the retina, and does not refer to motion sensitivity, spectral sensitivity, or any other type, unless otherwise stated.

Visual sensitivity is one of the basic functions of an eye. It determines in which lighting conditions, and therefore habitat, an animal can live. Animals that inhabit the deep sea tend to have a much better sensitivity than those in terrestrial habitats (Land & Nilsson, 2002). *Sepia officinalis* migrate between shallow waters in the summer to deeper waters during the winter months (Boletzky, 1983). This corresponds to a light intensity range of 10^{-8} - 10^{5} $\mu W/m^2$ in clear coastal waters (Clarke & Denton, 1962). Physiological measurements of sensitivity can be compared with the light intensities *S. officinalis* would normally encounter to determine how well they are suited to their habitat.

3.1.1 The cephalopod electroretinogram

To determine the physiological sensitivity of an animal's visual system, its responses to different light intensities must be measured. In this chapter, the electroretinogram (ERG) was used. The ERG is a recording of the retina's field potential. It can be easily picked up by placing a low resistance electrode in the retina of an excised eye. As the cephalopod retina has only one neuronal cell type, the ERG is relatively

simple (Messenger, 1981). The ERG of the isolated retina was selected to measure the animal's reaction to light because the impact of two of the mechanisms animals use to adapt to light intensities (pupil changes and any efferent input from the brain to change cell length and screening pigment location) were removed, thereby reducing the number of variables that could alter the ERG. Changes in cell length and in screening pigment migration could still occur but at a reduced level due to mechanisms intrinsic to the retina (Young, 1963; Gleadall *et al.*, 1993).

Although the cephalopod retina is relatively simple structurally, the ERG is somewhat more complicated. As mentioned earlier (Section 1.2); the cephalopod retina has two basic cell types, photoreceptors and supporting cells. Photoreceptors are composed of an inner and outer segment, separated by the basement membrane (Figure 1.3b). The ERG varies in polarity from negative at the outer segments, to positive at the inner segments. Hagins *et al.* (1962) showed that this was due to the receptor cell acting as a dipole in the squid retina. Duncan & Pynsent (1979a) provided further evidence for this theory by showing that this dipole could be related to voltage-sensitive potassium channels in the inner segment membrane. Tasaki and his co-workers (Tasaki *et al.*, 1963a; Tasaki *et al.*, 1963b; Tasaki *et al.*, 1963c), however, maintained that the negative response originated from the supporting cells, while the positive response came from the photoreceptors, as the two responses underwent different changes when exposed to the same stimuli. Their experiments were performed on octopus, and it is possible, though unlikely, that the negative ERG is generated by the supporting cells only in this species, but not in any other cephalopod species. The positive ERG was used throughout this thesis because it appeared to be more stable, and there is little disagreement over its origin. Its greater stability could be due to the fact that the inner segments of the photoreceptor are not known to lengthen in response to light, and so the electrode would always be in the same location. Outer segments, on the other hand, can lengthen in response to the amount of light they receive, and so the negative ERG would be less stable (Young, 1963). A second complicating

factor of the cephalopod ERG is that it can exhibit two positive components, one fast and one slow (Clark & Duncan, 1978). The slow component is dominant and was used in this study. These two components react differently to light adaptation, to perfusion with altered saline solutions, and are found at different locations within the retina (Clark & Duncan, 1978). It must be remembered when working with ERG's that they are "only indirectly a measure of retinular cell activity" (Ziedins & Meyer-Rochow, 1990), and so should be interpreted with care.

3.1.2 Factors that affect visual sensitivity

Three variables that are known to affect visual sensitivity were investigated here to determine if they influenced the sensitivity of *S. officinalis* in similar ways to those of other species. Firstly, sensitivity has been shown to change with the size of an animal (Blaxter, 1968, 1969). The length of photoreceptors and the pupil size in connection with the focal length of the lens of an eye are known to influence visual sensitivity (Land, 1981; Van der Meer, 1994; Nilsson & Warrant, 2001; Land & Nilsson, 2002). The length of the photoreceptors in *S. officinalis* increases as the animal grows (Sophia Farley, unpublished results), so it was possible that its sensitivity, as determined by the ERG of the isolated retina, changed also.

The overall shape of the ERG might also change in some way as the ML of *S. officinalis* increases with growth. Yinon & Auerbach (1969) discovered that the ERG of *Tenebrio molitor* changed with pupal and adult development. The change between pupa and adult was expected because the pupa underwent extreme reorganisation to form the adult. It was somewhat surprising that the ERG shape continued to change within the first two weeks of adult life. They did not carry out histological studies to see if any morphological changes corresponded to these changes (Yinon and Auerbach, 1969). There are at least two morphological changes in the retina of *S. officinalis*, as the animal grows: a) an increase in photoreceptor length, mentioned previously, and b) a decrease in the

proportion of inner segment length to outer segment length (S. Farley, unpublished results). Both these factors might affect the ERG shape, as well as other changes that are only visible at electron microscopy levels, which have not yet been studied across a size range of *S. officinalis*.

Secondly, the effect of two different wavelengths of light on the sensitivity of *S. officinalis* was investigated. The activation of a rhodopsin molecule initiates the phototransduction cascade, eventually causing the opening and closing of ion channels in the cell membrane. These changes in the cell membrane lead to voltage changes within the retina. These voltage changes affect the field potential measured, and it is changes of the field potential of the retina that are termed the ERG. Every type of rhodopsin has its own absorption spectrum (Archer, 1999). The sensitivity of an animal to different wavelengths of light varies depending on the maximum of that absorption spectrum and the ERG can be used to investigate this parameter. The maximum peak of the *S. officinalis* rhodopsin absorption spectrum lies at 492 nm (Brown & Brown, 1958), but the precise way this influences its sensitivity to other wavelengths is unknown. The physiological sensitivity of *Octopus* spp. was measured by Tomita (1968) and Tasaki *et al.* (1963c), and it matched Brown and Brown's (1958) data on the same genus.

The third factor that was investigated in this study, which could influence the sensitivity of *S. officinalis*, was that of flash duration. Matic & Laughlin (1981) stated that flash duration differentially affected the slope of the straight line section of the V/log I curve of vertebrates and invertebrates (the V/log I curve is a plot of response amplitude against log of the stimulus strength, usually sigmoid in shape, and is commonly used in sensitivity studies: Autrum, 1981). The slope of V/log I curves changed when sensitivity was measured with flashes of different durations in vertebrate, but not in invertebrate species (Matic & Laughlin, 1981). As the invertebrate species tested have all had compound eyes, this difference may not be an invertebrate/vertebrate split, but actually a simple/compound eye split. The cephalopod eye provides an ideal model to test this

theory, as it has invertebrate photoreceptors set in a simple eye. According to Matic & Laughlin (1981), the slope of the V/log I curve was due to the timings of the phototransduction cascade, and therefore, it was likely that *S. officinalis* should be more similar to invertebrates than to vertebrates.

To study the effects of these three factors on the sensitivity of *S. officinalis*, an appropriate variable obtained using the ERG had to be chosen. Within the scientific literature, sensitivity has been measured in many different ways, two of which were used in this chapter. Absolute sensitivity is defined as the reciprocal of the intensity that gives a constant response amplitude, generally taken to be the response amplitude at 50% of the maximum response (Autrum, 1981). A variation of this definition (see Section 3.2.4) was used to compare the sensitivity of *S. officinalis* under the different conditions studied in this chapter. Another frequently used measure of sensitivity is that of absolute threshold. It is defined as the minimum amount of light necessary to elicit the smallest measurable response (Autrum, 1981), and was used here to compare the sensitivity of *S. officinalis* with that of other species. More subtle changes in sensitivity can be characterised by plotting the response amplitude against the log of the stimulus strength, i.e. a V/log I curve. The V/log I curve is commonly used in papers on sensitivity (Autrum, 1981), and hence changes in its shape are known to represent different things, e.g. an increase in the slope of the linear part of the curve represents better contrast sensitivity (Eguchi & Horikoshi, 1984). The response amplitude is not the only characteristic of the photoresponse to change with different intensities of light (e.g. Takagi, 1994). The response latency and duration also provide information about the underlying mechanisms involved in sensitivity (Saibil, 1986). For further information on the different methods used to measure sensitivity, reviews written by Autrum (1981) and Land (1981) give useful summaries.

The main aims of this chapter were as follows:

- to measure the visual sensitivity of *S. officinalis* and compare it to that of other species,
- to examine whether the sensitivity of *S. officinalis* changed with the mantle length (ML) of the animal,
- to investigate if its ERG shape changed in a consistent manner with ML,
- to determine the effect of flash duration on absolute sensitivity and the V/log I curve of *S. officinalis*,
- to study the difference in the retinal sensitivity of *S. officinalis* to two wavelengths of light.

3.2 Materials and Methods

3.2.1 Animals

Sepia officinalis used in this study were either hatched from eggs and reared in the laboratory, or caught in the wild during the summer and early autumn months. They ranged in ML from <1 cm to 24 cm. When in the laboratory, they were kept under a 12 h light:dark cycle (it was light from 06:30 to 18:30) and fed daily. The animals less than 1 cm in ML were removed from eggs, before the following procedure was undertaken.

On the day of an experiment, an animal was taken from its holding tank between 08:30 and 09:30, and dark adapted for 30 min. After which it was anaesthetised with 2% ethanol in seawater and then decapitated. The dissection was carried out in dim red light (approximately 15 $\mu W/cm^2$). Both eyes were excised, the anterior portion and the lens removed, and the sclera detached from the posterior half of the eye (the sclera could not be removed from the eyes of 1 cm ML animals, as it tore the retina). In initial experiments, the retina was cut into pieces approximately 1 cm^2, and one piece was then placed in a

perfusion dish. Midway through these experiments, Dr. David Becker (University College London, UK) suggested using slices of tissue instead of pieces. Slices allowed an ERG to be found faster, and with more reproducibility than in a piece, so in later experiments, slices of retina were used. Slices were obtained by putting a piece of retina onto a Millipore filter disc (Black-gridded, 25 mm diameter, Millipore Corp, USA), and then chopping it into 300 μm thick slices using a McIlwain tissue chopper (The Mickle Laboratory Engineering Co. Ltd., UK). No differences were found in the ERG recorded from slices and pieces of tissue. Tissue not used immediately was kept in artificial seawater (ASW: 470 mM NaCl, 10 mM Hepes, 55 mM $MgCl_2$, 10 mM $CaCl_2$, 10 mM KCl and 10 mM Glucose; pH= 7.7, osmolality= 1000 mmol/kg) on ice until needed, for a maximum of 36 h. Experiments continued until 16:30-17:00 on any day an animal was killed, so measurements of sensitivity were always made when the animal was in the light phase of its circadian rhythm.

3.2.2 Electrophysiological set-up

The tissue was placed in a perfusion dish. Slices were held in position by a tissue anchor, and pieces of retina were pinned into a Sylgard (Farnell, UK) lined dish. They were perfused with chilled (18 – 20°C), oxygenated (95% O_2/ 5% CO_2) ASW. Stimulating light came from underneath the tissue, from a light emitting diode (LED; RadioSpares, UK). A D4030 pulse generator (Digitimer, UK) controlled the timing of stimulus flashes. The intensity was manipulated using neutral density (ND) filters (grey plastic purchased from StageElectrics, UK), which allowed a light intensity range of 0.05 to 154 $\mu W/cm^2$ to be tested.

An infra-red light, used with a video camera and monitor, allowed the electrode to be positioned correctly, without stimulating the photoreceptors. The ERG was recorded as the difference in potential between a low resistance electrode (1-2 MΩ) placed in the tissue

and an indifferent silver/silver chloride reference electrode that was in the perfusion dish. The signal was amplified using a BA-1S (npi Electronic GmbH, Germany), converted from analogue to digital using a 1401 (Cambridge Electronic Design, UK), and stored and analysed using the computer program Signal (Version 2.03, Cambridge Electronic Design, UK). Light intensities were measured using an optical power meter (TQ8210, Advantest, Japan) either directly before or after experiments were undertaken. The recording set-up was in a Faraday cage to reduce external, electrical noise from the ERG recordings and all of this equipment was in a dark room, which remained dark for the duration of the experiments.

3.2.3 The basic protocol used to measure the sensitivity of the retina

Once the electrode was placed in the tissue, the preparation was stimulated with a 10 ms flash of 95 $\mu W/cm^2$ yellow light (LED with peak emission of 590 nm, RadioSpares, UK). This stimulus was repeated once every five minutes, for a total of thirty minutes to check that the response amplitude of the evoked ERG had plateaued to a stable level (Clark & Duncan, 1978). A light intensity series protocol was then run, beginning with the lowest light intensity, keeping flash duration and interval constant (Table 3.1). Flashes of 10, 50, or 500 ms duration, and separated by 30, and 60 s intervals were applied to the tissue. Protocols, consisting of one flash duration with one flash interval, were interchanged, and separated by 300 s, and the number of different protocols delivered to a piece of tissue depended on how well the tissue recovered.

Light Intensity ($\mu W/cm^2$)	Yellow LED	Blue LED
	1.1	-
	2.0	-
	4.3	-
	8.0	0.05
	22.7	1.8
	30.7	3.5
	44.7	13.1
	95.3	53.3
	128.7	69.2
Maximum	154	104

Table 3.1: The light intensity ranges that both of the light emitting diodes (LED) passed with the neutral density filters used.

Sepia officinalis of different ML were tested using the above protocol to ascertain if ML influenced their sensitivity. The yellow LED was again used as the stimulus. This was carried out on tissue from animals from 5 different ML groups, <1, 2, 4, 12, and 23 cm (Table 3.2).

Group Name	Mean ML (cm)	se (cm)	N
1 cm	0.90	0.004	7*
2 cm	2.39	0.03	6
4 cm	4.36	0.12	6
12 cm	11.54	0.96	6
23 cm	23.00	0.37	5

Table 3.2: The mean mantle length (ML) and standard error (se) for the groups used for this experiment. * = only the results from 6 of these animals were used in the statistical analysis.

To investigate the agreement between the sensitivity measured using the ERG, and that predicted from the absorption spectrum of *S. officinalis* rhodopsin, a comparison of the ERG evoked when stimulated using the yellow LED and a blue LED (peak emission 500 nm; RadioSpares, UK) was made. This was done on retinal slices from animals of three ML groups: 1 cm, 7 cm and 22 cm. The basic protocol was modified as follows. The seven highest light intensities were used in each intensity run (Table 3.1). One LED was chosen at random, tested with one of the flash protocols, and then switched for the second LED. A gap of 300 s was left between stimulating with one LED before moving onto the next one. Once both LED's had been tested with one of the flash protocols, the sequence of LED's was repeated with the next flash protocol.

3.2.4 Analysis

When analysing the ERG's obtained at the various light intensities, Signal (version 2.03; CED, UK) was used to measure the response amplitude, latency and duration. SigmaPlot 2001 for Windows (version 7.0; SPSS Inc, USA) was used to draw graphs of these parameters. In all graphs, the error bars represented the standard error (se).

For the purpose of this chapter, Autrum's (1981) definition of absolute sensitivity (Section 3.1) was modified to be the reciprocal of the intensity that gave a 0.4 mV amplitude response (also following Tasaki *et al.*, 1963c). The reasons for choosing this value were that the maximum response amplitude was rarely reached, so 50% of it was inappropriate, and secondly that the ERG amplitude reached a value of 0.4 mV in the majority of the experiments undertaken.

All trends were compared using V/log I curves. In preliminary analysis (not shown), the flash interval was not found to affect the sensitivity of the tissue. To avoid the problem of repeated measures in statistical tests, only the flash protocol of 50 ms flashes every 60 s was used when investigating the effects of ML and stimulus wavelength. When

studying the effect of flash duration, the three flash durations separated by 60 s intervals were used in a repeated measures Analysis of Variance (ANOVA). To ensure that there were no unusual trends in the other flash protocols, the V/log I curves of each one were checked, but were not presented in the results section. SPSS (Version 11.0.1; SPSS Inc, USA) was used for statistical tests. Homogeneity of variance was always checked, using Levene's test, before a parametric ANOVA was carried out on the data.

To determine if the ML of an animal affected the shape of its ERG, statistical analysis was performed on the last test flash (i.e. 10 ms of 95 $\mu W/cm^2$ yellow light) given before a light intensity series was performed. Five different parameters, indicative of ERG shape, were measured using the computer programme, Signal. These were a) amplitude (maximum height the response reached), b) latency (the time between the start of the stimulus and the start of the response), c) duration (the time for which the response lasted), d) time to peak (the time from when the response started until it reached its maximum amplitude), and e) decay constant (the rate at which the response returned from its maximum value to zero), and they were compared using a one-way ANOVA.

3.3 Results

3.3.1 The effect of the mantle length of Sepia officinalis *on its sensitivity*

To ascertain if the ML of *S. officinalis* influenced its absolute sensitivity, the isolated retina was flashed with different intensities of light, and the recorded ERG's analysed. There was no significant difference between the absolute sensitivity of different sized animals using 50 ms flashes every 60 s (Table 3.3 and Figure 3.1).

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	0.001	4	0.000	2.510	0.070
Within Groups	0.002	23	0.000		
Total	0.003	27			

Table 3.3: The results of a univariate ANOVA to determine if mantle length had a significant effect on absolute sensitivity.

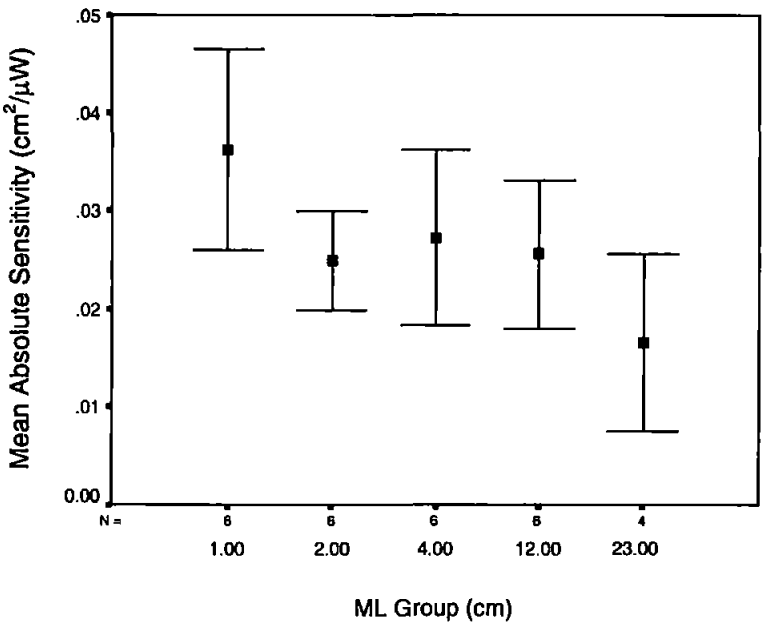


Figure 3.1: The mean (± 1 se) absolute sensitivities of the different mantle length (ML) groups tested (n for each group given in graph).

To gain an overview of the effect of ML on the response amplitude of the ERG to all light intensities tested, the results from the different flash protocols were drawn on V/log I axes (Figure 3.2). With all of the stimulating protocols used, the 1 cm animals always had the largest response amplitude of ERG's, and the 22 cm group always had the smallest. The three medium groups showed some degree of overlap, and the pattern they followed was not very clear. None of the graphs reached a plateau, which indicated that

the saturation intensity of these photoreceptors was not reached under the conditions used in these experiments.

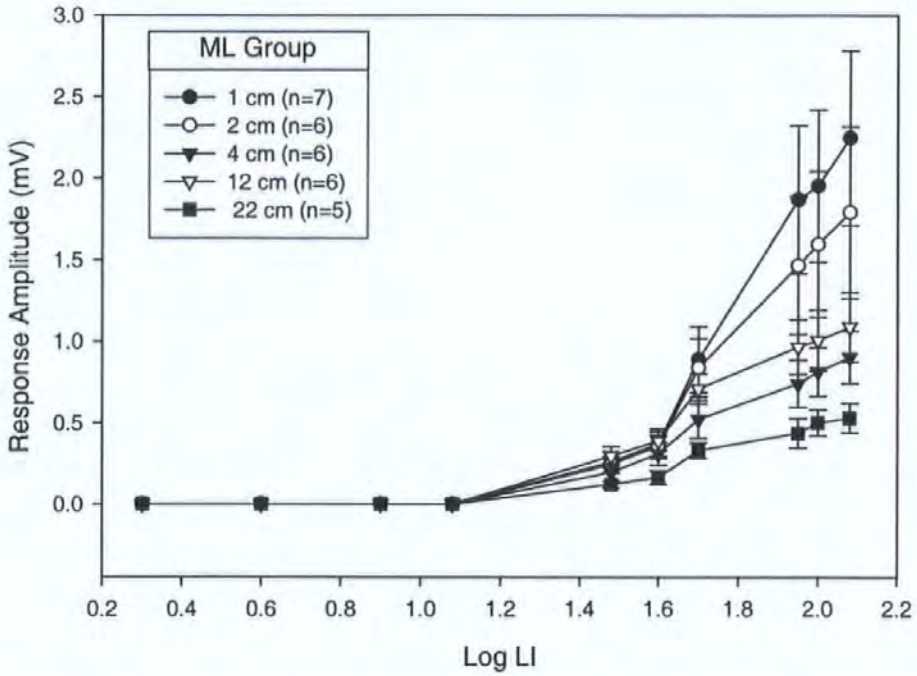


Figure 3.2: The V/Log I curves of the mantle length (ML) groups tested. The flash interval was 60 s, and flash duration was 50 ms, but the light intensity (LI) increased (error bars = +/- 1 se).

To examine the V/log I curves of Figure 3.2 in further detail, linear regressions were fitted to the mean response amplitudes of each ML group to the five highest light intensities used, i.e. to the straight-line section of the curves, as was commonly done in previously published papers (e.g. Ziedins & Meyer-Rochow, 1990). Overall, as the animals increased in ML the slope of best fit line decreased (Table 3.4). This implied that smaller animals had better contrast sensitivity than larger animals because a steeper slope means a greater increment threshold (Eguchi and Horikoshi, 1984), i.e. the same change in intensity will produce a larger response.

ML (cm)	Slope	r^2	Sig.
1	3.86	0.99	<0.01
2	2.87	0.99	<0.01
4	1.15	0.98	<0.01
12	1.34	0.94	<0.05
22	0.7	0.94	<0.05

Table 3.4: The slope of the best fit lines to the last five intensities shown in Figure 3.2 (df=3).

3.3.2 The change in ERG shape with the increasing mantle length of Sepia officinalis

Typical ERG responses from three of the ML groups tested in this study were presented in Figure 3.3.

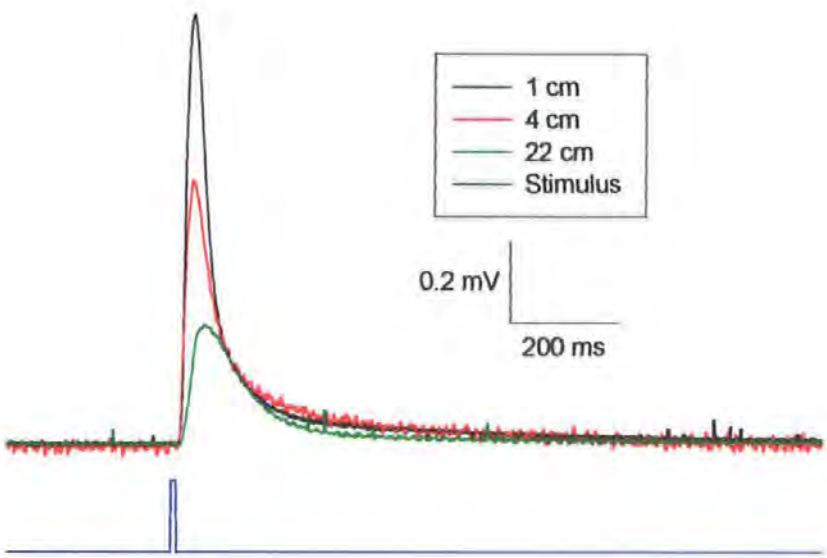


Figure 3.3: The mean ERG shape of three sized animals. The curves were the average of six animals. The light intensity of the stimulus used was 95 μW/cm^2 from an LED with peak wavelength emission of 590 nm, and this was the same for all three ML sizes.

Five parameters, indicative of ERG shape, were measured to investigate if the ERG shape changed with the ML of the animal. They were the latency, duration, maximum amplitude, time to peak and decay constant of the response. Each of these parameters were graphed against increasing ML using scatter plots to reveal any trends (Figure 3.4), and tested for

significant differences using a one-way ANOVA (Table 3.5). The response duration did not show homogeneity of variance, even after transformations, and so could not be tested with a parametric test. The amplitude data were log transformed to ensure homogeneity of the data. No significant trends were measured in any of these four ERG shape parameters with increasing ML (Table 3.5).

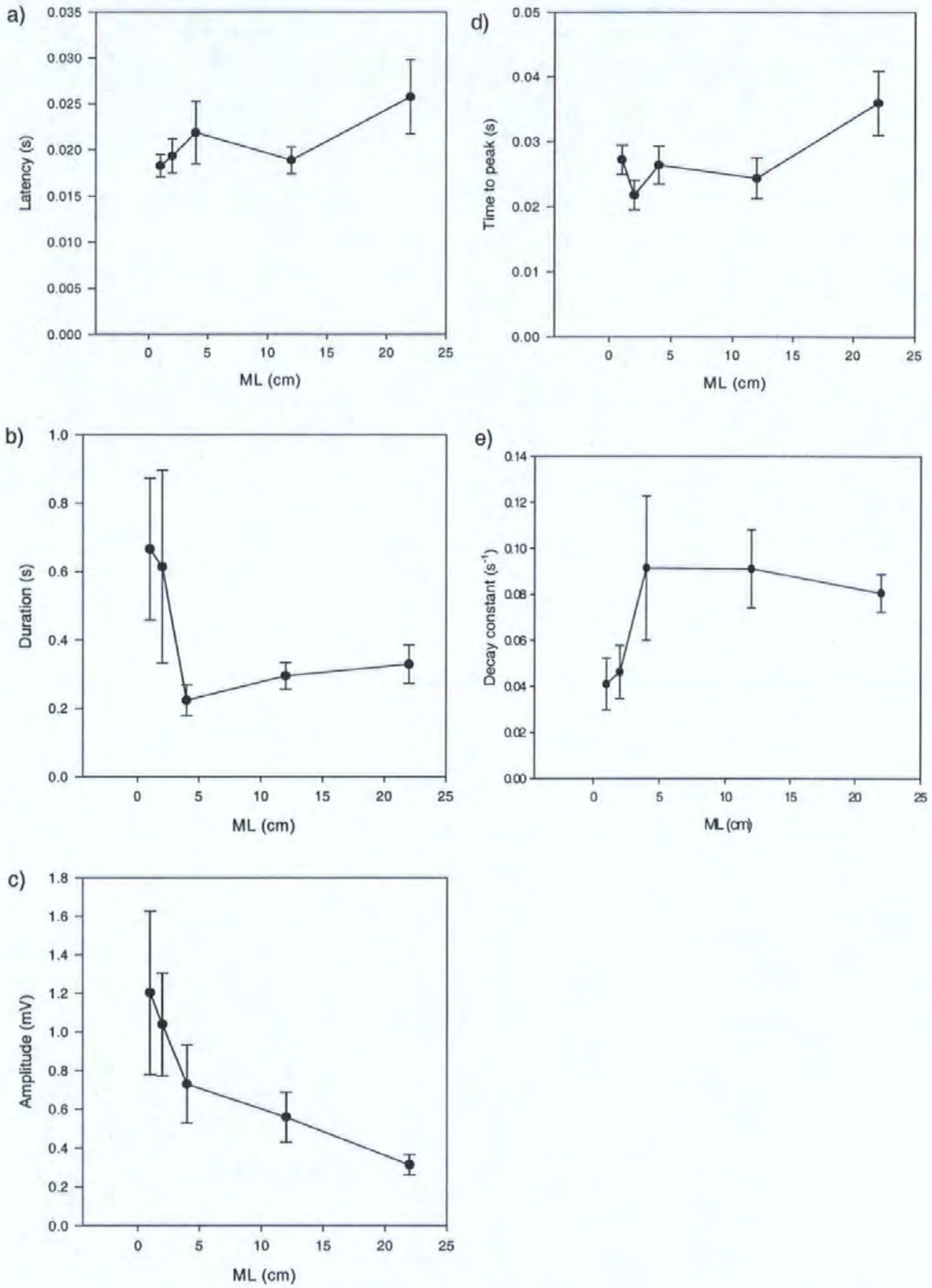


Figure 3.4: Scatterplots to investigate the change in shape parameters of the ERG with increasing ML of *S. officinalis*; a) response latency, b) response duration, c) maximum amplitude, d) time to peak, and e) decay constant (error bars= +/- 1 se).

		Sum of Squares	df	Mean Square	F	Sig.
LATENCY	Between Groups	0.000	4	0.000	1.348	0.283
	Within Groups	0.001	23	0.000		
	Total	0.001	27			
LOG	Between Groups	0.679	4	0.170	1.623	0.202
AMPLITUDE	Within Groups	2.405	23	0.105		
	Total	3.084	27			
TIME TO	Between Groups	0.001	4	0.000	2.651	0.059
PEAK	Within Groups	0.001	23	0.000		
	Total	0.002	27			
DECAY	Between Groups	0.014	4	0.004	1.760	0.171
CONSTANT	Within Groups	0.047	23	0.002		
	Total	0.061	27			

Table 3.5: The results of an ANOVA completed on the ERG shape parameters recorded from animals of five different mantle lengths.

The statistical results presented in Table 3.5 did not seem to tie in well with the graphical representation of the data (Figure 3.3 and Figure 3.4). When the data were examined more closely, two outliers were found. If these, and the 2 and 12 cm animals were dropped from the ANOVA, a statistically significant difference was found in the logarithm of the response amplitude (Table 3.6). None of the other four ERG shape parameters showed as large a change in their significance.

		Sum of Squares	df	Mean Square	F	Sig.
LATENCY	Between Groups	0.000	2	0.000	1.802	0.210
	Within Groups	0.000	11	0.000		
	Total	0.001	13			
LOG AMPLITUDE	Between Groups	0.786	2	0.393	6.704	**0.012**
	Within Groups	0.654	11	0.059		
	Total	1.431	13			
TIME TO PEAK	Between Groups	0.000	2	0.000	1.784	0.213
	Within Groups	0.001	11	0.000		
	Total	0.001	13			
DECAY CONSTANT	Between Groups	0.008	2	0.004	1.429	0.281
	Within Groups	0.033	11	0.003		
	Total	0.041	13			

Table 3.6: The results of a one-way ANOVA carried out on the means of four parameters related to ERG shape recorded from animals of three different mantle lengths.

3.3.3 The effect of varying flash duration on the sensitivity of Sepia officinalis

A one-way repeated measures ANOVA was conducted to compare the absolute sensitivity to three different flash durations. There was a significant effect for flash duration on the absolute sensitivity of *S. officinalis* (Wilks' Lambda= 0.14, $F_{(2,4)}$= 9.55, p= 0.05, multivariate eta squared= 0.86). The means and standard deviations are presented in Table 3.7. The absolute sensitivity of the retina increased, as the duration of the stimulus increased (Table 3.7).

Flash duration (ms)	Mean absolute sensitivity ($cm^2/\mu W$)	sd	n
10	0.022	0.008	5
50	0.028	0.012	5
500	0.034	0.010	5

Table 3.7 : The mean absolute sensitivities and standard deviations (sd) for 4 cm animals measured using different flash durations.

To examine more thoroughly the changes in response amplitude with flash duration, the data were plotted onto V/log I axes (Figure 3.5). The curves rarely reached a plateau, which showed that the photoreceptors seldom reached their saturation limit. As the flash duration increased, the slopes of the straight line section of the V/log I curves increased from 1.89 with 10 ms flashes (r^2=0.988, p<0.01) to 2.50 with 500 ms flashes (r^2=0.978, p<0.01), but decreased to 1.39 with 50 ms flashes. This change in slope of the V/log I curve with an increase in flash duration was not significant (One-Way ANOVA: $F_{(2,16)}$=2.11, p>0.05).

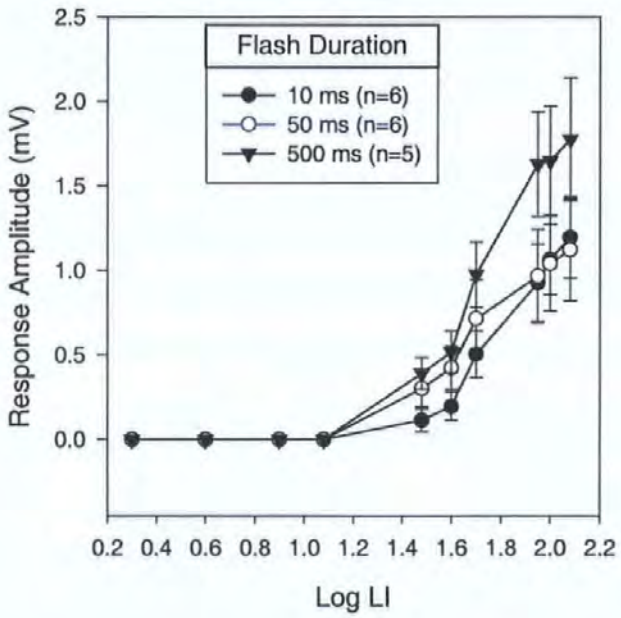


Figure 3.5: The V/Log I curves of 4 cm animals, obtained using different stimulus flash durations, but all with flash intervals of 60 s (error bars = +/- 1 se; LI = light intensity).

3.3.4 The effect of the stimulus wavelength on the sensitivity of Sepia officinalis

The retinal sensitivities of five medium sized animals (mean ± sd: ML = 6.9 ± 1.5 cm) were tested using a yellow and a blue LED. The absolute sensitivity of *S. officinalis* was significantly greater for the blue, than the yellow light (paired t-test, t=3.13, df=4, p<0.05) (Figure 3.6).

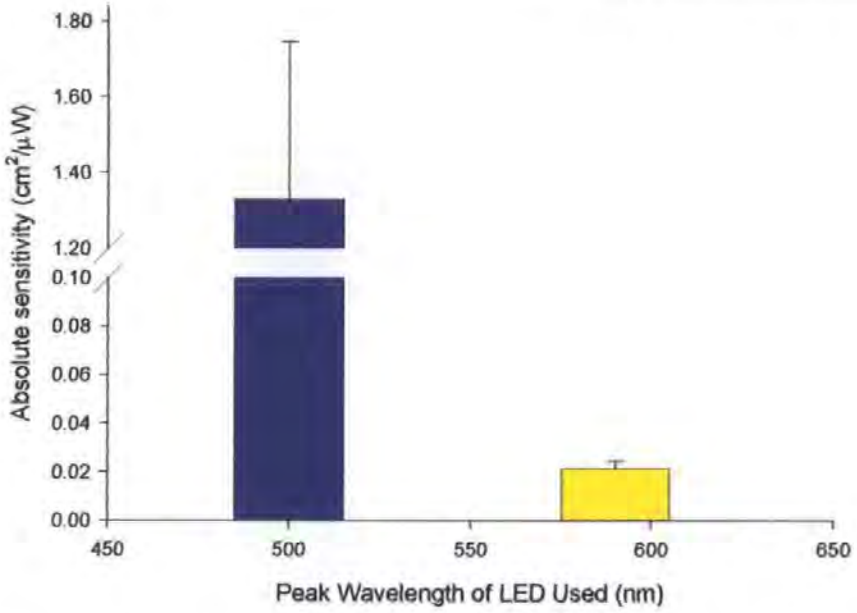


Figure 3.6: The absolute sensitivities of the retina of *Sepia officinalis* to two wavelengths of light tested with 50 ms flashes every 60 s (n= 5 for each bar, error bars = +/- 1 se).

The retina of *S. officinalis* was 100 times more sensitive to the blue wavelengths of light than to the yellow. To investigate how this increased sensitivity compared with the emission spectra of the LED's with the absorbance spectra of rhodopsin, the three spectra were plotted on the same axes and the areas they covered compared (Figure 3.7). Although this figure portrayed a simplified version of the data, it showed that all the wavelengths emitted by the blue LED would be absorbed by *S. officinalis* rhodopsin. The curve of the yellow LED, however, only partially overlapped with the rhodopsin curve. As the output from the LED's differed (Table 3.1), a direct numerical comparison between the areas beneath these curves could not be made.

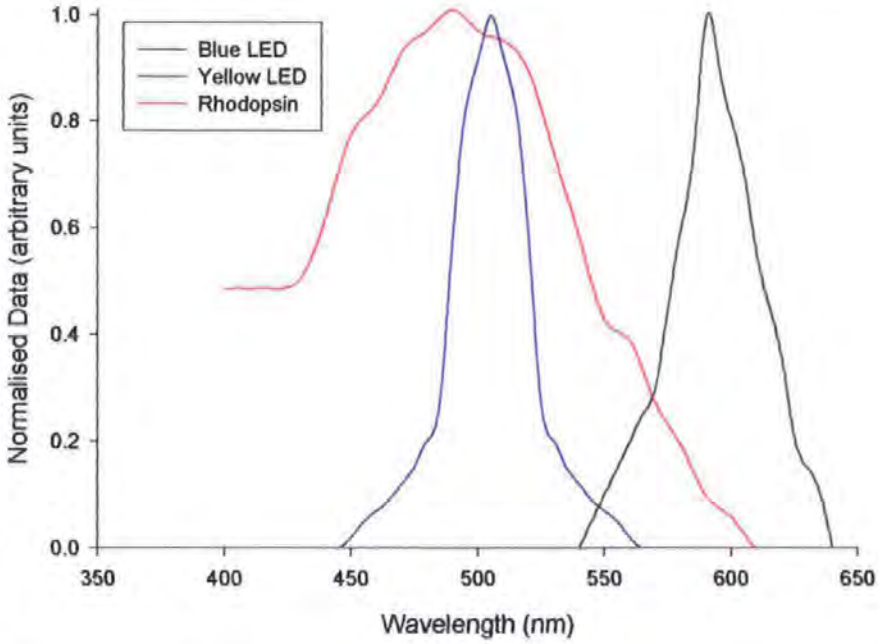


Figure 3.7: The emission spectra of the LED's used in this experiment with the absorption spectrum of *S. officinalis* rhodopsin. The data for the LED's were taken from manufacturer's technical information and the rhodopsin curve from Brown and Brown (1958). The data for each spectrum was normalised before plotting it.

The response to the two stimulus wavelengths were plotted on V/log I curves (Figure 3.8) and again the difference in sensitivity could be seen. The reason for the different starting intensities of the LED's was that their maximum intensities were different (Table 3.1). From the graphs, it was clear that the retina of *S. officinalis* reached its saturation level with blue light, but the response amplitude was still increasing rapidly with yellow light. Over the range of light intensities tested, the V/log I curve obtained using a yellow wavelength stimulus had the steeper slope, which implied that the retina of *S. officinalis* had better contrast sensitivity when stimulated with this light. The retina of *S. officinalis* has less contrast sensitivity, but better absolute sensitivity when tested with blue wavelengths of light.

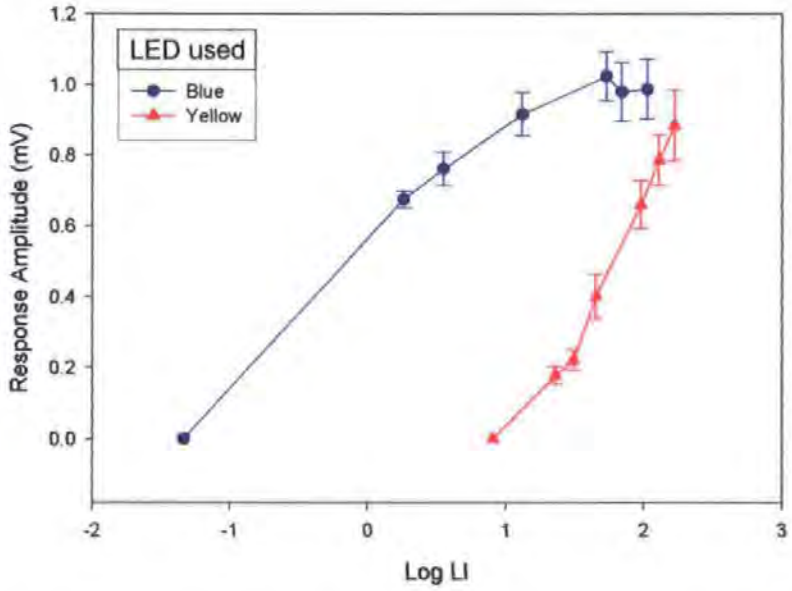


Figure 3.8: The V/log I curves obtained using two stimuli of differing wavelengths to illuminate the retina of *S. officinalis* (n = 5 for each point, error bars = +/- 1 se).

The latencies of the responses elicited with the yellow stimulus were always larger than for the blue, but the response durations were generally shorter (Figure 3.9). The error bars for the response duration data (Figure 3.9b) were very much larger than the response latency (Figure 3.9a) and the response amplitude (Figure 3.8). This simply reflected that the response duration was much more variable than the response latency and amplitude.

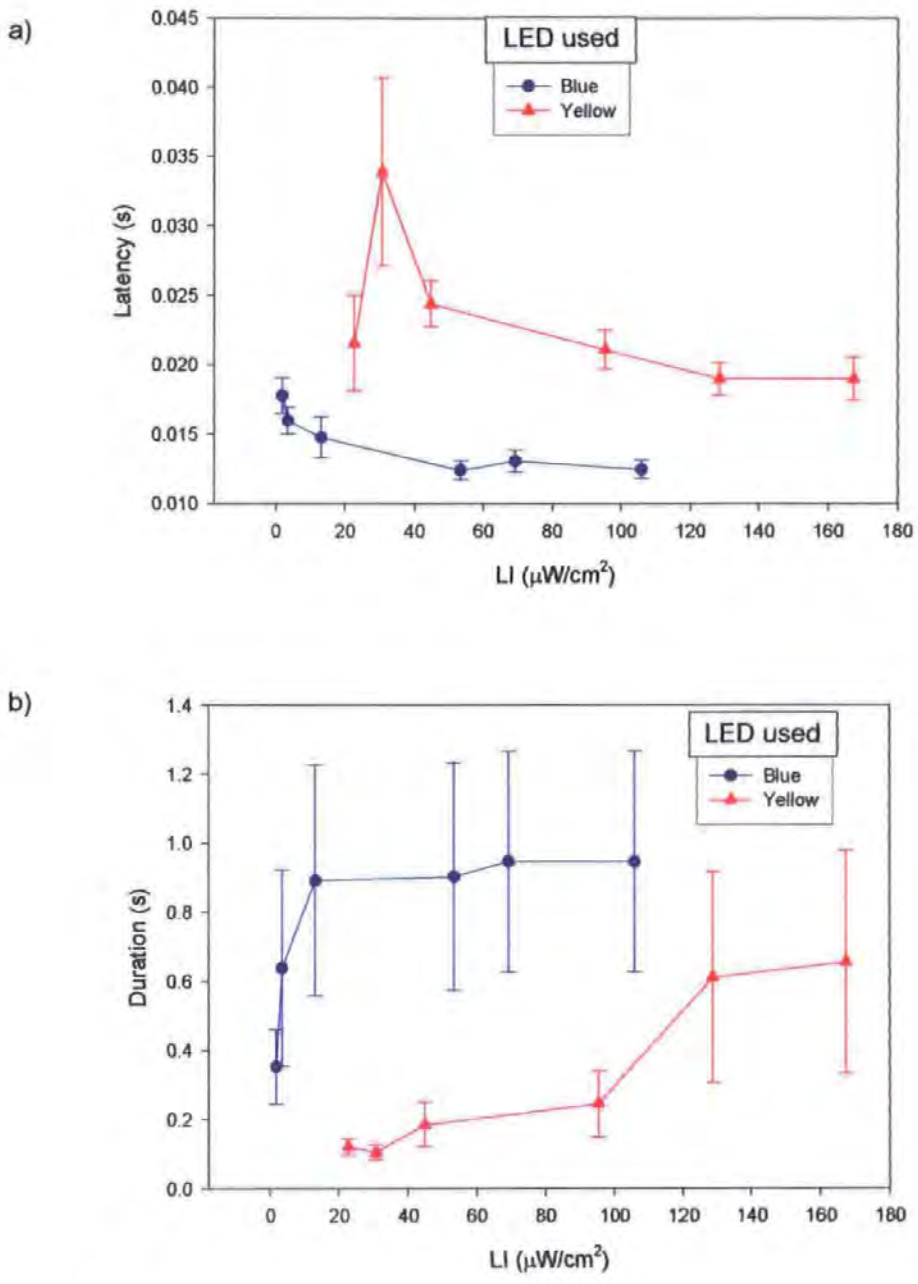


Figure 3.9: The kinetics of the ERG of *S. officinalis* when stimulated with two wavelengths: a) the latencies of the responses to increasing light intensity of two different wavelength stimuli; b) the durations of the same responses plotted in a). (n=5 for each point, error bars = +/- 1 se).

3.3.4.1 The change in sensitivity of three mantle length groups of *Sepia officinalis* to two stimuli of different wavelengths

Three ML groups of *S. officinalis* were tested using the two LED's and their absolute sensitivities measured (Figure 3.10). The trend of decreasing sensitivity with size was the same as that outlined in section 3.3.1 when using the yellow LED, but the trend when stimulation was with blue wavelengths of light was unexpected. The increase in sensitivity to blue light with 7 cm animals was unusual.

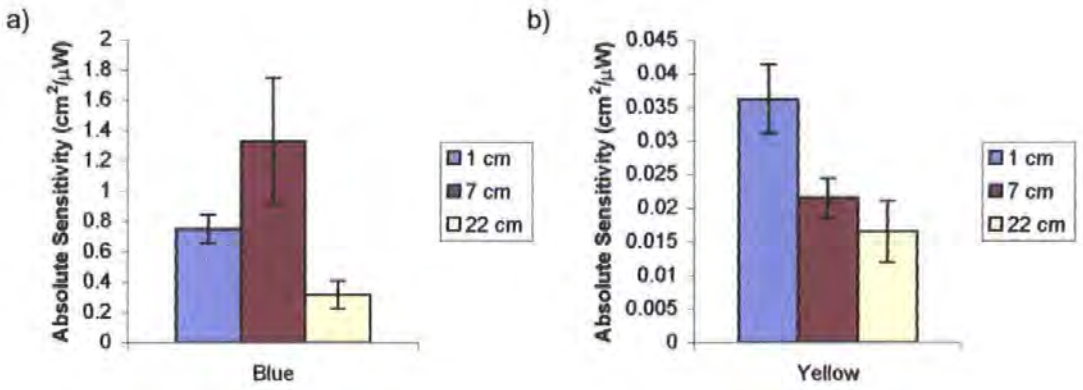


Figure 3.10: The change in absolute sensitivity of *S. officinalis* in three size groups to two different wavelengths (a=Blue LED; b=Yellow LED) (n=5 for each point, error bars= +/- 1 se).

The ML of *S. officinalis* significantly affected its absolute retinal sensitivity to the two wavelengths of stimulus used (Table 3.8). The data obtained using the blue LED were log transformed to ensure homogeneity of the data, but no transformation was necessary for the data obtained using the yellow LED.

		Sum of Squares	df	Mean Square	F	Sig.
LOG BLUE	Between Groups	0.991	2	0.496	6.839	0.010
	Within Groups	0.869	12	0.072		
	Total	1.860	14			
YELLOW	Between Groups	0.001	2	0.001	5.403	0.021
	Within Groups	0.001	12	0.000		
	Total	0.002	14			

Table 3.8: Results of a one-way ANOVA to show the effect of two wavelengths on the absolute sensitivity of different sized animals. The data obtained using the blue stimulus was log transformed to ensure its homogeneity.

Student-Newman-Keuls tests were performed on these data to determine which of the mean absolute sensitivities were significantly different from the others (Table 3.9). One important fact to draw from Table 3.9a is that the absolute sensitivity of the 7 cm animals to blue light was not significantly different to that of the 1 cm animals. The 1 cm animals were more sensitive to both stimulus wavelengths than the 22 cm animals, but the 7 cm animals did not always group with one or the other. To summarise Table 3.9 and Figure 3.10, 1 cm animals, which were pre-hatchlings, were more sensitive than 22 cm adult animals, when tested with both yellow and blue light.

a)

ML	N	Subset for alpha = 0.05	
		1	2
22.00	4	-0.6018	
1.00	6		-0.1457
7.00	5		0.0560
Sig.		1.000	0.265

b)

ML	N	Subset for alpha = 0.05	
		1	2
22.00	4	0.0165	
7.00	5	0.0215	
1.00	6		0.0363
Sig.		0.454	1.000

Table 3.9: Student-Newman-Keuls tests to show the means for homogenous ML groups in their absolute sensitivity to two different LED's, a) blue LED (data log transformed), b) yellow LED.

3.4 Discussion

This study measured the sensitivity of the excised retina of *Sepia officinalis*, and how it changed with three variables, namely ML, stimulus wavelength and stimulus duration. There was no significant change in absolute sensitivity with ML (Table 3.3). The only variable of ERG shape to change significantly with ML was response amplitude (Table 3.5), larger animals reached smaller maximum amplitudes than the smaller animals (Figure 3.4). The photoreceptors of *S. officinalis* proved to be more sensitive to blue light (500 nm) than yellow light (590 nm) (Figure 3.6), which agreed qualitatively with the published absorption spectrum of their rhodopsin (Brown & Brown, 1958). Increasing the flash duration increased the absolute sensitivity (Table 3.7) and had a slight impact on the slope of the V/log I curve (Figure 3.5).

3.4.1 The sensitivity of Sepia officinalis *in relation to the light intensity of its habitat and in relation to the sensitivity of other animals*

The levels of blue light to which *S. officinalis* were shown to be sensitive (i.e. its absolute threshold = 0.1 $\mu W/cm^2$, Figure 3.8) agreed approximately with the minimum light intensities of the waters in which they live (Clarke & Denton, 1962). Absolute comparisons between many different animals could not be made due to the number of different light intensity units used in the literature, but the sensitivity of *Eledone cirrhosa* (the lesser octopus) was measured using the same units. Cobb & Williamson (1998a) measured the absolute threshold of the epistellar body of *E. cirrhosa* to be <1 $\mu W/cm^2$. The agreement between these two numbers matched previous studies, as Mauro & Baumann (1968) showed that the sensitivity of the extraocular photoreceptors of *Eledone moschata* was comparable to that the sensitivity of the retinal photoreceptors of honeybee

drones. *Sepia officinalis* proved to be less sensitive than *Pagothenia borchgrevinki* (an Antarctic notothenиid fish), whose absolute threshold was 2 x 10^{-4} $\mu W/cm^2$ (Morita *et al.*, 1997). This was expected as *P. borchgrevinki* is found at much deeper depths in the ocean than *S. officinalis*. The absolute threshold of *S. officinalis* matched that of *Strombus luhuanus* (gastropod), which also has an absolute threshold of 0.1 $\mu W/cm^2$ (Gillary, 1974). The sensitivity of the retinal photoreceptors, therefore, did not prove to be very different from that of other molluscs, although it was possible that *S. officinalis* could respond to light intensities lower than this. Although every attempt was made to reduce the background light intensity affecting the preparation, there was still a tiny amount present (approximately 0.1 $\mu W/cm^2$), so the preparation was never fully dark-adapted. Photoreceptors must be fully dark-adapted to respond to very low light intensity stimulation (Autrum, 1981).

The dynamic ranges of various species were compared to that of *S. officinalis*, as these are measured in log units, and so do not require as precise measurements of light intensity as do absolute thresholds. The dynamic range of an eye is defined as the range of light intensities over which response amplitude changes, i.e. the straight line section of a V/log I curve (Autrum, 1981). The eyes of other species have dynamic ranges varying from 2.4 to 7 log units (Yinon & Auerbach, 1969; Eguchi & Horikoshi, 1984). *Sepia officinalis* fell towards the lower end of this range, as even with the blue LED, their range measured 2 log units (Figure 3.8). For the yellow wavelength stimulus, the dynamic range was only 1 log unit. It must be remembered that the V/log I curve for the yellow LED never reached a plateau, so the dynamic range was probably larger. Also with both wavelengths, it was likely that *S. officinalis* might have responded to light intensities lower than those that were tested. These adjustments to the measured dynamic ranges would be unlikely to extend them much beyond 3 log units, so it would appear that the dynamic range of *S. officinalis* was less than that of most animals. This short range is likely to be

due to their habitat and lifestyle. They remain in deep waters (down to 150 m) for most of the year (Boletzky, 1983). During these times, they are unlikely to encounter large changes in ambient light intensities. It is during the summer months, when they come inshore to breed, that they are most likely to encounter larger diurnal variations in the ambient light. Weeks & Duncan (1974) found that there were large seasonal differences in the sensitivity of *Sepiola atlantica*. It is likely that the range measured here is enough for *S. officinalis* to cope with the light intensities they normally encounter, when pupillary movements, screening pigment movements, changes in cell length and seasonal variations are also considered.

Sepia officinalis proved to be far more sensitive to blue wavelengths of light than to yellow wavelengths, as determined using the physiological measurement of the ERG (Figure 3.6). This agreed qualitatively with the absorption spectrum of rhodopsin published by Brown and Brown (1958) for this species, the peak of which was at 492 nm. Not only were there large differences in the absolute sensitivities to the two wavelengths tested (Figure 3.6), but there were also large differences in the maximum response amplitudes (Figure 3.8), and the latencies and durations (Figure 3.9). The responses to yellow light were slower and shorter than for the blue light. This indicated that yellow (590 nm) light was not as efficient in stimulating the rhodopsin of *S. officinalis* as blue (500 nm) light. The slope of the V/log I curve plotted using data from the blue LED in Figure 3.8 was shallower than the yellow one. This implied that *S. officinalis* had less contrast sensitivity in blue light, but better absolute sensitivity, than in yellow light over the light intensity range tested, although this difference in contrast sensitivity may have relied on the intensities tested. If a larger range of intensities were tested, different V/log I curves and their respective slopes, and hence contrast sensitivities, might have been seen. This difference in sensitivity did not constitute colour vision, as it was highly unlikely that the cephalopod retina and visual processing system would be able to distinguish between a low intensity blue flash and a high intensity yellow flash. It was expected that *S. officinalis*

would be more sensitive to blue wavelength light, because water is most transparent to the wavelengths of 470 – 490 nm (Lythgoe, 1985). Many marine invertebrate species have their peak sensitivity, like *S. officinalis*, close to this range (Cronin, 1986).

3.4.2 The effect of increasing mantle length on the sensitivity of Sepia officinalis

Mantle length had an almost significant effect on the absolute sensitivity of *S. officinalis*, when 1, 2, 4, 12, and 22 cm animals were tested (Table 3.3), and it had a significant effect when 1, 7 and 22 cm animals were compared (Table 3.9). In both these cases, larger animals were less sensitive than smaller ones (Figure 3.1, Figure 3.10). This was unexpected, as cephalopod photoreceptors increase in length, as the ML of an animal increases (S. Farley, unpublished results). An increase in the volume of a photoreceptor is thought to increase its chance at catching a photon and thereby improving the eye's visual sensitivity (Land, 1981; Van der Meer, 1994; Nilsson & Warrant, 2001). Ziedins & Meyer-Rochow (1990) found that the sensitivity of *Petrolisthes elongatus* (a half crab) did not change with age. A study on the changes in morphology of the eye of *P. elongatus* was published in the same year (Meyer-Rochow *et al.*, 1990) and it implied that the sensitivity of its eye probably did change with age. It is unknown why the physiology did not match the morphology in both of these cases. Changes in visual sensitivity with increasing body size in other animals can be due to other factors, e.g. in *Carassius auratus* (goldfish) the visual sensitivity increases with increasing body size, but this is thought to be due to the increase in density of rods in its retina (Powers *et al.*, 1988). The photoreceptor density within the cephalopod retina does not change as the animal grows (Packard, 1969).

Land (1981), in his review of invertebrate vision, gave the following equation for sensitivity, based on anatomical and histological features of the eye:

$$S = (\pi/4)^2 (A/f)^2 d^2(1-e^{-kx})$$

Where S= sensitivity, A= aperture of the eye, f= principal focal length, d= receptor diameter, k= extinction coefficient of the photopigment, and x= photoreceptor length.

Using already published data, Land calculated that the sensitivity of *Octopus* to be 4.23, taking a photoreceptor length of 200 μm. This would be similar to *S. officinalis* with the same sized eye. If cell length was the only variable to change and it increased by 150 μm, then the eye would increase in sensitivity to 5.15. Although this is not the situation that would arise in the wild, where focal length and aperture would also change with the size of the animal, it was quite similar to the situation that was investigated here, as only the excised retina was tested in experiments, and so only cell length could change. Therefore, according to Land's formula the sensitivity should have increased, which was not found here. The likely reason for the difference between these two findings was that the physiology of the system was measured here, which included some characteristics of the retina that were not considered in Land's formula. Land's (1981) formula predominantly measured morphological features, but both these and physiological features are important to the intact animal in its natural environment.

Although by increasing the length of a photoreceptor, more rhodopsin is added to it, increasing its photon catching capability, an increase in cell length also affects the resistance of the cell. This increase in cell length equates to adding resistors in parallel, which actually reduces the resistance of the system (Johnston & Wu, 1995). So a longer cell has a lower resistance. Ohm's Law states that V= IR, (where V= voltage, I= current and R= resistance) so if I remained constant, then a decrease in resistance would decrease the voltage recorded, which was seen (Figure 3.4c). In an unpublished study Sophia Farley (University of Plymouth, UK) found that cephalopod photoreceptor length increases with increasing ML but she did not fully described the relationship between these two variables. It, therefore, remains unknown how well the increase in cell length is correlated with the decrease in sensitivity. This change in cell resistance gives one possible reason why the physiology results presented here did not match previous theoretical ones.

A second possible physiological factor that could affect the sensitivity of an animal is dark noise. Dark noise can be defined as the spontaneous activity present within a photoreceptor in the complete absence of light (Laughlin & Lillywhite, 1982), and it is present in every photoreceptor to some degree. Baylor *et al.* (1980) showed that it was linearly related to cell length in *Bufo marinus* (the tropical toad), and due to the basic nature of dark noise (Barlow *et al.*, 1993), this relationship is likely to be similar in most animals. The cephalopod eye might be at its optimal sensitivity when it is smaller, as then the gain in photon capture by its photoreceptor is balanced well with the dark noise such a photoreceptor length generates. As an animal grows, and hence it photoreceptors grows, the noise level increases, thereby decreasing its sensitivity. But as the animal is bigger, it can protect itself, and it eats larger food items, and so has no need to improve its sensitivity. It could be that the longer photoreceptors in the eye of *S. officinalis* are a by-product of the growth of the animal, and although they are of no benefit to the animal, there is also no cost in keeping them. Again, it is unknown how well the pattern of changes in dark noise with increasing ML correlates with that of sensitivity. Both the resistance of the cells and the presence of dark noise illustrated possible reasons why the sensitivity as determined by the ERG did not match the theoretically predicted change.

There are other possible reasons, which might act together to reduce the sensitivity with increasing ML. Although no detailed study has been published on the morphological changes in the cephalopod retina with animal size, there is anecdotal evidence to suggest that the basement membrane changes in structure (Nelson, 2003). This would alter the resistance of the system, possibly causing the ERG to change in amplitude. Another possible reason is linked to a discovery by Chrachri & Williamson (2000). They found that the one neurotransmitter has opposite effects in the optic lobes of juvenile and adult cephalopods. It is possible that something similar happened in the retina of *S. officinalis*, where the same neurotransmitter was present, but in the smaller animals it increased the amplitude of the ERG and in the larger animals it decreased it. A final possibility is that

there are different ion channels in different sized animals, which would alter the current in the retina, thereby changing the ERG amplitude. It is known that some genes are translated at specific times during the development of every organism (Campbell, 1993); e.g. genes controlling gap junctions in the *Patella vulgata* embryo (de Laat *et al.*, 1980), and so smaller animals might possess ion channels that allow a larger current flow. All of these different possibilities would need to be studied individually to determine which ones are responsible for the changes in the sensitivity of the ERG of *S. officinalis*.

As none of the tested ML groups of *S. officinalis* reached a plateau in their V/log I curves (Figure 3.2), some questions about the differences in the sensitivity of these groups remained. There are two possibilities as to how these curves finish. One is that the different groups reach the same plateau levels but at different times, i.e. the slopes of the linear part would be different, indicating that younger animals would have better contrast sensitivity. The other alternative is that they increase at approximately similar rates, but reach different plateaus. This means that their contrast sensitivity would be the same, but larger animals would have a greater dynamic range. It is most likely that a compromise between these two extremes occurs, with the smaller *S. officinalis* having a steeper curve (better contrast sensitivity), and a higher end point, but with bigger animals having a larger dynamic range.

3.4.3 The effect of increasing mantle length on the shape of the ERG of Sepia officinalis

Previous work on insect species has shown a dramatic change in the ERG shape with pupation (e.g. Autrum, 1958), but one study that continued to record the ERG from adults noticed more subtle differences as the animals grew in size (Yinon & Auerbach, 1969). As there are ontogenetical changes in the morphology of the cephalopod retina (S. Farley, unpublished results), it was possible that its ERG shape would also change. As already mentioned, the retinal cell length of this species increases with increasing ML, and

these changes in length are likely to lead to changes in cell resistance and capacitance (Johnston & Wu, 1995). These parameters could have affected the ERG shape in many ways, but were only found to affect the response amplitude of the ERG. Another factor that might change, but that had never been investigated is that of the intercellular connections in the plexiform layer. Cohen (1973b) and Yamamoto (1984) have shown that the photoreceptors are connected via gap junctions and desmosomes in this layer. It is possible that these change as the animal grows, but that they do not affect the ERG shape significantly.

3.4.4 The effect of changing stimulus flash duration on the retinal sensitivity of Sepia officinalis

The absolute sensitivity of *S. officinalis* was influenced significantly by the stimulus duration (section 3.3.3). The reason for the increase in sensitivity with increasing flash duration might be because the retina received more photons in a 500 ms flash than it did in a 10 ms flash. This, however, cannot be the entire story, as at any given point in time the tissue could not have determined if it was to receive more light, and change its response accordingly. Part of the answer lies in the latency, or delay, of the response. As shown in Figure 3.4, the mean response latency to a flash was 20 ms, and the time to peak was approximately 25 ms. Using these values, the time to reach 0.4 mV amplitude would be approximately 40 ms. This delay in response to the stimulus could explain the difference between the 10 ms flash and the other two. A 10 ms stimulus would have finished before the response begun, so the tissue had already received all the light it would in that flash, and therefore it probably took higher light intensities for the response to reach a 0.4 mV amplitude. But the other two flash durations were longer than 40 ms, so the tissue was stimulated for the entire critical period before reaching the 0.4 mV amplitude, allowing this to happen at a lower light intensity. It is harder to explain the difference

between the 50 and 500 ms flash duration, but from Table 3.7, it was clear that there was a smaller difference in sensitivity between these two durations.

The slope of the V/log I curve increased with increasing stimulus duration, but not by a significant amount (Figure 3.5). The slope has been found previously to change with flash duration in vertebrates (Kleinschmidt & Dowling, 1975), but not to do so in invertebrates (Matic & Laughlin, 1981). This implies that cephalopods are more similar to invertebrates than to vertebrates in this respect. Matic & Laughlin (1981) suggested that the reason for the difference between vertebrates and invertebrates was because of differences in the timings at which the peak of the response is reached, and when feedback to switch off the phototransduction cascade is initiated. If the time to peak response is fast in relation to the switch off of the cascade, then the pulse duration should not affect the V/log I curve. This is probably the case in most invertebrates, including *S. officinalis*. However if the peak amplitude is not reached before the shut down mechanism is begun, then the pulse duration will have more of an effect on the V/log I curve as was found with vertebrate species.

3.4.5 Conclusions

The conclusions of this study were that *S. officinalis* showed a reduction in absolute sensitivity and in the maximum amplitude of the response to the light intensity used with an increase in ML. This trend was unexpected, but could be partially explained by a probable decreased resistance in the photoreceptor cells and an increase in dark noise due to the increase in photoreceptor length. This change in absolute sensitivity was found using two stimuli of different peak emission wavelengths, 500 nm and 590 nm. *Sepia officinalis* was 100 times more sensitive to blue light than to yellow light, which agreed qualitatively but not quantitatively with the biochemical results of Brown and Brown (1958). The stimulus duration affected the absolute sensitivity, which implied that in some way the timing of the *S. officinalis* phototransduction cascade and shut down mechanism was more similar to invertebrates than previously tested vertebrates.

Chapter 4 Light-Adaptation in the Retina of *Sepia officinalis* as Determined by Changes in the Electroretinogram

4.1 Introduction

Adaptation, in its simplest form, is a change in sensitivity due to a background level of stimulation (Laughlin, 1989). By altering the sensitivity of a system, adaptation allows the detection of changes over a much wider range (Jindrova, 1998). It is essential for the survival of animals that encounter a wide range of stimulus strengths. For example, if an animal's eye retained its night-time sensitivity during the day, it would not be able to function correctly, as the photoreceptors would be saturated at the higher light intensities. To prevent this from happening, adaptation occurs. It can occur at every level in a system, from neural networks to protein structures to macroscopic changes, such as pupil dilation (Laughlin, 1989).

In cephalopods, adaptation of the visual system consists of changes at many levels. The most obvious adaptation mechanism is that of the pupil. It dilates in low light levels to allow more light in, but contracts in brighter light, to limit the amount of light reaching the photoreceptors. Changes in pupil size can reduce the amount of light reaching the cephalopod retina by 0.7 log units (Muntz, 1977). There is also a screening pigment associated with the retinal cells, which serves pupillary type functions (Autrum, 1981). The screening pigment covers most of the cell during bright periods, but migrates to the base of the cell during darkness, changing the amount of light reaching the photoreceptors by 0.6 log units (Daw & Pearlman, 1974). The final large-scale change lies in the length of the photoreceptor. In cephalopods, photoreceptors lengthen in response to decreases in the ambient light intensity, which increases the probability of a photon encountering a photoreceptor (Land, 1981). Changes in cell length accounts for, at most, 1 log unit reduction in stimulation (change in length of 25%; Young, 1963). Pupil dilation, pigment migration, and cell lengthening allow the cephalopod visual system to cope with a total of

2.3 log units of change in light intensity, but most coastal cephalopod species encounter changes in light intensity of at least 10 log units, probably more (Clarke & Denton, 1962). This is a similar situation to that found in non-cephalopod animals (Perlman & Normann, 1998). The remaining adaptation is likely to be provided by neural and photoreceptor mechanisms, but as of yet there have been few studies of these in cephalopods (Muntz, 1999).

This study investigated the adaptation capabilities of the isolated retina of *Sepia officinalis*. The effect of an increase in the background light intensity (light adaptation) on the retinal sensitivity of a cephalopod species has not yet been reported. Dark adaptation has been investigated in some cephalopod species (Weeks & Duncan, 1974; Duncan & Pynsent, 1979b; Lange *et al.*, 1979), but this is a different process to light adaptation, with different underlying mechanisms (Fain *et al.*, 2001; Meyer-Rochow, 2001). Dark adaptation is the recovery of the sensitivity of a tissue that was exposed to light, and so is measured with no background stimulation. Light adaptation, on the other hand, is the change in sensitivity when a light is shining on the preparation. Daw & Pearlman (1974) investigated both of these processes in the intact *Loligo pealei* (longfin inshore squid). When the intensity of the background light was increased, the sensitivity decreased, but this paper concentrated on the screening pigment movements that occurred within the eye when it was adapted and so contained very few physiological data.

In other classes and phyla of animal, the adaptation capabilities of the eye have been examined in much greater detail (e.g. insects: Zuker, 1996 and Laughlin, 1989; fish: Northmore, 1977; vertebrates: review by Perlman & Normann, 1998). Perlman & Normann (1998) provided a detailed review about the controlling mechanisms of light adaptation in vertebrate photoreceptors. Much of the analysis shown in this review was based on the V/log I curve (see Chapter 3). It illustrated that by examining changes in the shape of this curve due to the application of a background light on the retina, some inferences about the underlying mechanisms of adaptation could be made. There are two

general mechanisms that photoreceptors use to adapt to light. The first is known as response compression, defined as the non-linearity between the internal transmitter concentration and membrane potential (Perlman & Normann, 1998), and is seen as a compression in the V/log I curve (e.g. in *Necturus maculosus*: Normann & Werblin, 1974). The second is known as biochemical adaptation, defined as adaptation when 'intrinsic mechanisms are activated within the photoreceptors', and is seen as a shift in the V/log I curve, with no reduction in the amplitude (Normann & Perlman, 1979). By measuring the V/log I curve of *S. officinalis* under different background light intensities, it was determined which of these mechanisms was more likely to occur in cephalopod photoreceptors.

In all photoreceptors, it is the activation of rhodopsin by light that initiates the phototransduction cascade. This cascade is composed of several different enzymatic stages (Figure 1.2), all of which are deactivated in turn to cause the cascade to cease operating. It is these feedback mechanisms that, in part, determine the adaptation capabilities of a cell. Calcium ions are known to play a very important role in this feedback process (Gomez & Nasi, 1997). Fain *et al.* (2001) considered that it was the only chemical necessary for adaptation, but there might be at least one other secondary messenger involved (Jindrova, 1998). Calcium was shown to have an effect on the dark adaptation of *Sepiola atlantica* by Weeks & Duncan (1974), and so is thought to play a role in the shut down mechanism in cephalopods. An investigation into the physiological measurement of light adaptation in *S. officinalis*, and the effect of the extracellular calcium concentration of the retina on this process, was the main focus of this chapter.

The aims of this chapter were:

- to examine the effect of background light on the sensitivity of the retina of *S. officinalis*,

- to determine if the light adaptation capabilities of *S. officinalis* changed between two mantle length (ML) groups, and
- to investigate how the concentration of extracellular calcium of the retina affected both visual sensitivity and light adaptation in *S. officinalis.*

4.2 Materials and Methods

The conditions under which the animals were kept, the preparation of the animals, and methods for recording the electroretinogram (ERG), used were the same as those detailed in Chapter 3. Experiments were always done between 09:00 and 17:00, i.e. in the usual light phase of the animal's circadian cycle. In brief, an animal was anesthetised, decapitated, and the eyes removed. The retina was chopped into 300 μm slices, two of which were placed in the perfusion chamber at any one time. The potential difference between an inert electrode in the chamber and a glass microelectrode of low resistance in the retinal slice was recorded and it constituted the ERG. The analogue signal was amplified, converted to digital and saved on a computer. The method in Chapter 3 was varied as followed. Two ML groups of *S. officinalis* were used, 7.0 (± 1.2) cm and 1.6 (± 2) cm (mean ± sd), each of which had six animals. Only slices of tissue were used, and the stimulus was the blue LED (RadioSpares, UK). Most of the experiments were completed in normal artificial seawater (ASW: see Chapter 3 for the recipe). When the effect of the concentration of calcium ($[Ca^{2+}]$) on sensitivity and adaptation was examined, the recipe for normal ASW (10 mM Ca^{2+}) was altered as follows: low $[Ca^{2+}]$ ASW had 0.5 mM $CaCl_2$, and high $[Ca^{2+}]$ ASW had 20 mM $CaCl_2$. The $MgCl_2$ concentration was altered to keep the osmolality of the solution constant (Gomez & Nasi, 1997).

A white light was used for adaptation, when needed. It shone on top of the preparation, and its intensity was controlled using neutral density (ND) filters (Edmund Optics Ltd., UK) (Figure 4.1).

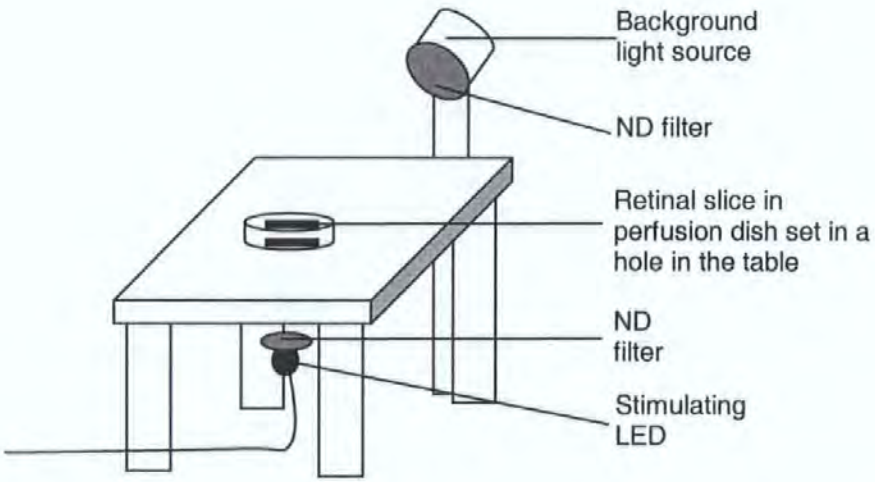


Figure 4.1: A diagrammatic representation of the positions of the two light sources used in these experiments in relation to the preparation (not drawn to scale).

4.2.1 Stimulus protocol used to investigate adaptation

To examine light adaptation in *S. officinalis*, a background light of changeable intensity was used to illuminate the tissue, while its sensitivity was measured. A 10 ms control test flash of approximately 13 $\mu W/cm^2$ every 5 min was used for the first 30 min, with no background light (Clark & Duncan, 1978). Then a light intensity series was completed (Table 3.1). The background light was switched onto its lowest level, and after two minutes of no stimulation, the light intensity series was repeated. The background light intensity was then increased, and the last step repeated (following Daw & Pearlman, 1974). In total, four background light intensities were tested (Table 4.1).

The control test flash was then applied to the tissue every 5 min until the amplitude of the recorded response had levelled out, and the next stimulating protocol was run. The stimulating protocols used were 10 ms flashes every 30 s, 50 ms every 60 s and 500 ms

every 60s. Different flash durations were tested because this variable was found to influence sensitivity (see Section 3.3.3). This entire procedure was performed on both ML groups.

	Background Light Intensity ($\mu W/cm^2$)
None-light switched off	0.1
	0.3
	1.9
Maximum used	5.5

Table 4.1: The four background light intensities used in these experiments, as measured at the preparation.

4.2.1.1 Histological examination of retinal slices adapted to varying light intensities

To determine any morphological changes in the retinal slices that might accompany exposure to the background light intensities used to measure adaptation, some tissue was fixed for histological studies. For one series of experiments with an animal of 6.5 cm ML, instead of recording from a slice, many slices were adapted to the different background light intensities, and fixed for histology. To accomplish this, 40 slices were placed in a perfusion chamber, taking care that none overlapped any of the others. Minimum perfusion was used to avoid movement of the slices. At each critical stage of the experiment two slices were removed and fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS). For example, two fresh slices were fixed, then two after 30 min in the dark, then two after 2 min with minimum background light, then two after 5 min minimum light, etc. This was continued for each of the light intensities used in the preceding section, and then two slices were removed each five minutes of recovery, until 1 h had passed from when the adapting light was switched off. All slices were left to fix

overnight, and then stored in 4% PFA in PBS and 0.05% sodium azide. When ready to be mounted, the slices were washed three times in PBS. The aqueous mountant, VectaShield (Vector Laboratories, UK), was used with special coverslips that had rubber gaskets (Imaging chamber Probe clip with adhesive from Sigma, UK), to enable 300 μm slices to be mounted without damage. They were viewed using a Nikon inverted microscope, and photographs taken using a digital camera (Nikon coolpix 950, Japan).

4.2.2 Protocol used to measure the effects of the extracellular calcium concentration of the retina on its sensitivity and adaptation

To examine the effects of calcium concentration on sensitivity and adaptation in 1.5 cm ML *S. officinalis*, the above protocol was modified and applied when the retinal slices were perfused with ASW of differing [Ca^{2+}]. Pinto & Brown (1977) and Bader *et al.* (1976) showed that extracellular perfusion of different calcium concentrations onto the retina had the same influence on its reactions as injection of the altered solution into retinal photoreceptors. For this reason, it was assumed that by extracellular perfusion of the retina with the solutions of altered calcium concentration, the intracellular concentration of calcium of all photoreceptors would also change.

Firstly, to see how low and high [Ca^{2+}] ASW affected the sensitivity of *S. officinalis*, series of seven light intensities (Table 3.1) were given using 10 ms flashes every 30 s, with each series separated by 120 s. The light intensity series was repeated three times in normal ASW, and then another four times (i.e. 20 min) as the slice was perfused with either low or high [Ca^{2+}] ASW, after which slice was perfused with normal ASW. The light intensity series was run one final time in the normal ASW, and then the tissue was flashed with the control flash once every 5 min for 30 min to determine if there was any recovery.

Secondly, to examine the effect of changing the calcium concentration on the animal's ability to adapt to light, the above protocol was repeated on six different animals

of the same ML, but after 20 min (i.e. after the seventh light intensity series in total) in the altered ASW, the background light was switched on with the 2.5 ND filter (0.3 $\mu W/cm^2$) in place for 120 s (i.e. a background of 0.3 $\mu W/cm^2$), and the light intensity series run again. The recovery was monitored as before. Only one background light was tested, as light intensities greater than this sometimes irreversibly reduced the response amplitude of the ERG in preliminary studies. A control was also run, where the slice was always perfused with normal ASW, but the stimulus protocol was as stated. Once one such treatment (control, low $[Ca^{2+}]$, or high $[Ca^{2+}]$ ASW) was completed, the slice was discarded, and another slice used. Each animal employed for this set of experiments had the three different ASW's tested on different slices.

4.2.3 Analysis

The effect of background light on the ERG was analysed in a similar way to the ERG's in Chapter 3, using Signal (version 2.03; CED, UK) and SigmaPlot 2001 for Windows (version 7.0; SPSS Inc, USA). The response amplitude, duration and latency were the main factors investigated, along with the absolute sensitivity and the V/log I curves. The effect of background light on sensitivity was compared with all three protocols used, 10 ms flashes every 30 s, 50 ms flashes every 60 s and 500 ms flashes every 60 s. Only the 50 ms flashes every 60 s data were presented and used in any statistical tests performed to eliminate any statistical complications caused by repeated measures.

When examining the effect of ASW of differing $[Ca^{2+}]$ perfusing the retina on its sensitivity and adaptation, the results from the light intensity series run after perfusion of the altered ASW for 20 min were used. The series at intermediate times were probably when the intra- and extra-cellular concentrations were equilibrating. SPSS (Version 11.0.1; SPSS Inc, USA) was used to carry out statistical tests where required. Levene's

test for homogeneity of variance was used before a parametric analysis of variance (ANOVA) was performed on any data set. In all graphs, the error bars represent the standard error (se).

The final piece of analysis was to examine the photographs taken of slices fixed after different exposures to light and for this Adobe Photoshop (Adobe PhotoDeluxe Business Edition, Adobe Corporation Inc.) was used.

4.3 Results

4.3.1 The effect of light adaptation on the retinal sensitivity of Sepia officinalis

The absolute sensitivity of 7.0 cm ML *S. officinalis* significantly decreased as the background light intensity increased (One-way ANOVA on logarithmic transformed data: $F_{(3,14)}=42.57$, $p<0.001$; Figure 4.2). A very small response was recorded with the maximum background light intensity used, which did not reach 0.4 mV every time, so the absolute sensitivity was not always determined. The overall trend in absolute sensitivity with an increase in background light intensity resembled an exponential decay (Figure 4.2).

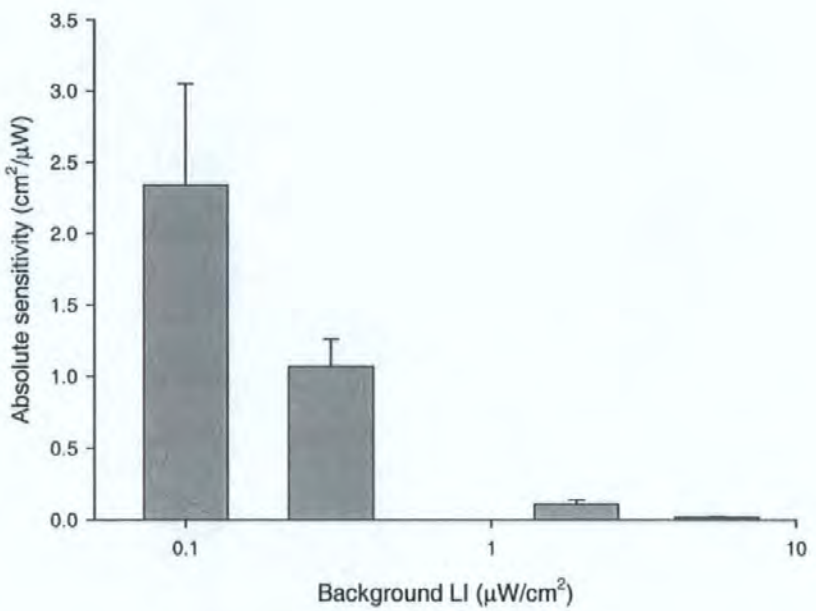


Figure 4.2: The change in absolute sensitivity with increasing background light intensity (LI) for 7.0 cm animals (n=6; error bars= +/- 1se). The stimulus used was 50 ms every 60 s.

Increasing the background light intensity decreased the response amplitude of the ERG and changed the shape of the V/log I curves, obtained with the stimulus light intensities used in these experiments (Figure 4.3). The slopes of the linear sections of these curves decreased significantly (Kruskall Wallis test, H=15.51, df=3, p=0.001) from 1.14 with 0.1 $\mu W/cm^2$ background light (r^2= 0.99, df=4, p<0.001) to 0.06 with 5.5 $\mu W/cm^2$ of background light (r^2=0.49, df=4, p>0.05). It was noteworthy that at the two lowest background light intensities tested, the flash interval of 60 s was not long enough for the cells to recover, and so the amplitude decreased or plateaued at the highest stimulating intensities. With the two highest background light intensities, the response amplitude of the ERG continued to rise with an increasing stimulus intensity, indicating that the saturation intensity of the photoreceptors under these conditions was not met.

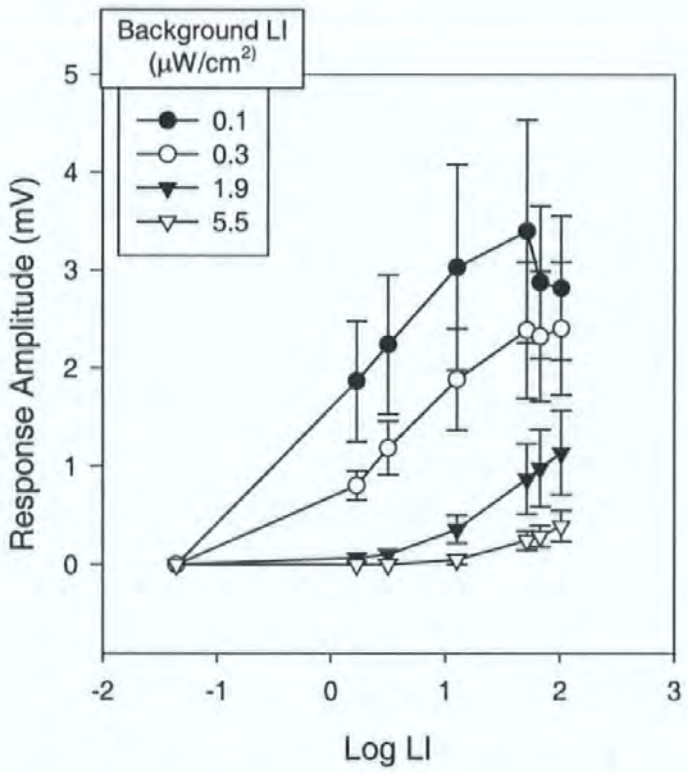


Figure 4.3: The change in the V/log I curve of 7.0 cm mantle length animals with increasing background light intensity (LI). The stimulus was a flash of 50 ms every 60 s (n=6; error bars= +/- 1 se).

As the adapting light grew in brightness, the kinetics of the light-evoked ERG changed. The latency of the ERG responses increased, with increasing background light intensity (Figure 4.4). The duration of the ERG response decreased with increasing background light intensity, but the pattern was not very clear, particularly when the large error bars were considered (Figure 4.4b).

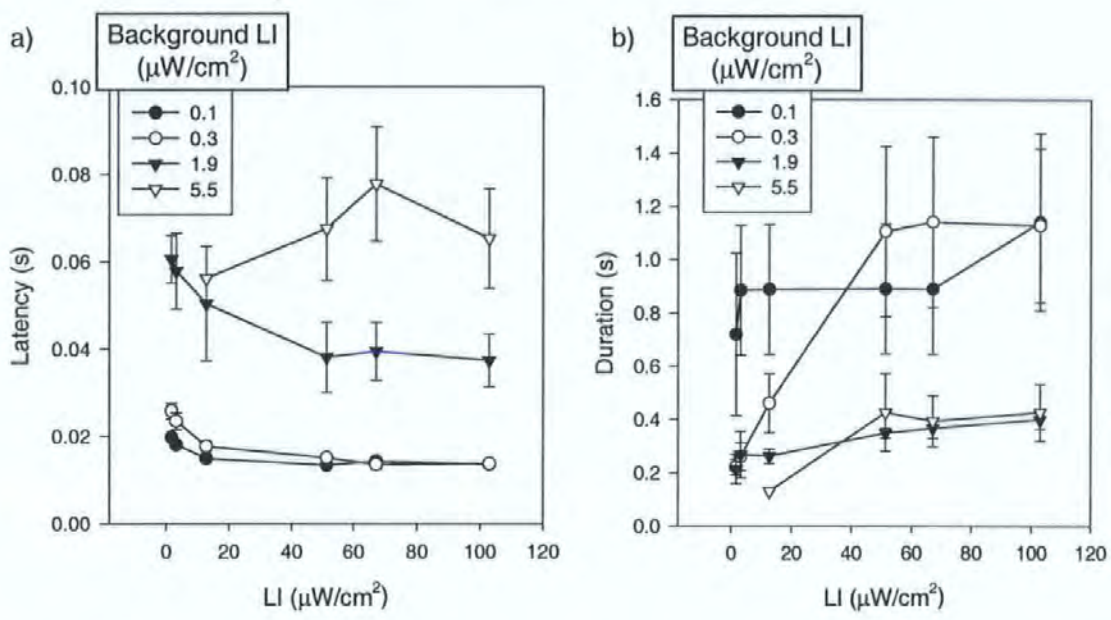


Figure 4.4: The change in the response kinetics of the ERG with increasing background light intensity (LI): a) the latency of the response with increasing stimulus light intensity; b) the response duration with increasing stimulus light intensity (n=6 for each point; error bars = +/- 1 se).

4.3.1.1 Comparison of the light adaptation within two mantle length groups

The adaptation capabilities of two ML groups (1.6 and 7.0 cm ML) were found to be significantly different from each other (Figure 4.5 and Table 4.2). The application of a 0.3 $\mu W/cm^2$ background light reduced the absolute sensitivity of the 1.5 cm animals by 8 times, but it only halved that of the 7 cm animals (Figure 4.5). The 7 cm animals continued to respond when illuminated by a background light intensity of 1.8 $\mu W/cm^2$, but the smaller animals did not. This illustrated that there was a difference in the adaptation

mechanisms of *S. officinalis* of different sizes. The data were non-homogenous, even after transformation, so a non-parametric interaction ANOVA was performed on the three lowest background light intensities used. There were significant differences between the two ML groups, but not between the three background light intensities tested (Table 4.2).

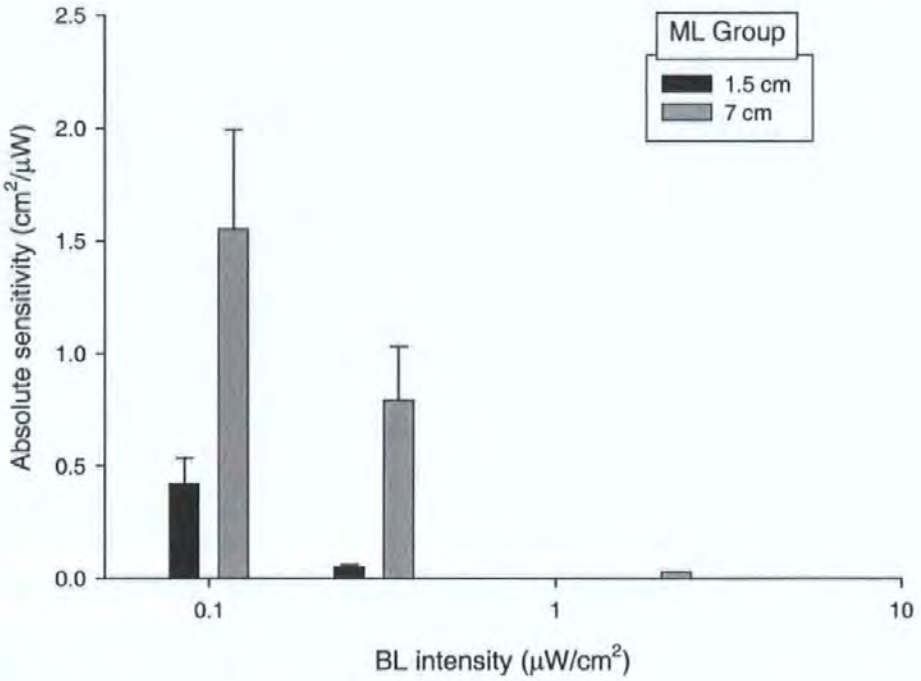


Figure 4.5: The difference in absolute sensitivity in two mantle length (ML) groups of *S. officinalis*, when recorded with a background light (BL) of different intensities (n=6; error bars = +/- 1se).

Source	Sum of Squares	df	H	P
Sample	1361.17	5	12.26	<0.05
ML	831.36	1	7.49	<0.01
BL	492.04	2	4.43	>0.05
Interaction	37.77	2	0.34	>0.05

Table 4.2: The results of a non-parametric ANOVA on the absolute sensitivities of two mantle length (ML) groups measured under three background light (BL) intensities.

4.3.1.2 Photographs of retinal slices after various exposures to light

Retinal slices of *S. officinalis* at each stage of the background light experiment were fixed, mounted and photographed to determine the effects of the light intensities used on the slices. Three of the photographs were reproduced in Figure 4.6. They showed that the screening pigment did not migrate within the time and with light intensities used. They also showed that the slices did not appreciably deteriorate within the time frame of an experiment.

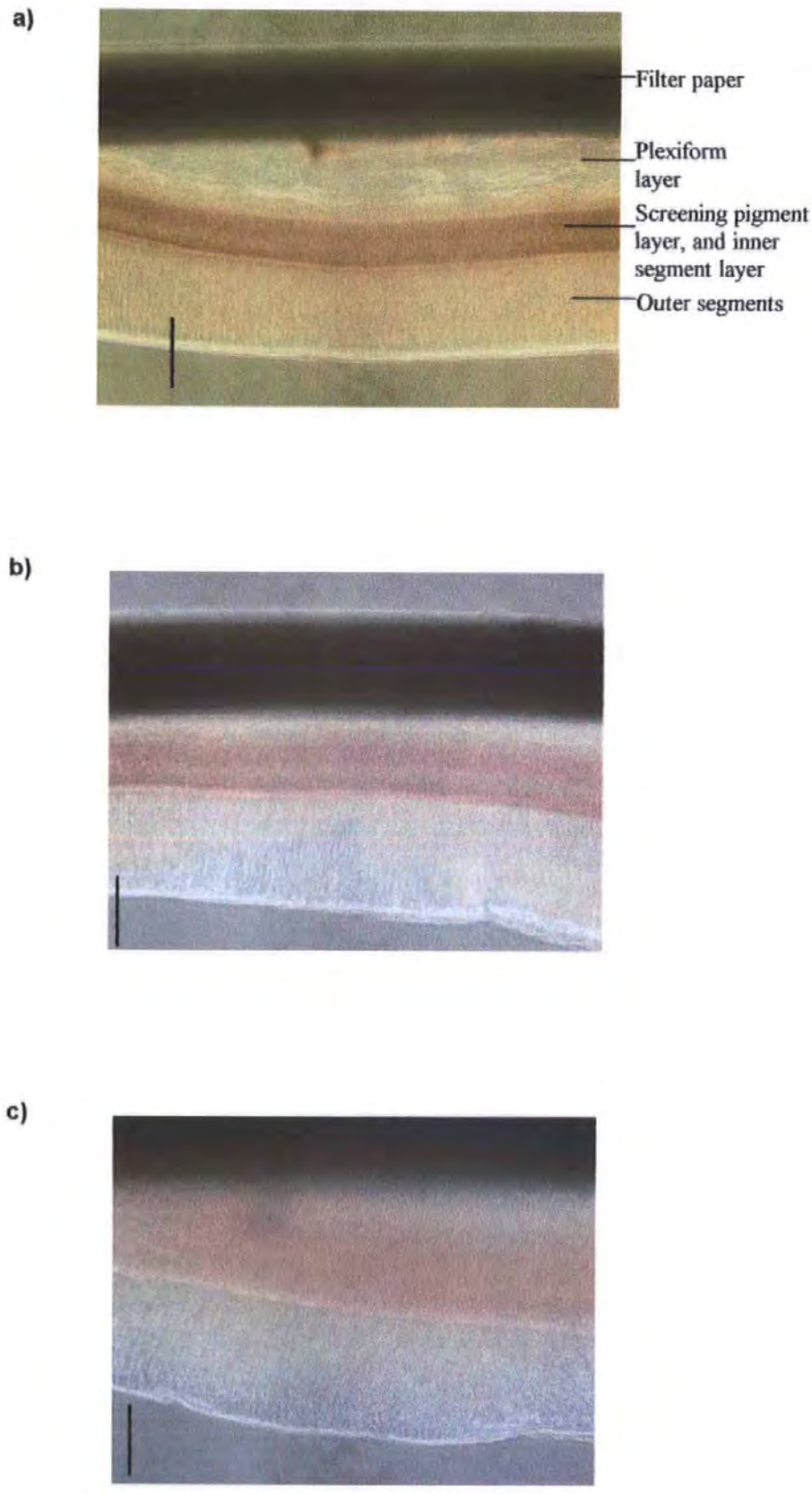


Figure 4.6: Slices of retina fixed at different stages of the background adaptation experiment, a) a freshly chopped retinal slice, b) a retina slice after 15 min in adapting light, c) a retinal slice with 1 h recovery in the dark, having spent 15 min in adapting light (scale bar = 100 μm).

4.3.2 The effect of altering the calcium concentration of the solution perfusing the retina on its sensitivity

Calcium is known to play an important role in the adaptation mechanisms of the majority of species (Rayer *et al.*, 1990; Jindrova, 1998), but its precise action in the cephalopod phototransduction cascade is unknown. To investigate its role in the photoreceptors of *S. officinalis*, the calcium concentration of the retina was altered, while its sensitivity was measured. The absolute sensitivity of *S. officinalis* was not affected by the calcium concentration of the solution perfusing the retina (Figure 4.7 and Table 4.3).

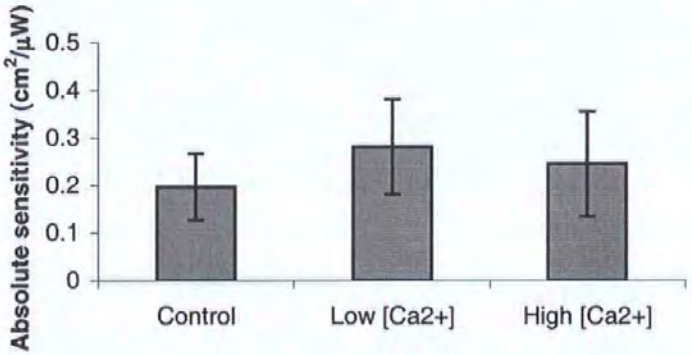


Figure 4.7: An error bar chart of the absolute sensitivity of the retina of *S. officinalis* when it was perfused with three solutions of differing calcium concentrations ($[Ca^{2+}]$) (n=6; error bars= +/- 1 se).

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	0.020	2	0.010	0.173	0.843
Within Groups	0.789	14	0.056		
Total	0.808	16			

Table 4.3: An ANOVA performed on the absolute sensitivity of slices that were perfused with different concentrations of calcium.

When the V/log I curves of these data were examined, it was seen that the calcium concentration of the ASW perfusing the retina influenced the sensitivity of the tissue, but not its absolute sensitivity (Figure 4.8). It was at higher intensities that differences in the treatments became apparent. The V/log I curves recorded from slices in low [Ca^{2+}] ASW reached a plateau with the light intensities used, but the other two did not, particularly the curve recorded from slices in high [Ca^{2+}] ASW, which was still increasing at a rapid rate (Figure 4.8). The slopes of these V/log I curves were significantly different (One-way ANOVA, $F_{(2,17)}=7.75$, $p<0.01$). When a Student-Newman-Keuls test was used to analyse the patterns, it was found that the slope of the high [Ca^{2+}] curve was significantly different from the other two (Table 4.4).

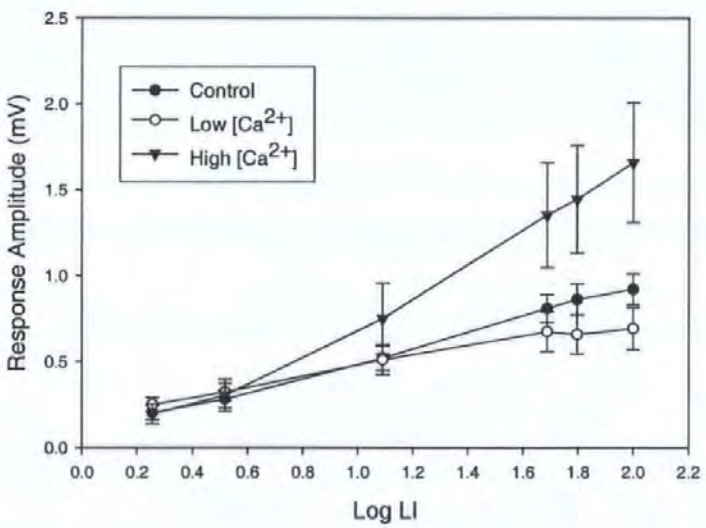


Figure 4.8: The V/log I curves for retina perfused with ASW of three different calcium concentrations ([Ca^{2+}]) (n=6; error bars= +/- 1 se).

The [Ca^{2+}] of ASW	N	Subset for alpha = 0.05	
		1	2
Low	6	0.26	
Control	6	0.42	
High	6		0.84
Sig.		0.30	1.00

Table 4.4: The results of a Student-Newman-Keuls test performed on the slopes of the V/log I curves of the retina perfused by ASW of three different calcium concentrations ([Ca^{2+}]). The means for groups in homogenous subsets are displayed.

The latency of the response decreased significantly with increasing light intensity in the same way for each of the calcium concentrations tested (Table 4.5; Figure 4.9). A two-way interaction ANOVA showed that there was no interaction in the effects of stimulus intensity and extracellular calcium concentration of the retina on the latency of the evoked response (Table 4.5). At each light intensity tested, the slices in low [Ca^{2+}] ASW and the control ASW had the larger latencies, that were statistically indistinguishable from one another, and the slices in high [Ca^{2+}] had the smallest mean latency (Table 4.6).

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Model	2.539E-02	18	1.411E-03	501.398	0.000
Light intensity	9.789E-04	5	1.958E-04	69.587	0.000
[Ca^{2+}]	5.989E-05	2	2.994E-05	10.642	0.000
Light Intensity * [Ca^{2+}]	1.008E-05	10	1.008E-06	0.358	0.961
Error	2.532E-04	90	2.814E-06		
Total	2.565E-02	108			

Table 4.5: The results of a two-way interaction ANOVA on the latency of the evoked ERG, using different stimulus intensities, and different concentrations of calcium ([Ca^{2+}]) to perfuse the retina.

[Ca^{2+}]	N	Subset	
		1	2
High	36	0.014	
Control	36		0.015
Low	36		0.016
Sig.		1.00	0.14

Table 4.6: The results of Student-Newman-Keuls tests performed on the response latencies of ERGs in different concentrations of calcium ([Ca^{2+}]). Any latencies that were grouped in the same subset were not significantly different from each other at the p=0.05 level.

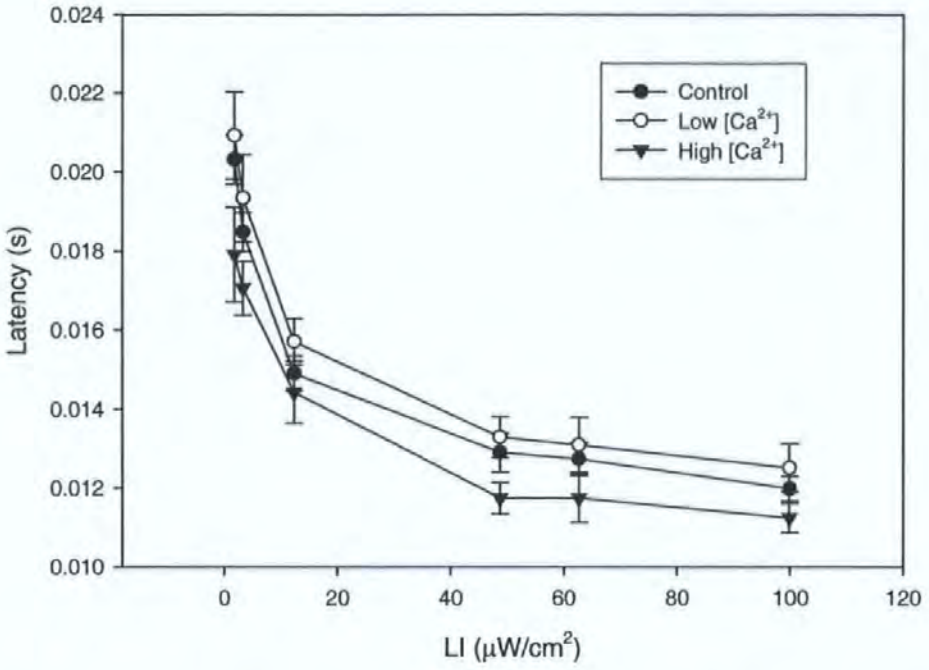


Figure 4.9: The change in the latency of the response with increasing light intensity (LI) when the retina was perfused with ASW of three different calcium concentrations ($[Ca^{2+}]$) (n=6; error bars= +/- 1 se).

The response duration increased with increasing stimulus light intensity for the three different calcium treatments (Figure 4.10). The response durations were log transformed before a two-way interaction ANOVA was performed on the data to ensure homogeneity of variance. Both the stimulus intensity, and the retinal concentration of calcium had significant effects on the response duration, but there was no significant interaction between these two factors (Table 4.7a). It was interesting that both the low $[Ca^{2+}]$ and the high $[Ca^{2+}]$ treatments increased the response duration significantly from the control (Table 4.7b).

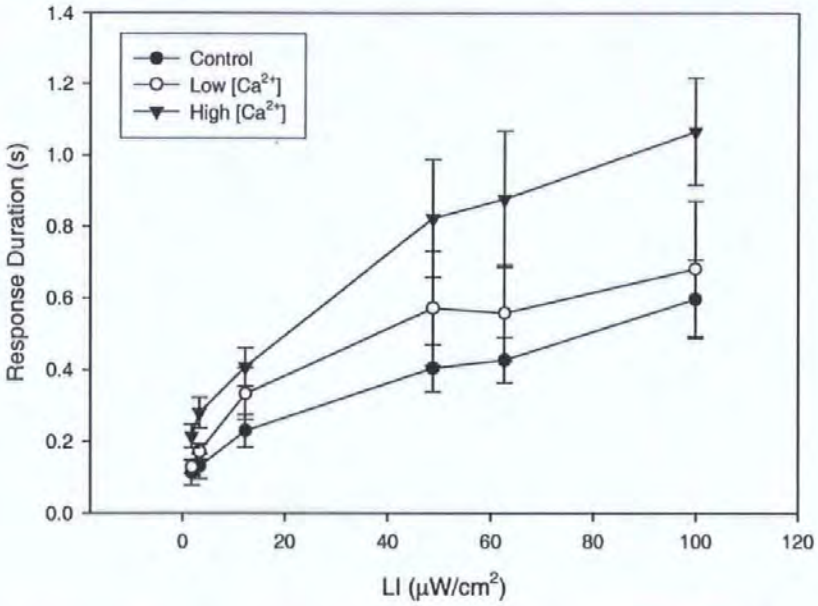


Figure 4.10: The effect of light intensity (LI) on the response duration of the ERG recorded from retinal slices perfused with ASW of three different calcium concentrations ([Ca^{2+}]) (n=6; error bars= +/- 1se).

a)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Model	34.885	18	1.938	48.202	0.000
[Ca^{2+}]	1.617	2	.808	20.104	0.000
Light intensity	7.962	5	1.592	39.608	0.000
[Ca^{2+}]* Light intensity	5.258E-02	10	5.258E-03	0.131	0.999
Error	3.619	90	4.021E-02		
Total	38.503	108			

b)

[Ca^{2+}]	N	Subset		
		1	2	3
Control	36	-0.620		
Low	36		-0.507	
High	36			-0.323
Sig.		1.000	1.000	1.000

Table 4.7: a) The results of a two-way interaction ANOVA with the logarithm of the flash duration as the dependent variable, and the calcium concentration of the ASW perfusing the retina ([Ca^{2+}]), and stimulus light intensity as the fixed factors. b) The results of a Student-Newman-Keuls test to show the homogenous means in each subset.

4.3.3 The effects of calcium concentration of the solution perfusing the retina on its ability to adapt to light

Illuminating the preparation with a dim background light, whilst it was still under the effects of the different perfusion solutions, resulted in a decrease in the absolute sensitivity in all calcium treatments (Table 4.8). The high [Ca^{2+}] solution reduced the absolute sensitivity by the least amount, while the low [Ca^{2+}] solution reduced it by a much larger amount than the control. This change in the decrease in absolute sensitivity, when the tissue was adapted to a background light, showed that calcium must play some role in this process. There was a significant difference between the percentage decrease in absolute sensitivity of the retina under different calcium concentration treatments (One-Way ANOVA, $F_{(2,16)}=5.84$, $p=0.014$). A Student-Newman-Keuls test showed that the significant difference was between the retinal slices in the low [Ca^{2+}] ASW and those in the other two treatments (Table 4.4). The difference between the decrease in sensitivity of the slices in high [Ca^{2+}] ASW and the control ASW was not significant.

Treatment	Mean % decrease in absolute sensitivity	n
Control	67.6[a]	6
Low [Ca^{2+}]	89.3[b]	6
High [Ca^{2+}]	57.0[a]	6

Table 4.8: The mean percentage decrease in absolute sensitivity when a background light was shone on a preparation perfused with solutions of different calcium concentrations. The two treatments that share the same superscript letter were not significantly different at the $p<0.05$ level, when tested using a Student-Newman-Keuls test.

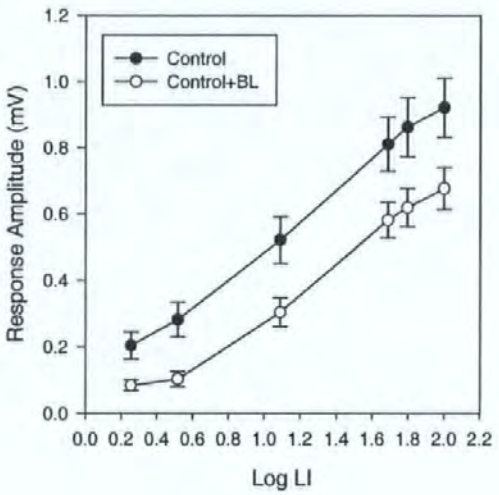
The V/log I curves under different retinal calcium concentrations and under the influence of a background light were shown in Figure 4.11. Both the control and high

[Ca^{2+}] treatments simply shifted the curves to the right. Shining an adapting light on a preparation in the low [Ca^{2+}] ASW, compressed the V/log I curve, so that it reached a plateau at the lower amplitude of 0.35 mV, compared to 0.7 mV without background light (Figure 4.11b). It also decreased the slope of the straight line section of the curve by a larger amount than in the high [Ca^{2+}] ASW and control treatments (Table 4.9 and Figure 4.11b), but not by a significant amount (One-way ANOVA, $F_{(2,17)}=2.83$, $p>0.05$).

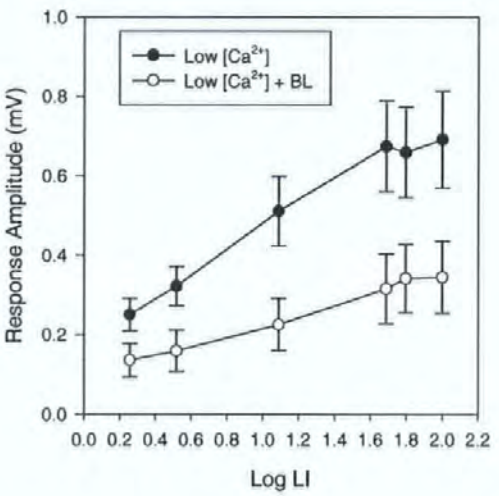
	Control	Low [Ca^{2+}]	High [Ca^{2+}]
No BL	0.42 (± 0.05)	0.26 (± 0.06)	0.84 (± 0.17)
+ BL	0.37 (± 0.05)	0.13 (± 0.03)	0.73 (± 0.16)
Difference	0.04 (± 0.02)	0.13 (± 0.03)	0.10 (± 0.03)

Table 4.9: The slopes of the lines of the V/log I curves in Figure 4.11. They show the effect of different calcium concentrations of the retina, combined with background light, on the retina of *S. officinalis*.

a)



b)



c)

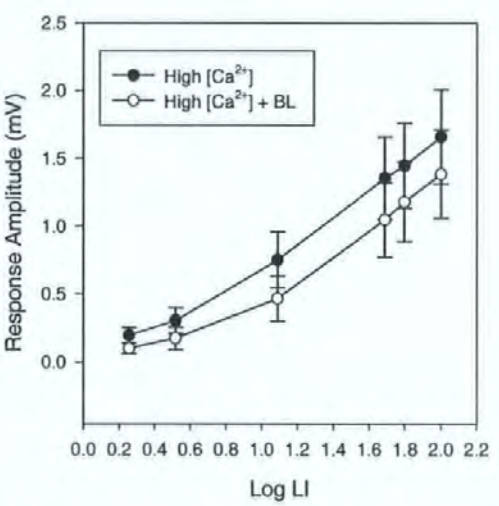


Figure 4.11: Changes in the V/log I curves when a background light (BL) was applied to retinal slices in ASW of different calcium concentrations ([Ca^{2+}]) (n=6); a) control, b) low calcium concentration and c) high calcium concentration. Note that the stimulus was delivered to the tissue for 10 ms every 30 s (error bars= +/- 1 se).

For both the response latency and duration, the background light affected the retinal slices control and high [Ca^{2+}] in the same manner by increasing latency by the same amount for each light intensity (i.e. the graphs remained parallel), and by reducing the response duration in a manner proportional to the light intensity (i.e. the graphs diverged, Figure 4.12a, b, d, and e). The low [Ca^{2+}] treatment resulted in different trends for the response duration, but not the response latency (Figure 4.12c+f). The response durations recorded were not affected by the low [Ca^{2+}] and adapting light, as was evident from Figure 4.12f where the curves only diverged from each other to a small degree.

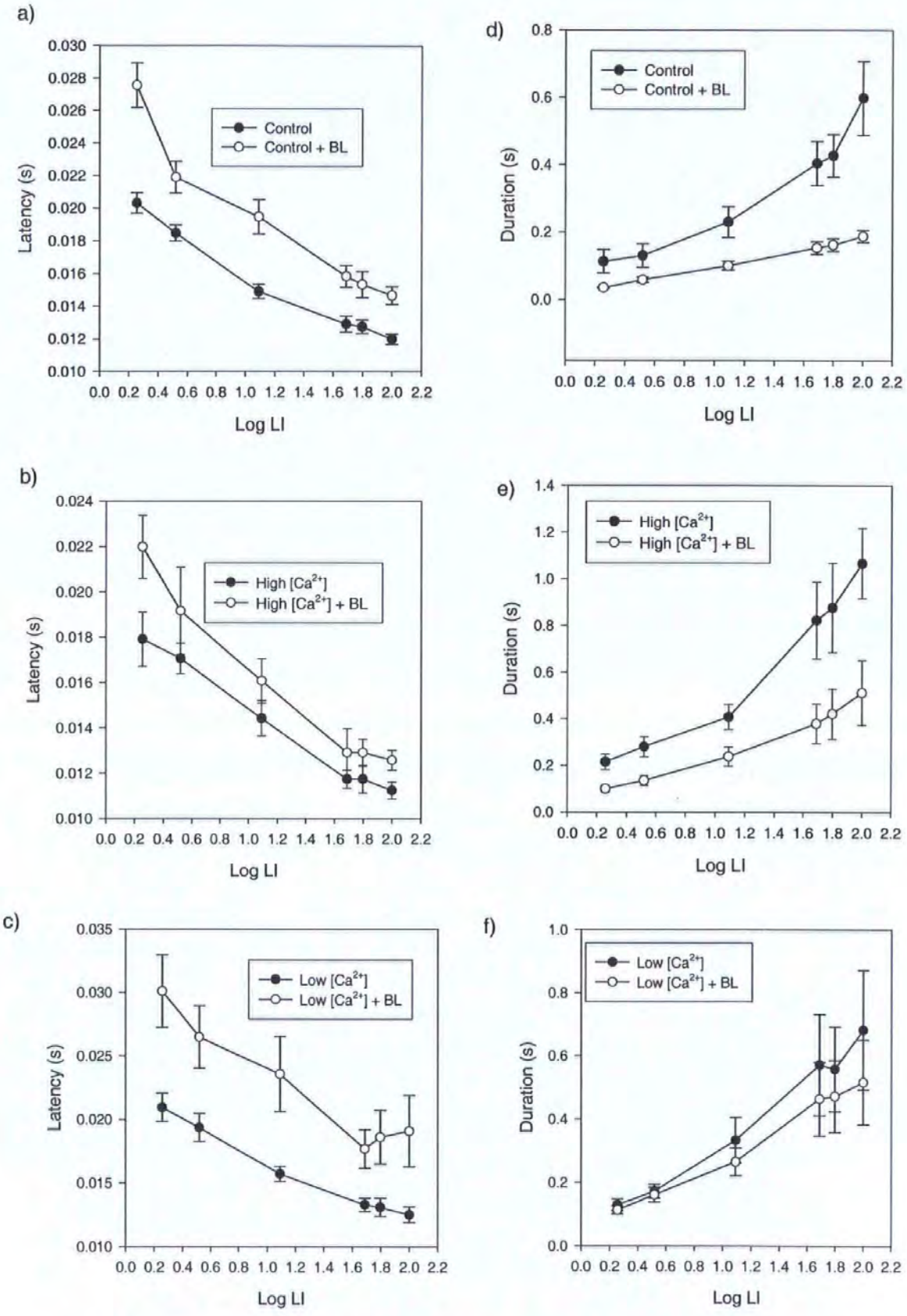


Figure 4.12: The effect of background light on the response latency (a-c) and the response duration (d-f) of the ERG recorded from retinal slices in ASW of three different concentrations of calcium ($[Ca^{2+}]$) (a,d = control; b,e = high $[Ca^{2+}]$ ASW ; c,f = low $[Ca^{2+}]$ ASW) (n = 6, error bars = +/- 1 se).

The slices were allowed to recover for 30 min after these various treatments were completed. In five of the six controls, the response amplitude returned to the levels recorded before the background light was shone on the preparation within this 30 min. When the background light was applied to tissue in low [Ca^{2+}] ASW, the response amplitude never returned to its initial height, but in four of the six slices the response amplitude was increasing towards its original values. For the slices in high [Ca^{2+}] ASW, the response amplitude remained larger after background light application and with 30 min washing in normal ASW. The different concentrations of calcium had marked effects on the sensitivity of the tissue within 20 min of perfusion, it took longer that 30 min of washing in normal ASW to return the slices to their initial adaptive state.

4.4 Discussion

Light adaptation in *S. officinalis* was investigated in this study by examining changes in the evoked ERG to identical flash stimuli but with different levels of background illumination. The retina of *S. officinalis* adapted to light intensities up to 5.5 $\mu W/cm^2$, and this process was affected by the calcium concentration of the solution perfusing the retina. With a moderate amount of background light, the ERG response amplitude to the stimulus decreased to almost zero (Figure 4.3). Smaller *S. officinalis* did not adapt with the same efficiency as medium sized animals, as the same background light intensities reduced their response amplitudes by a greater amount (Figure 4.5). Screening pigment movements did not occur within the experimental parameters tested (Figure 4.6) and so were not responsible for the changes in retinal sensitivity observed with the application of background light. The concentration of calcium in the ASW used to perfuse the retinal slices affected both the sensitivity (but not absolute sensitivity) and the adaptation capabilities of this species (Figure 4.8 and Figure 4.11).

4.4.1 Changes in the sensitivity of S. officinalis, *when its retina was adapted to increasing intensities of light*

Background light reduced the absolute sensitivity of *S. officinalis* by two orders of magnitude (Figure 4.2). This trend was similar to that found in other animals (various lepidopteran species: Eguchi & Horikoshi, 1984; *Locusta migratoria*, *Valanga irregularis*, and *Calliphora stygia*: Matic & Laughlin, 1981; *Pagothenia borchgrevinki*: Morita *et al.*, 1997). The application of a background adapting light changed the shapes of some of the V/log I graphs (Figure 4.3). From the description given by Perlman & Normann (1998) in their review of sensitivity and light adaptation, it was most likely that response compression occurred in the cephalopod retina when it was stimulated with 50 ms flashes (also found in *Necturus maculosus* by Normann & Werblin, 1974; and in *Gekko gekko* by Kleinschmidt & Dowling, 1975).

The slopes of the V/log I curves of *S. officinalis* decreased significantly with an increase in background light, but a larger range of stimulus light intensities would need to be tested to confirm this. (Figure 4.3). Similar experiments in other invertebrate species found that the slope of the V/log I curve increased (Matic & Laughlin, 1981; Eguchi & Horikoshi, 1984), but in vertebrates the slope either stayed the same or decreased (Normann & Werblin, 1974; Kleinschmidt & Dowling, 1975). In this respect, therefore, *S. officinalis* with a significant decrease in slope (Section 4.3.1 and Figure 4.3), more closely resembled vertebrates than invertebrates. This might be explained by different species having different phototransduction cascades and using different mechanisms associated with these cascades to adapt to background light. Within invertebrate species, there are at least two documented phototransduction cascades, one typified by *Limulus polyphemus*, that uses inositol 1,4,5-triphosphate (IP_3) mediated calcium release and the other typified by *Drosophila melanogaster*, that uses diacylglycerol (DAG) and its downstream products (Fein & Cavar, 2000). Both Matic & Laughlin (1981) and Eguchi & Horikoshi (1984)

examined insect species with phototransduction cascades similar to *D. melanogaster*, but it is likely that the cephalopod cascade more closely resembles that of *L. polyphemus* (Nelson, 2003). Invertebrate species that use a phototransduction cascade similar to *L. polyphemus* might respond to an adapting light in a way more similar to vertebrates, as was found here with *S. officinalis*. The adaptation capabilities of more invertebrate species need to be examined to test this hypothesis fully.

The latency of the evoked ERG increased with increasing background light intensity (Figure 4.4a). This was unexpected, as it meant that the response kinetics of the activation of the photoresponse slowed when an adapting light was shone on the retina. This was the opposite way round for the response duration, which decreased dramatically with an increase in the intensity of background light (Figure 4.4b). This meant that the response kinetics of the deactivation of the phototransduction cascade were increased with an adapting light. In both vertebrates and invertebrates, light adaptation has been shown to speed up the kinetics of both the activation and deactivation phases of the light response (e.g. in *Limulus polyphemus* Chapman & Lall, 1967, and in *Apis mellifera* Raggenbass, 1983; for reviews see: Fain & Matthews, 1990; Rayer *et al.*, 1990; Jindrova, 1998). A possible explanation for the difference in *S. officinalis* is as follows. The presence of a background light had already activated some of the rhodopsin, so the phototransduction cascade was producing some of the chemicals that feedback to switch off the response. The effect of these chemicals would have to be overcome, when a second stimulating flash was given, and so the response to a flash would take longer to reach a detectable level. For the deactivation phase of light response, the presence of a threshold of the feedback chemicals could cause the phototransduction cascade to shut down quicker, and so terminate the response faster, decreasing the response duration. It was also possible that due to the retina being isolated, it did not receive the correct feedback from the brain that would enable it to react appropriately to the adapting light.

Adaptation in cephalopods is controlled by pupil dilation (Muntz, 1977), screening pigment migration (Daw & Pearlman, 1974), cell lengthening (Young, 1963), and intracellular mechanisms (Muntz, 1999). Photographs of retinal slices, taken after varying exposures to adapting light, showed that the screening pigment did not move under the conditions used for these experiments (Figure 4.6). This meant screening pigment migration was not responsible for the changes in the ERG found in this chapter with adapting light. There were two main reasons for the screening pigment not moving. The first was due lack of efferent input from the brain. Gleadall *et al.* (1993) showed that efferent input, in the form of dopamine, was necessary for the screening pigment to move correctly. Secondly, the background light intensities that elicited a change in the ERG of *S. officinalis* were low, 0.3 - 5.5 $\mu W/cm^2$, compared to the 270 $\mu W/cm^2$ used by Daw and Pearlman (1974) to elicit pigment migration. It was possible, therefore, that the light intensities used in this chapter were simply too low to stimulate the screening pigment to move. The photographs of slices excluded the possibility of screening pigment migration affecting the results, but adaptation was influenced by the ML of the animal (Figure 4.5). This result implied that cell length was an important factor in determining this capability. Cell length is the one of the few known factors within the cephalopod eye to change with an increase in body size (S. Farley, unpublished results). Young (1963) demonstrated that one of the mechanisms used by cephalopods to adapt to light was lengthening of the photoreceptor cells. The influence of ML on the adaptation of *S. officinalis* (Figure 4.5) reinforced Young's paper, by showing that photoreceptor length was important to the process of adaptation in cephalopods.

Daw & Pearlman (1974) showed that intact *L. pealei* still had a measurable sensitivity with 5 $\mu W/cm^2$ of background light, and that this sensitivity was further halved with a background light of 270 $\mu W/cm^2$, but in Figure 4.2 the absolute sensitivity of the excised retina of *S. officinalis* was reduced by 99% in the presence of 5.5 $\mu W/cm^2$ of

background light. The reason for this large difference was probably due to the preparations used, i.e. excised retina and whole animal. This does not invalidate the results here, but shows the limitations of the retinal tissue, as only the sensitivity of the excised retina was measured here, and not the sensitivity of the entire visual system. This change in sensitivity with an adapting light showed that the retina of *S. officinalis* had approximately the same ability to adapt to light as the *Pagothenia borchgrevinki* (Antarctic nototheniid fish), whose absolute threshold of sensitivity increased by 2 log units when the excised retina was exposed to a background light of 12 lux (approximately 4.4 $\mu W/cm^2$) (Morita *et al.*, 1997).

4.4.2 The effect of altering the extracellular retinal concentration of calcium on the sensitivity and light adaptation of S. officinalis

Calcium is known to influence the activities of some of the enzymes and certain membrane channels involved in the phototransduction cascade (Fain & Matthews, 1990; Rayer *et al.*, 1990), and so its concentration could play a role in determining the sensitivity of photoreceptors. The precise role of calcium within the invertebrate photoresponse remains undetermined (Piccoli *et al.*, 2002). Changing the concentration of calcium in the perfusion solution did not affect the absolute sensitivity of *S. officinalis* (Table 4.3). It did, however, influence the maximum response amplitude of the evoked ERG at higher stimulating intensities, with an increase in calcium concentration, increasing the response amplitude (Figure 4.8). At low stimulating intensities, the response amplitudes for each of the calcium concentrations were the same, due to the limited number of ion channels that were opened by the stimulus. Brighter stimuli opened more of the light-sensitive channels, and so with more extracellular calcium ions available to flow through these channels, a larger potential difference (i.e. ERG) was generated (Rayer *et al.*, 1990; Hardie, 1991b). The differentiating step between the responses to low and high stimulus intensities was the number of ion channels they opened. When a relatively few channels were opened by the

low light intensity flashes, the amount of calcium in the extracellular solution did not influence the ERG. When all the channels were opened by the higher intensity flashes, the concentration of calcium ions available to flow through these open channels probably determined the response amplitude of the ERG in part, as the greater the flow of ions, the greater the potential difference generated.

Weeks & Duncan (1974) and Clark & Duncan (1978) used a different technique to investigate the changes in the ERG of *Sepiola atlantica* under similar conditions. In both of these papers, lowering the calcium concentration of the solution perfusing the retina resulted in an increase of the response amplitude, contrary to the results presented here (Figure 4.8). The reason for this difference could be related to the permeability of the tissue and the time for which the different [Ca^{2+}] solutions were left to perfuse over the tissue. Both of the previous studies used half-eye cup mounts, but retinal slices of 300 μm thickness were used here. This reduced quantity of tissue would allow the altered [Ca^{2+}] ASW to penetrate the tissue quicker, and so it should affect a response in a shorter length of time. Secondly, Clark & Duncan (1978) perfused the retina with lowered [Ca^{2+}] ASW for 10 –15 min. The protocol used in this chapter was to perfuse the tissue in the altered ASW for 20 min and the sensitivity of the retina was recorded once every five minutes under these changing conditions. It was found that initially in the low [Ca^{2+}], the amplitude of the response rose, but it then fell (also found by Millecchia & Mauro, 1969; Bolsover & Brown, 1985). The responses to the intensity series at 20 min was used in the results presented here, as it was considered that these showed the true effect of different solutions. It was probable that Clark & Duncan (1978) and Weeks & Duncan (1974) reported the initial change in a relatively large piece of tissue, and not the long-term change. The initial increase in the response amplitude of the ERG when the retina was in low [Ca^{2+}] ASW was probably due to releasing the deactivation mechanism of the photoresponse from the effect of calcium (Bolsover & Brown, 1985). Once the concentration of calcium both inside the cell, including the intracellular stores, and outside

the cell had equilibrated to the lower level, there were fewer calcium ions available to flow through the ion channels, so the response amplitude decreased. This decrease in response amplitude was also due to the effect that calcium has on the activation of the phototransduction cascade. Without enough calcium in the extracellular solution of the retina, this cascade functions at sub-optimal levels (Bolsover & Brown, 1985).

The latency of the evoked ERG increased proportionally with decreasing calcium concentration (Figure 4.9). This agreed with other studies on photoreceptors, where the activation kinetics (measured in this chapter as response latency) were dependent on the calcium concentration with low calcium slowing the activation kinetics down, and high calcium speeding them up (Bader, 1976; Zuker, 1996). The effect of the calcium concentration of the retina on the response duration of the ERG was somewhat more complicated, as both increasing and decreasing its concentration lengthened the response duration (Figure 4.10). This increase in response duration with both altered $[Ca^{2+}]$ ASW highlights the complexity of the role of calcium, which has a part in both the activation of the phototransduction cascade and the feedback mechanisms that deactivate it. One of the reasons for the increase in response duration with the high $[Ca^{2+}]$ ASW was because the high $[Ca^{2+}]$ ASW caused the response amplitude to increase (Figure 4.8), and it simply took longer for this larger response to deactivate. The probable reason the low $[Ca^{2+}]$ ASW prolonged the duration was that there was less calcium available to feedback into the shut down mechanism and so to deactivate the photoresponse (Rayer *et al.*, 1990). Clark & Duncan (1978) found that the duration of the response was greater with lower concentrations of calcium, but they did not test any concentration higher than 10 mM, unlike this study, where 20 mM calcium was tested.

Brown & Lisman (1975) established that by increasing the intracellular calcium concentration of *L. polyphemus* photoreceptors, they reacted as if they had been adapted to a background light. To ascertain if this was similar to the situation in the retina of *S. officinalis,* the results from sections 4.3.1 and 4.3.2 were collated to form Table 4.10.

Treatment	Sensitivity	Amplitude	Latency	Duration	Relevant Figures
Light	Decrease (decrease)	Decrease (decrease)	Increase (decrease)	Decrease (decrease)	Figure 4.2, Figure 4.3, Figure 4.4
High [Ca^{2+}]	No change (decrease)	Increase (decrease)	Decrease (decrease)	Increase (decrease)	Figure 4.8, Figure 4.9, Figure 4.10
Low [Ca^{2+}]	No change (increase)	Decrease (increase)	Increase (increase)	Increase (increase)	

Table 4.10: A summary of the effects that background light and changes in calcium concentration [Ca^{2+}] had on the retina of *S. officinalis* (the words in brackets were the expected responses if increased calcium mimicked light adaptation of the retina, as determined from Brown & Lisman, 1975; Clark & Duncan, 1978; Zuker, 1996).

In two main ways *S. officinalis* proved to be different to other species, a) applying increasing background light intensities to a retinal slice increased the latency of its response, where it often decreases it (Millecchia & Mauro, 1969; Zuker, 1996) and b) increasing the extracellular calcium concentration of the retinal slice increased the response amplitude of its ERG (Table 4.10). As was already stated (section 4.4.1), only the adaptation capabilities of the excised retina were examined here. Changes in the excised retina are only one part in the overall adaptation that occurs within an animal's visual system. If there had still been efferent input into the retina, and the presence of a pupil limiting the amount of light reaching the photoreceptors, then the ERG might have changed in a more predicted way. It appeared from Table 4.10 that decreasing the calcium concentration of the retina of *S. officinalis* more closely resembled light adaptation, unlike the situation in *L. polyphemus*, where increasing [Ca^{2+}] mimics light adaptation (Brown & Lisman, 1975).

To investigate if the addition or omission of calcium in the perfusion solution either increased or decreased the effect of background light on the ERG, a background light was shone on preparations already in the altered ASWs, and their sensitivities measured again. With the high [Ca^{2+}] ASW, the background light decreased the response amplitude and latency by a smaller amount than with the control ASW (Figure 4.11). This suggested that higher calcium concentrations lessened the effect of the background light. With the lowered calcium concentration, the light had a much more dramatic effect implying that the tissue did not adapt as well to this light intensity (Figure 4.11, Figure 4.12). These data showed that the concentration of calcium had an important role to play in adaptation. Without enough calcium in the system, the adaptation seemed to be of the response compression type (following Perlman & Normann, 1998). But when there was at least 10 mM Ca^{2+}, the adaptation was of the biochemical type (Figure 4.11). This implies that the concentration of calcium determined the type of adaptation the tissue underwent, with a certain amount of calcium necessary to feedback into the phototransduction cascade to initiate biochemical adaptation.

Although comparisons between the light adaptation capabilities and the general underlying mechanisms (i.e. response compression, or biochemical adaptation) were made between cephalopods and vertebrates, this was not done for the influence of the calcium concentration on the sensitivity and light adaptation in *S. officinalis*. In both vertebrates and invertebrates calcium is known to affect the phototransduction cascade, but far more detail is known about the vertebrate one (Rayer *et al.*, 1990). For example, increasing the levels of calcium in a vertebrate photoreceptor increases the peak velocity of light-activated phosphodiesterase (Barkdoll *et al.*, 1989) and the activity of guanylate cyclase activity (Hodgkin & Nunn, 1988), but within the invertebrate phototransduction cascade, there has only been one quantitative study on the effects of [Ca^{2+}] completed, and that was on inositol-triphosphate (Fein *et al.*, 1984). Because of the differences in the two cascades,

and in the amount of study done on them, no comparisons were made between the effect of calcium on sensitivity in vertebrates, and those reported here.

4.4.3 Conclusions

The light adaptation of the excised retina of *S. officinalis* was investigated and found to be similar to the response compression type, also seen in vertebrate species. Smaller animals were more affected by an increasing background light than medium sized animals, implying that the length of the photoreceptors influenced adaptation. The extracellular concentration of calcium affected the sensitivity and light adaptation of *S. officinalis*, but not always in the expected ways. In its reaction to increased calcium levels, this species proved to be slightly different to other cephalopod species in that a low calcium concentration reduced the amplitude of the response. The light adaptation and retinal reactions to changes in the calcium levels showed that *S. officinalis* does not share precisely the same feedback mechanism in the phototransduction cascade as other animals. These differences were also partly due to the preparation used. By shining a background, adapting light on the excised retina of *S. officinalis*, the calcium levels appeared to decrease, which is contrary to findings in other species.

Chapter 5 Physiological Evidence for the Presence of Functioning Gap Junctions in the Retina of *Sepia officinalis*

5.1 Introduction

Gap junctions are membrane channels between two cells that connect their cytoplasms (Spray & Bennett, 1985). One gap junction is composed of two hemi-channels (or connexons), one from each cell involved in the junction. Each hemi-channel is composed of six proteins (Saez *et al.*, 1993). These proteins are called connexins in vertebrates and innexins in invertebrates (Zoidl & Dermietzel, 2002). Structurally, the six proteins can be in an opened or closed formation between which they can alternate quickly (Saez *et al.*, 1993). When opened, they form a channel approximately 1.5 nm in diameter, through which any molecule smaller than this in size, or less than 1,000 in molecular weight will pass (Saez *et al.*, 1993; Kumar & Gilula, 1996). They allow the passage of charged molecules (current) and metabolites such as second messengers directly from one cell to another (Hardie, 1991a; Vaney *et al.*, 1998). These molecular movements rely on the passive diffusion of chemicals, either along a potential or a chemical gradient. Within the vertebrate retina, gap junctions exist between many of the cell types (Vaney, 1997), including between photoreceptors (Raviola & Gilula, 1973). These intercellular channels are now recognised to play an important part in vertebrate visual processing (Vaney, 1997). The advantage of such connections, is that they decrease the noise in each rod, as they allow a population of rods to share the random noise, but it also has a disadvantage in that it blurs the target by increasing the receptive field of individual cells (Werblin, 1991). The number of fully functioning gap junctions in the vertebrate retina changes depending on the time of day and the adaptational state of the eye (Kurz-Isler & Wolburg, 1986; Kurz-Isler *et al.*, 1992). There is far less information on any changes of the presence of functioning gap junctions in the cephalopod retina under different light regimes.

Two lines of investigation have shown that gap junctions exist in the retina of cephalopods. Cohen (1973b) and Yamamoto & Takasu (1984) performed histological

studies on the retina of squid and octopus species using electron microscopy and found gap junctions in the plexus layer linking the axons of the inner segments. Tasaki *et al.* (1963a) provided electrophysiological evidence that some type of connections must exist between photoreceptors, for they found that the retinal potentials they recorded in the octopus spread outside the area illuminated by a spot of light. A possible mechanism for this would be the electronic coupling of photoreceptors through gap junctions. There is no information on whether the numbers of functional gap junctions in a cephalopod retina change with time of day and adaptation state in a similar way as those in vertebrate retinas change.

5.1.1 Methods used to investigate the presence of functioning gap junctions

There are three physiological methods commonly used to identify the presence of gap junctions in tissues: the double voltage patch clamp, sharp electrodes for intracellular recordings and dye injection. The double voltage patch clamp technique requires two cells that are in close proximity to have electrodes attached to their membranes. The voltage of one cell is then monitored as current is injected through the other. If the two cells are electrotonically coupled via gap junctions, the current injection will be reflected by a change in the other cell's membrane voltage (Gho, 1994). This technique is difficult to perform, especially if the cells used are small, such as is the case with cephalopod retinal photoreceptors. A variation of this technique is to use intracellular electrodes instead of patch electrodes and so record the occurrences within the cells under similar conditions (Griff & Pinto, 1981).

Dye injection is somewhat easier, in that only one cell needs to be manipulated. In this method, a dye of small molecular mass is injected into a cell. If the dye is of the correct molecular size, and if gap junctions are present and open, the dye will spread to the connected cells. Some scientists have taken this further and used a combination of dyes of different sizes (Blennerhassett & Caveney, 1984; Vaney *et al.*, 1998). Gap junctions

typically have a pore size of 1.5 nm and allow only molecules smaller than 1,000 molecular weight to pass through them (Zoidl & Dermietzel, 2002). By choosing two dyes, one smaller than this, and one larger, the extent of gap junction coupling can be measured. El-Fouly *et al.* (1987) altered this technique, and found that simply scraping the tissue with a wooden probe allowed the dye to enter some cells. They called this technique "scrape-loading" and established that it worked just as efficiently as dye injection.

In combination with these methods of investigation, the open/close state of gap junctions has been manipulated using different processes. The presence of functional gap junctions can be controlled at several different stages within the cell (Saez *et al.*, 1993). They range from the longer term processes of transcription and translation of the connexin (or innexin) genes to the more medium term ones affecting post-translational processing and the insertion and removal of the proteins into the cell's membrane, to the short term ones of the assembly of proteins into gap junction channels and gating processes, such as phosphorylation (Spray & Bennett, 1985). This chapter is concerned with the artificial manipulation of gap junction state, so chemicals known to block gap junctions were used. Gap junction blockers fall into 5 broad groups: lipophiles, acidifiers, tumour promoters, antibodies, and other molecules (Rozental *et al.*, 2001) (Table 5.1). Many of these chemicals have deleterious side effects and their precise modes of action are unknown, so not all are commonly used to block gap junctions.

Group	Examples	References
Lipophiles	Heptanol	Guan *et al.* (1997)
	Octanol	McMahon *et al.* (1989); Gho (1994)
	Oleamide	Guan *et al.* (1997); Boger *et al.* (1998)
Acidifiers	HCO_3^-	Schmitz & Wolburg (1991)
Tumour promoters and antibodies	Antibody against 27 kDa rat liver protein	Fraser *et al.* (1987)
Other molecules	glycyrrhetinic acid	Davidson & Baumgarten (1988); Yamamoto *et al.* (1998)
	carbenoxolone	Vaney *et al.* (1998); Kamermans *et al.* (2001)
	Dopamine	Teranishi *et al.* (1983); Lasater & Dowling (1985)

Table 5.1: The types of chemicals known to act as gap junction blockers, including example references of where they have been used.

The main aim of this chapter was to provide physiological evidence that functioning gap junctions exist in the cephalopod retina and to determine if their operation could be controlled by the application of chemicals or by the adaptation state of the retina. To do this, three different techniques were used:

- changes in the light-evoked electroretinogram (ERG) were recorded, as the retina was perfused with gap junction blockers;
- intracellular electrodes were used to inject dyes into single photoreceptor cells and the resulting patterns analysed;
- scrape-loading and variations of this technique were used to introduce two dyes into photoreceptor cells and the staining patterns were analysed.

5.2 Materials and Methods

5.2.1 Animals

The majority of *Sepia officinalis* used in these experiments had been reared from hatchlings, but occasionally ones caught by trawling were used. The mean mantle length (ML) (± standard deviation; sd) of the group used to investigate changes in the ERG was 69.5 (± 15.3) mm (n = 22 animals). For experiments involving the application of dyes, both with intracellular electrodes and by scrape-loading, the ML ranged from 0.9 cm to 24 cm (n = 27 animals). While in captivity, animals were fed daily and kept on a 12 h light:dark cycle. The light phase lasted from 06:30 to 18:30, and animals were killed between 08:30 and 09:30 on the day experiments were undertaken.

5.2.2 Chemicals

All chemicals were purchased from SigmaAldrich, UK, unless otherwise stated. Several proven gap junction blockers were used to investigate if the presence of functional gap junctions affected the light-evoked ERG. The concentrations of these chemicals used on the retina of *S. officinalis* were chosen according to publication of Rozental *et al.* (2001) and were as follows:

- Carbenoxolone (50 and 100 μM),
- Dopamine (10 and 50 μM),
- Heptanol (500 μM, 1 and 2 mM),
- Octanol (250, 500 μM and 1 mM),
- Oleamide (50 μM).

Oleamide, octanol and heptanol were made up as stock solutions in ethanol and diluted in ASW so that the final concentration of ethanol was never more than 1.5%. This method did not work for carbenoxolone, so it was dissolved in a phosphate buffered-artificial

seawater (PBS-ASW). Finally, to prevent oxidation of dopamine while in ASW, 50 μM sodium metabisulphite (NaMBS) was added to the solution (Sutor & ten Bruggencate, 1990). Tetrodotoxin (TTX, 1 μM from Tocris, UK) was used to investigate the influence of voltage-sensitive sodium channels on the light-evoked ERG.

The dyes used to investigate the level of coupling between photoreceptors were as follows: Lucifer yellow (Sigma Aldrich, UK), N-(2-aminoethyl) biotinamide hydrochloride (neurobiotin) (Vector Labs, USA), and Fluoroscein 5(6) isothiocyanate-dextran (FITC-dextran) (3000 MW; Molecular Probes, the Netherlands). Other chemicals used in this process were, paraformaldehyde (PFA), bovine serum albumin (BSA), triton X, CY2, and CY3 (CY2 and CY3 from Amersham BioSciences, UK). Octadecyl indocarbocyanine (DiI: Molecular Probes, The Netherlands), in paste form, was also used to examine the resulting staining in the retina, when the optic nerves were filled using a retrograde mechanism.

5.2.3 Chemical screening using the electroretinogram

The methods used to kill the animals and to slice their retinas were detailed in Chapter 3, but briefly were as follows. Before killing, animals were dark adapted for 30 min. They were anaesthetised in 2% ethanol in seawater and killed by decapitation. Both eyes were dissected out, the anterior portion and sclera removed and the retina chopped into 300 μm slices, using the McIlwain tissue chopper (The Mickle Laboratory Engineering Co. Ltd., UK). Slices were either used straight away or kept on ice in artificial sea water (ASW: see Chapter 3 for recipe) until needed. Slices were never used more than 36 h after dissection.

The basic electrophysiology set-up was the same as that used in Chapter 3. For the experiments of this chapter, the blue LED (peak wavelength= 500 nm; RadioSpares, UK) was used as the light stimulus. Two slices were placed in the perfusion chamber, and held

in position using a tissue anchor. One slice was used for the experiment and the other was a replacement, in case an ERG was difficult to record from the first. They were perfused using normal ASW. The electrode was placed in the tissue with the aid of an infra-red light and a video camera with a monitor. Once a positive ERG of an amplitude greater than 0.5 mV was located, the experiment began. Throughout these experiments the tissue used was in the dark, unless otherwise stated.

A stimulus flash of 10 ms every 120 s was given to avoid any possibility of light adaptation. The light intensity varied between 12.1 and 12.5 $\mu W/cm^2$ and was checked at the beginning of each day using an optical power meter (TQ8210, Advantest, Japan), just before experiments were started. The slice was perfused in normal ASW for at least 40 min until the flash evoked response amplitude had levelled out (Clark & Duncan, 1978). The ASW perfusing the retina was then changed to one of the test solutions, by placing the perfusion tubing from one solution bottle into the bottle containing the test solution. All bottles of solution were kept on ice, to keep the temperature constant, and were bubbled with 95% O_2/ 5% CO_2. The test solution was perfused over the slice for 60 min, or until the response amplitude of the ERG had reduced to almost 0 mV (i.e. blocked), whichever happened first. If there were any effects of the test solution on the light-evoked ERG, the perfusion solution was then switched back to normal ASW to determine if the effects could be reversed. This wash with normal ASW usually continued for 60 min, but on some occasions it was left for longer to see if the response would recover. The stimulus flash of 10 ms duration and 12.5 $\mu W/cm^2$ light intensity was continued at the same rate throughout the perfusion of different solutions over the retinal slices.

Controls were run to ensure that the response amplitude of the light-evoked ERG remained constant for a sufficient length of time to allow testing of different chemicals, and also to ensure that none of the chemicals used as delivery vehicles for the gap junction blockers affected the response. To determine the stability of the ERG over time, the ERG

was recorded from a slice with normal ASW only perfusing it for 3-4 h. Every chemical used in conjunction with the gap junction blockers, but that was not a thought to be a blocker itself, was tested individually at the concentrations used with the blockers. So ethanol, NaMBS, and PBS-ASW were tested using the same protocol as the gap junction blockers to see if they had an effect on the ERG. TTX, at 1 μM concentration in ASW, was used to examine the effects of voltage-sensitive sodium channels on the light-evoked ERG. TTX blocks voltage-sensitive sodium channels only (Moore *et al.*, 1967), but heptanol and octanol block both sodium channels and gap junctions (Haydon & Urban, 1983; Guan *et al.*, 1997). For all the controls and for the different concentrations of potential gap junction blockers, at least three repeats were performed on slices from different animals.

5.2.4 Dye injection

To investigate whether individual cells were linked to neighbouring cells via gap junctions, intracellular electrodes were used to fill cells with Lucifer yellow and neurobiotin. The tips of high resistance, sharp electrodes were back filled with 5% Lucifer yellow and 2% neurobiotin. The electrodes were then filled further with 3 M lithium chloride. The electrode was brought to the surface of the tissue, as was indicated by a large rise in the noise level of trace. The light response of the tissue was monitored using a 10 ms flash of 95 $\mu W/cm^2$ from an LED (peak wavelength emission=590 nm, RadioSpares, UK). A piezo-electric stepper (MPM-20, WPI Ltd, UK) was used to advance the electrode forward by very small distances until the electrode impaled a cell. Once a cell was penetrated with an electrode, as indicated by a drop in the resting potential and a light response larger in response amplitude than that of the ERG of the surrounding tissue, current injection (iontophoresis) was used to fill the cell with dye. The success of the dyes

entering and filling the cell was checked using a fluorescent light source with the appropriate filter block for Lucifer yellow (Table 5.2)

Dye	Excitation peak (nm)	Emission Peak (nm)
Lucifer yellow	428	536
CY-3	550	570
FITC-dextran	496	520
CY-2	489	500

Table 5.2: The excitation and emission peaks for the dyes used in this chapter. These wavelengths determined the filter blocks used when viewing the stained tissue. The peaks were obtained from the catalogues of the companies, from whom the dyes were purchased.

5.2.5 The scrape-loading technique and its modifications

To determine if cells were coupled via gap junctions, groups of photoreceptors were filled with different dyes by scrape-loading them and the staining patterns examined. The technique of El-Fouly *et al.* (1987) was modified using suggestions from Dr. David Becker (University College, London). Initially, pieces of retina (usually half of an eye, but this depended on the size of the animal, for small animals approximately 1 cm ML, whole eyes were used) were scrape loaded using blunted electrodes that were filled with 5% Lucifer yellow and 2% neurobiotin. The dyes were left on the tissue and the preparation placed in the fridge overnight. The following day the tissue was fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) and then reacted using the following steps (adapted by Dr. Abdul Chrachri from the protocol produced by Vector Laboratories, USA).

-Washed in PBS for 10 minutes, three times,

-Blocked and permeabilised in 2% BSA and 1% triton X in PBS, either for 2 h or overnight,

-Washed in PBS for 30 minutes, two times,

-Incubated in streptavidin linked CY3 1:400 dilution (see Table 5.2 for excitation/emission wavelengths) either overnight in the fridge, or for 2 h in the dark at room temperature,

-Washed in PBS for 15 minutes, three times,

-Viewed using a microscope with the fluorescent filters appropriate for the dyes used.

One dye should have remained within certain cells only, and the other should have passed through gap junctions to the neighbouring cells. Several variations of this method were tried, but none were entirely successful. Seventeen trials were performed on animals of various mantle lengths and using slight differences in the technique. One of the major differences was to use GelFoam (Pharmacia Upjohn, USA) to deliver the dyes to the tissue: here a tiny piece of GelFoam was cut, soaked in a solution containing both dyes, and then placed on some optic nerves of the retina. Another variation was to replace Lucifer yellow with FITC-dextran. This was because FITC-dextran was specifically designed to withstand fixation in aldehydes (Molecular Probes Catalogue, 2003). Both Lucifer yellow and FITC-dextran have approximately similar excitation and emission frequencies (Table 5.2) and so the same filter block could be used.

Other variations in the treatment that were used in an attempt to improve the success of this technique were as follows. On some occasions, the eye of an animal was cut in half, one half was then dark-adapted and the other was light adapted and differences in the staining patterns examined. The animal was dark adapted for 30 min before killing it and keeping the tissue in the dark after this. For light adaptation, the animal was in light before killing it, and the dissection was completed in the light. The tissue was then put in the dark, as light bleached the dyes used. Also following Pottek & Weiler (2003), on one occasion some tissue was light-sensitised. Light-sensitised tissue was tissue that was dark-adapted, and then exposed to a moderate light intensity for 15 minutes. As a second

alternative, the halves of some other eyes were treated with 2 mM heptanol, to close the gap junctions chemically. Again the retina was examined for any differences in staining patterns. Finally, on one occasion CY2 was used instead of CY3, as it fluoresces green, unlike CY2 that fluoresces red (Table 5.2), and so was used to check that the neurobiotin-CY2 staining was real, and that the red fluorescence seen not just an artefact due to the natural fluorescence found within the retina. Another small alteration to the technique was to apply the GelFoam to the photoreceptor side of the retina, as opposed to the optic nerve side.

Retrograde labelling of the photoreceptors using DiI was also used to check the process of filling the cells with dye. DiI was purchased in paste form and applied to the back of excised retina. The tissue was kept alive for 24 h to allow faster diffusion of the dye. It was then fixed in 4% PFA in PBS, after which the dye continued to diffuse, but at a slower rate. The chamber in which the sample was held was kept moist with PBS and held at room temperature. The surface of the retina was checked periodically for any visible fluorescence. After two weeks, some of the retina was cut into slices as for the ERG studies (detailed in Chapter 3), to determine if any individual cells fluoresced.

All photographs were taken using a digital camera (Nikon coolpix 950, Japan), attached to an inverted microscope (Nikon inverted microscope).

5.2.6 Analysis

The response amplitude of the light-evoked ERG formed the basis of the analysis of the results from the first section of this chapter. Other parameters of the evoked ERG, such as shape, were not investigated, as they were not thought to be affected by gap junctions. Altering the number of functional gap junctions should result in a change the resistance of individual cells. And as V= IR, where V= voltage, I= current, and R= resistance, it is likely that a change in the cell resistance would result in a change in the response amplitude (V). The response amplitude was measured using the computer programme

Signal (version 2.03; CED, UK) and changes were examined using SigmaPlot 2001 for Windows (version 7.0; SPSS Inc, USA) and SPSS (Version 11.0.1; SPSS Inc, USA). To calculate the change in response amplitude, the amount by which the response amplitude decreased was divided by the initial plateau value and then multiplied by 100 to get the percentage change.

Any photographs taken of retina stained with dye were viewed and analysed, when necessary, using Adobe Photoshop (Adobe PhotoDeluxe Business Edition, 1999, Adobe Systems Incorporated). Photoshop was used to overlay photographs of the same piece of tissue, taken with the different fluorescence filters, so that any differences in the staining patterns of the two dyes could be easily seen.

5.3 Results

5.3.1 The effects of gap junction blockers on the maximum amplitude of the light-evoked ERG

5.3.1.1 Controls

Several controls were performed, to examine how the response amplitude of the light-evoked ERG changed over the duration of an experiment and to investigate if any of the chemicals used to dissolve the gap junction blockers had an effect on the ERG. These experiments showed that most of the slices remained healthy for up to 4 h (Figure 5.1). Eight slices from different animals, four tested on the same day the animals were killed and four tested on the day after the animals were killed, were perfused in normal ASW alone to monitor any changes that occurred without the presence of other chemicals over a 4 h period. In 75% of the slices, the response amplitude of the ERG took 40-50 minutes to level out. The retinal slices responded to flashes of light with an ERG, which was present generally for 4 h, but its response amplitude did not remain constant throughout this time.

It changed by a maximum of 20%, in those slices from which an ERG was recorded for at least 4 h. On only two occasions, did the response amplitude of the ERG reduce to zero within the 4 h. This was the maximum time subsequently allowed for perfusing chemicals on the slices, because any deterioration after this would not necessarily be due to the applied chemical, but simply to the worsening condition of the slice.

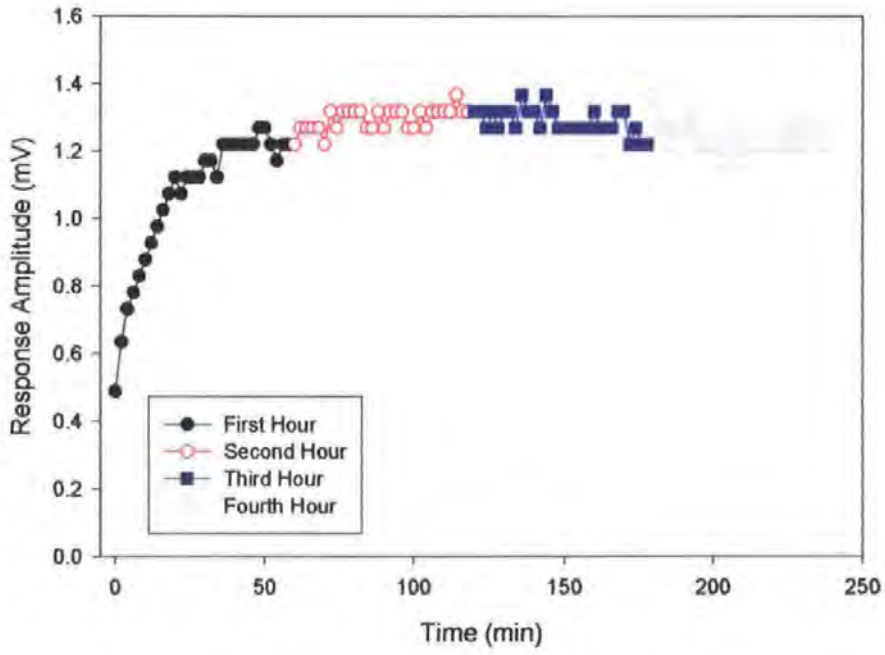


Figure 5.1: The effect of time on the response amplitude of the ERG recorded from a slice of tissue.

As ethanol was used as a delivery vehicle for octanol, heptanol and oleamide, its effect on the response amplitude of the ERG alone was tested. At the highest concentration used (1.5%), it had no effect on the maximum response amplitude of the ERG after 1 h perfusion in four of the five slices tested. In the fifth slice, the response amplitude increased by 200%, so 1.5% ethanol never caused a decrease in the ERG amplitude.

Carbenoxolone did not dissolve in normal ASW, so a substitute of PBS-ASW was used instead. When a slice was perfused with PBS-ASW alone, the response amplitude of

the ERG of four slices did not change, but it decreased by 50% in two slices. The effects of carbenoxolone were investigated only on those slices on which the PBS-ASW had no effect on the response amplitude of the ERG.

To prevent the oxidation of dopamine while it was in ASW, 50 μM NaMBS was used. Usually, when the NaMBS was tested by itself in ASW, it had no effect on the ERG, but on 3/8 times the response amplitude decreased. This proportion was similar to the 2/8 times the response decreased, while being perfused with normal ASW alone.

TTX is a voltage-sensitive sodium channel blocker (Moore *et al.*, 1967). It was used here to determine whether the action potentials generated by the inner segment layer of cephalopod photoreceptors formed a significant part of the ERG. This was because there is evidence to suggest that heptanol and octanol affect sodium channels, as well as gap junctions (Haydon & Urban, 1983; Guan *et al.*, 1997). By ruling out that voltage-sensitive sodium channels play a role in determining the ERG of *S. officinalis*, it can be said that any effects these long-chained alcohols had on the ERG amplitude were not related to them blocking voltage-sensitive sodium channels. TTX, at 1 μM concentration in ASW, did not affect the ERG shape, including amplitude, on the five occasions it was tested (Figure 5.2).

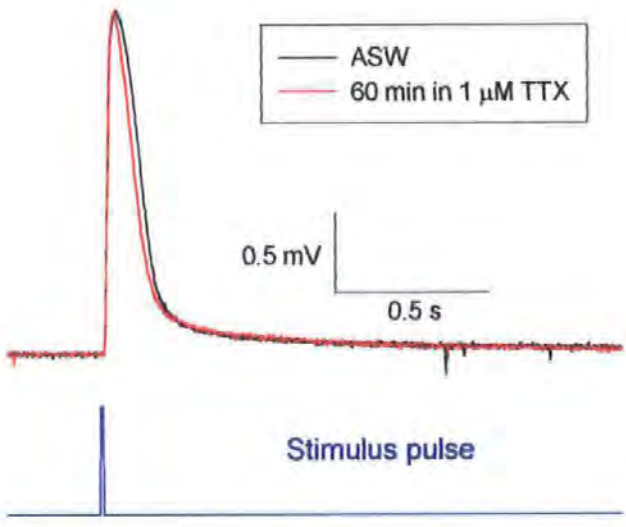


Figure 5.2: The electroretinogram of *Sepia officinalis* in normal ASW and after 60 min perfusion in 1 μM tetrodotoxin in ASW.

5.3.1.2 Gap junction blockers

Carbenoxolone and oleamide did not dissolve satisfactorily in the normal ASW used for perfusion, even when ethanol was used as a delivery vehicle. This problem was not overcome with the oleamide. It was still tested twice, but had no effect on the response amplitude of the ERG. Carbenoxolone, at 50 and 100 μM concentrations in PBS-ASW, had no effect on the maximum response amplitude of the evoked ERG of the six slices on which it was tested. Due to the problems in dissolving these chemicals, there was some uncertainty if they had no effect because they were not sufficiently dissolved, or because they failed to affect the gap junctions. Due to this ambiguity, these chemicals were not tested further.

Heptanol was tested at 500 μM, 1 mM and 2 mM concentrations in ASW. The lowest concentration caused a decrease in the maximum response amplitude of the evoked ERG by approximately 66% (67%, 71% and 60% in each of the 3 slices tested), which showed an almost complete recovery within 60 min (Figure 5.3). This happened with three slices, but in two slices 500 μM had no effect on the response amplitude of the ERG. Heptanol, at a concentration of 1 mM, only partially blocked the response amplitude (an average of 72% reduction: 66%, 90%, and 60% individually), and the ERG took longer to recover to its original amplitude than when the retina was perfused with the lower concentration of 500 μM. This again happened in 3/5 slices tested. The highest concentrations completely blocked the response in all four of the slices it was tried and the response amplitude took even longer to recover, once the tissue was perfused with normal ASW again (Figure 5.3). Heptanol reduced the response amplitude of the ERG to less than 10% of its size in ASW alone significantly more times with 2 mM heptanol, than with 500 μM and 1 mM heptanol (Fisher exact probability test, $p=0.042$).

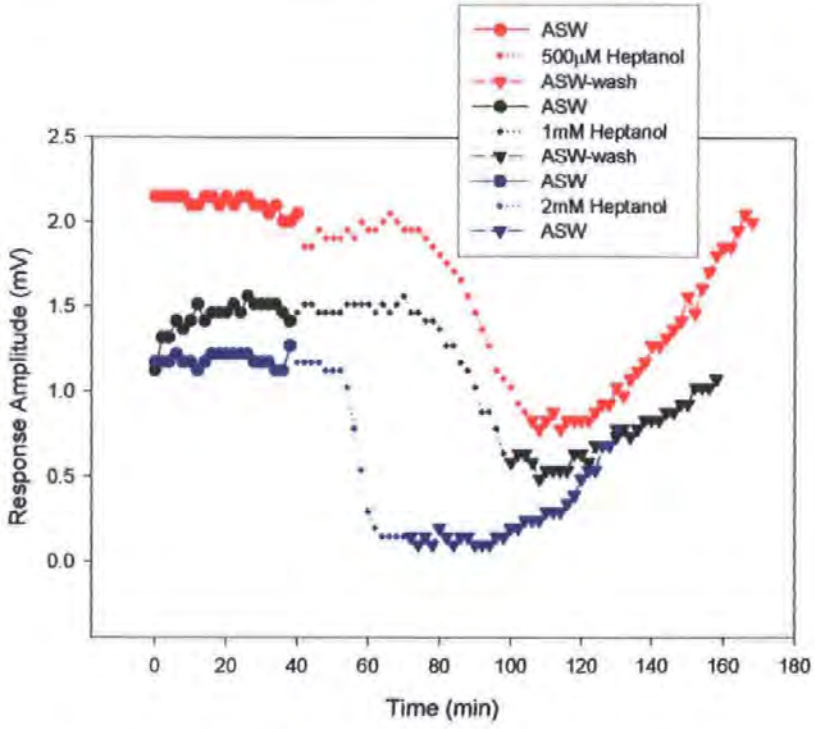


Figure 5.3: The effects of different concentrations of heptanol on the ERG amplitude.

Octanol was tested at 250 µM, 500 µM and 1 mM. With the two lower concentrations of this chemical, there was no effect on the ERG amplitude in 7/8 slices used. At the highest concentration, the response decreased, on average by 75 % (response amplitude decrease for each slice: 94%, 94%, 41%, 54%, and 88%), with all of the five slices tested and did not begin to recover within 60 min for three of the slices (Figure 5.4). On one occasion a slice was left longer with normal ASW to perfuse it, and the response amplitude of the ERG started to increase after 100 min of perfusion in ASW. There was a significant influence of octanol concentration on the response amplitude of the ERG (Fisher exact probability test, $p= 0.042$), as 1 mM octanol reduced the response amplitude of the ERGs from 4 slices of retina to less than 10% their original size, but octanol did not have such a dramatic effect at the two lower concentrations tested.

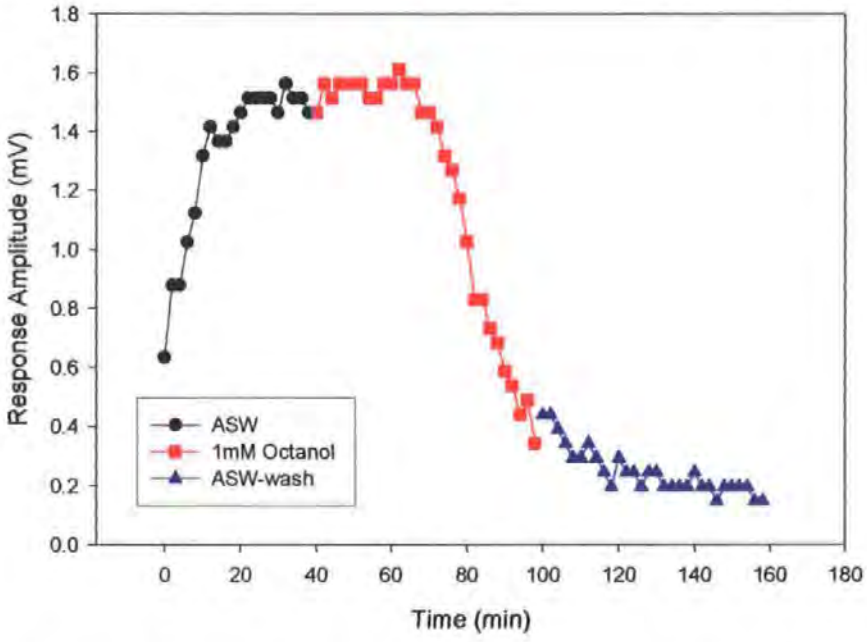


Figure 5.4: The effect of 1 mM octanol on the response amplitude of the ERG.

Comparing the previous two figures (Figure 5.3 and Figure 5.4), heptanol appeared to be more potent, as it decreased the maximum response amplitude of the ERG at 500 μM concentration in ASW, but octanol did not. This was contrary to published findings that the longer the carbon chain of the alcohol, the more potent it is (Rozental *et al.*, 2001), and is considered in more depth in the discussion (section 5.4.1). However, at 1 mM concentration of both chemicals took effect after 20 min of perfusion and had approximately the same effect of reducing the evoked ERG maximum amplitude by 70-75%, but the response took longer to recover when the slice had been perfused with octanol.

Dopamine was perfused at two concentrations, 10 μM and 50 μM in ASW, over retinal slices, as their ERGs were monitored. The maximum response amplitude of the ERG increased slightly and then decreased (Figure 5.5). In 80% of the slices on which 10 μM dopamine was tested, the response amplitude of the ERG increased and in three of these slices there was a subsequent decrease in the ERG amplitude. Although this initial increase was quite small (10-20%), it occurred in 60% of all the slices perfused with

dopamine, both 10 and 50 μM concentrations, implying that it was not just a random event. The response amplitude of the ERG always recovered from its decrease relatively quickly, once the dopamine, at both concentrations, was washed out of the perfusion dish. The complex effect of dopamine on the response of the retina is discussed later (Section 5.4.1).

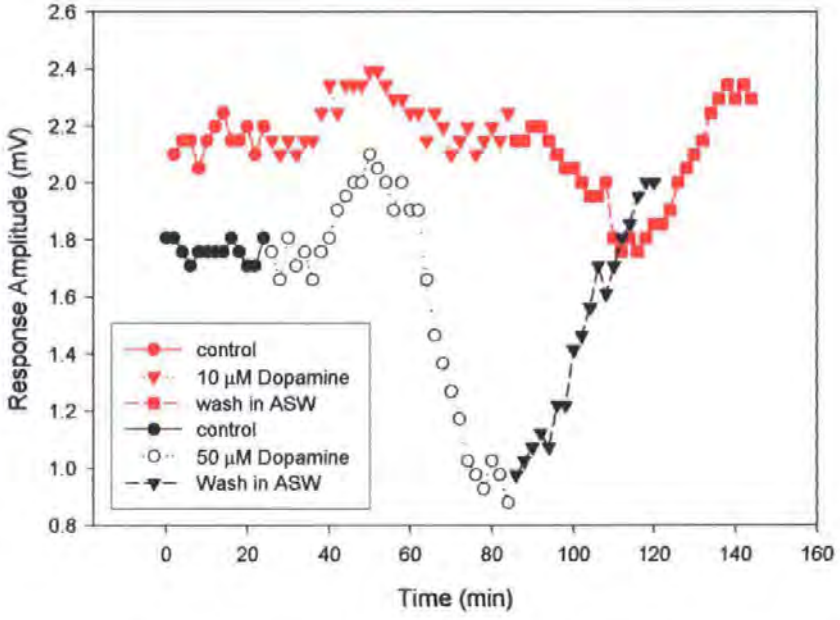


Figure 5.5: The effects on the ERG response amplitude of perfusing 10 and 50 μM dopamine over the retina of *S. officinalis*.

5.3.2 Dye injection results

Previous experiments, using the whole-cell clamp technique, in this laboratory showed that Lucifer yellow did not diffuse readily from one photoreceptor to its neighbours (Abdul Chrachri, unpublished results). Several attempts were made to inject Lucifer yellow and neurobiotin into cells penetrated by an intracellular electrode. The results were not as expected, as they did not produce a single filled cell, but resulted in a collection of dye within the inner segment layer of the retina. As the Lucifer yellow staining did not show the expected pattern, the slices were not processed further to allow the neurobiotin staining to be checked.

5.3.3 Using modifications of the scrape-loading technique to introduce dyes to the photoreceptor cells of S. officinalis

Several attempts were made to refine the technique of scrape-loading to obtain the expected results, without success. No conclusive results about the presence or absence of functional gap junctions in the retina of *S. officinalis* were obtained using variations of the scrape-loading technique.

The experiments can be split into two groups. The first group was performed on larger animals. Here retinal slices were scrape loaded with Lucifer yellow and neurobiotin and then viewed as whole mounts. The second group was performed on smaller animals, typically pre-hatchlings and hatchlings. These had FITC-dextran and neurobiotin loaded into the retina using GelFoam. They were sliced, after fixation, to check if the dyes were in individual cells. There were eight animals used in each group, and there were some differences made to the basic techniques within these two groups.

The first three attempts showed some areas of the retina stained with Lucifer yellow, but there was no neurobiotin staining found when looked on as a whole mount. Labelling of the photoreceptor cells with DiI was set up, and the cells allowed to fill for two weeks. The results of the DiI showed that if the animals were not fully dark-adapted, there was some screening pigment at the top of the photoreceptors that blocked the view of the fluorescence underneath (Figure 5.6). When the tissue was sliced (as in Chapter 3), the dye was clearly seen in the cells. It was then decided that presence of screening pigment at the tips of the photoreceptors was one possible reason for not seeing the dye in the whole mounts of some of the earlier preparations.

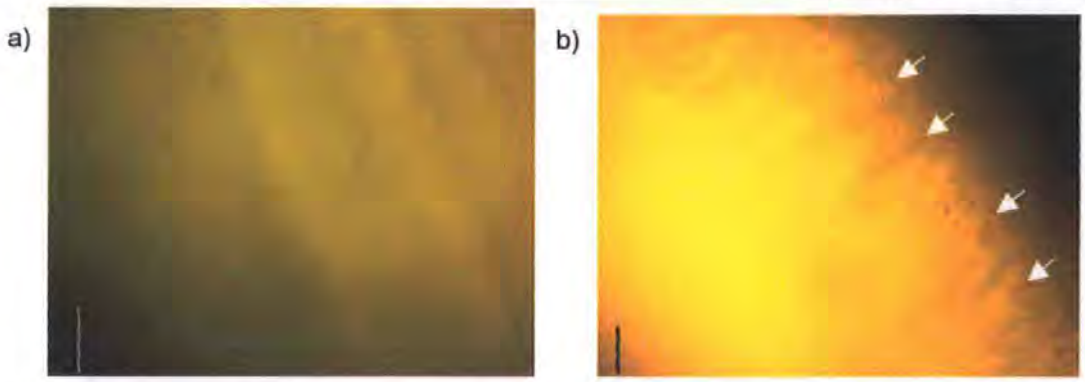


Figure 5.6: a) A whole mount view of a DiI stained retina, looking at the photoreceptor side of the retina. b) A slice stained with DiI, dots of screening pigment are visible at the photoreceptor tips, indicated by white arrows (scale bars = 100 μm).

A second check of the scrape-loading technique was done as follows. It was thought that perhaps the technique was working, but almost too well, in that the neurobiotin-CY3 was evenly distributed among all the photoreceptor cells of the retina due to the possible presence of a large number of properly functioning gap junctions, and so there was no clear positive staining of a group of cells. All tissue used before this was dark-adapted, the condition perhaps likely to have opened, functioning gap junctions. To investigate if the adaptational state of the tissue had an influence on the pattern of neurobiotin-CY3 staining, some light and dark-adapted, and light-sensitised (see Section 5.2.5) tissue was used in this preparation. This was more promising as there was differential staining between the FITC and CY3 (Figure 5.7), but still not what was expected, as there was no real difference between any of the treatments. It was expected that in dark-adapted tissue, and light-sensitised tissue, there would be more cells stained red with neurobiotin-CY3 than yellow with FITC-dextran. But with the light-adapted retina, only those cells stained yellow would also be stained red. This was not found in the experiments completed for this chapter (Figure 5.7). There was always a central core of yellow (FITC-Dextran), surrounded by a large amount of red staining (neurobiotin-CY3).

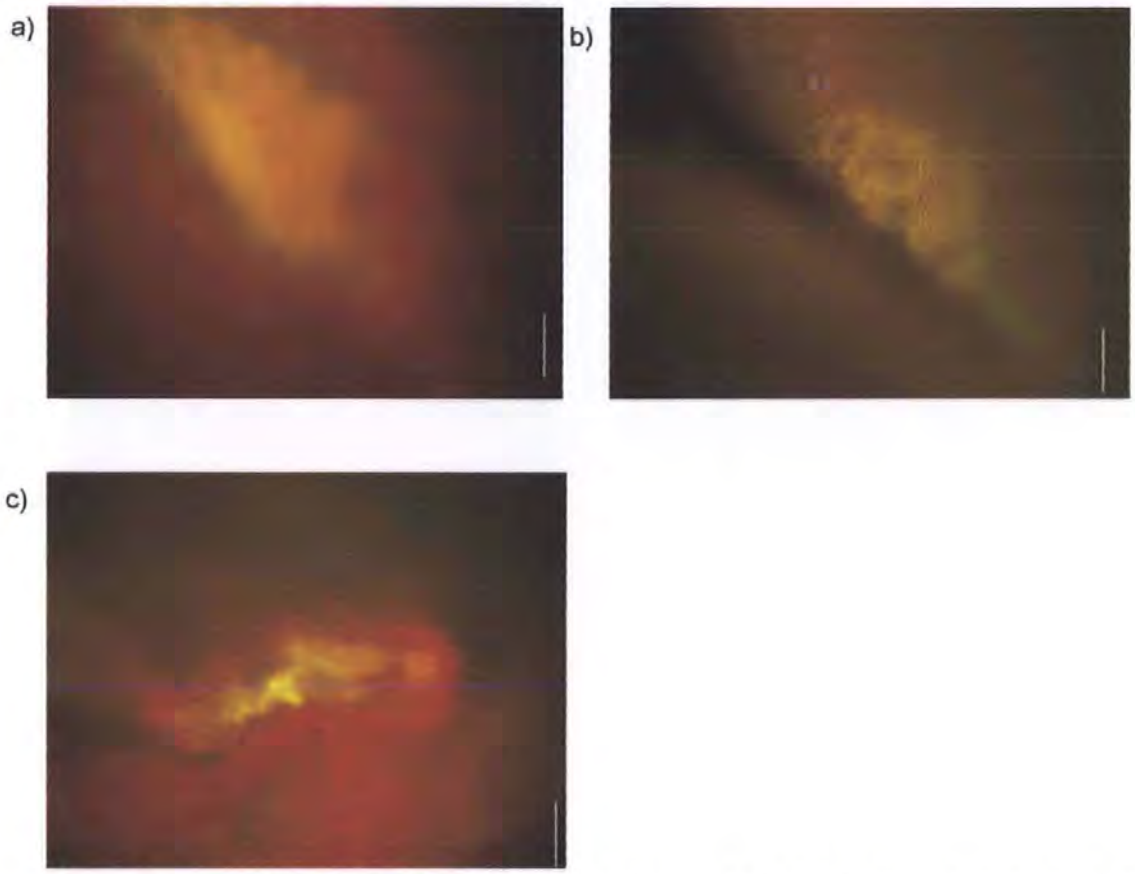


Figure 5.7: Whole mount views of the photoreceptor side of the retina: a) dark adapted, b) light adapted, and c) light sensitised (scale bar = 100 μm) (red=CY3, yellow=FITC).

To examine if the dye pattern would change, when gap junctions were artificially closed, the gap junction blocker heptanol was used. Heptanol, at 2 mM concentration, was applied to five pieces of retina, and at 5 mM to one piece for 60 min before the application of the dyes. As had already been shown in section 5.3.1.2, heptanol had an effect on the ERG of *S. officinalis*. Guan *et al.* (1997) showed that heptanol was a gap junction blocker, so even if heptanol had other effects in the retina of *S. officinalis*, it should also have closed the gap junctions. This did not appear to be the case, as there was still diffuse neurobiotin-CY3 staining, when it should just have more closely matched the FITC staining (Figure 5.8).

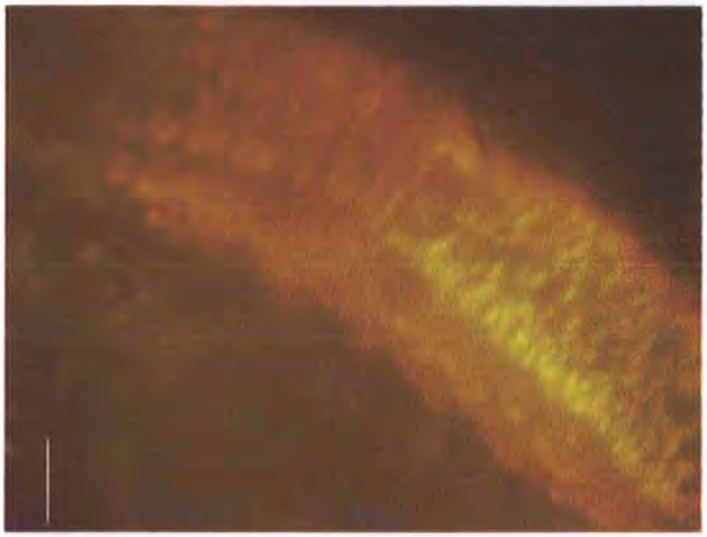


Figure 5.8: The staining patterns of a slice that was incubated in 5 mM heptanol. The yellow/green colour represents FITC-Dextran, and the red neurobiotin-CY3 (scale bar = 100 μm).

In all cells there seemed to be a faint background glow of red that was possibly due to the rhodopsin, or else to faint levels of neurobiotin/CY3 in all cells. To clarify this point, some pieces of tissue had neurobiotin alone put on them, and were then stained up using CY2, a green alternative to the red CY3. The results of this were that there was still a faint green glow due to the presence of neurobiotin in all the cells. Another check performed was to use FITC-dextran only, and to check the tissue for red fluorescence. This also showed a faint level of red fluorescence, which was probably due to the rhodospin (Figure 5.9). These two types of fluorescence could not be differentiated.

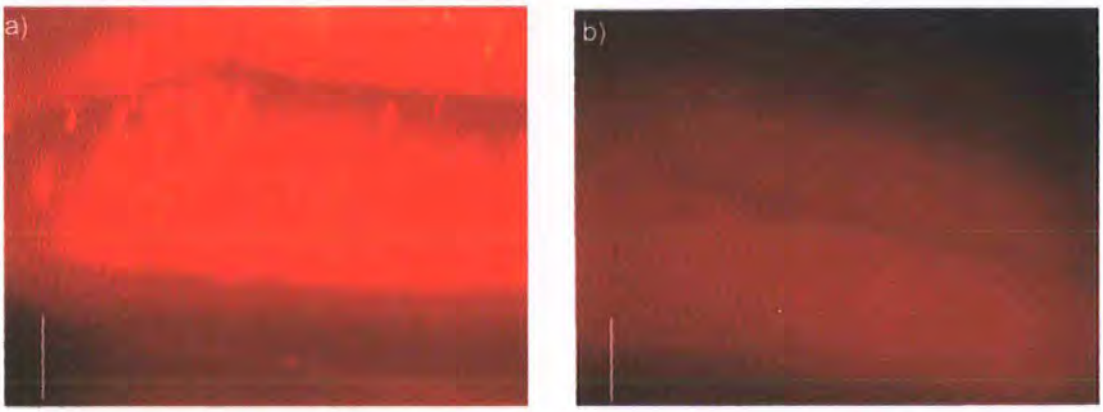


Figure 5.9: Two slices of retina, a) was stained with neurobiotin-CY3, b) was not (scale bar = 100 μm), but both have some degree of red fluorescence.

The conclusion of this section was that no definitive histological proof of the presence of functioning gap junctions was found, when using two dyes of differing molecular weight to investigate their presence. On many occasions, there were different staining patterns of the FITC-dextran and the neurobiotin-CY3, but this did not change under different experimental treatments. It helped to slice the tissue, once it had been fixed, to allow the staining patterns to be clearly seen. To ensure that the staining patterns were seen in whole mounts, it was better to fully dark-adapt the animal before beginning the experiment. FITC-dextran proved to be more successful than Lucifer yellow, due to its larger size, and as it could be fixed using paraformaldehyde.

5.4 Discussion

This study collected physiological proof for the existence of functional gap junctions in the retina of *S. officinalis*. The response amplitude of the ERG was affected by the presence of chemicals, previously proven to be gap junction blockers (Figures 5.3, 5.4 and 5.5). Several attempts were made to visualise the amount of cell coupling via gap junctions using dyes of different molecular weights, but none was entirely successful. Gap junctions were likely to exist between the photoreceptor cells, as shown by the differential staining of FITC-dextran and neurobiotin-CY3 obtained in the retina of *S. officinalis* (Figures 5.7 and 5.8).

5.4.1 The effect of gap junction blockers on the maximum response amplitude of the evoked ERG

The use of the ERG as a screening mechanism for gap junctions was a novel approach. This method has not been used in vertebrates, as the ERG is a very complex

response that represents the responses of at least five different neuronal cell types, with all of their different inter-cellular connections (McIlwain, 1996). The reason this technique was chosen to use with *S. officinalis* was that its positive ERG only represents the action of photoreceptors (Messenger, 1981), because there are no other neurons present in the cephalopod retina (see Chapter 1). Curtin *et al.* (2002) found that the presence or absence of functioning gap junctions affected the ERG of another invertebrate, *Drosophila melanogaster*, so it was possible that changes in the gap junctions would affect the ERG of *S. officinalis*.

Several controls were performed (section 5.3.1.1) to show that the ERG amplitude was relatively stable over time (Figure 5.1) and that chemicals used to dissolve the relatively insoluble gap junction blockers did not affect the ERG. Of these controls, the application of TTX was the most important. TTX blocks voltage-sensitive sodium channels (Moore *et al.*, 1967) and here it had no effect on the ERG (Figure 5.2). This showed that current flowing through voltage-sensitive sodium channels did not play an important role in determining the ERG. This was a necessary point to establish, as heptanol and octanol affect gap junctions, and can also affect sodium channels (Haydon & Urban, 1983; Guan *et al.*, 1997). TTX has already been shown not to affect the ERG of *Limulus polyphemus* (Kass & Barlow, 1984) and *Sepiola atlantica* (Duncan & Weeks, 1973), illustrating that *Sepia officinalis* is the similar to other cephalopods and invertebrates in this respect.

Heptanol, octanol and dopamine had dose-dependent effects on the response amplitude of the light-evoked ERG (section 5.3.1.2). Both heptanol and octanol decreased the amplitude of the ERG, but not in an entirely expected way. Heptanol is known to be less potent than octanol in *Aedes albopictus* (mosquito) (Weingart & Bukauskas, 1998; Rozental *et al.*, 2001), but the inverse was found to be true here. Octanol at a concentration of 500 μM in ASW had no effect, but the same concentration of heptanol partially blocked the response (Figure 5.3 and Figure 5.4). However, if the length of time

the effect of the chemical took to wash away was examined, it could be seen the octanol had a more lasting effect. Heptanol is twice as soluble as octanol in water (2 g/l and 1 g/l respectively, Sigma-Aldrich technical help), so the actual concentrations of octanol that were perfused over the slice were less than it was thought to be. Heptanol would be a more reliable chemical, of these two alcohols, to use as a gap junction blocker in cephalopods.

Dopamine caused an increase in the response amplitude of the evoked ERG initially, which was closely followed by a decrease in this variable (Figure 5.5). Dopamine is known to have many effects within the cephalopod retina. Makman *et al.* (1986) showed, using fluorescence histochemistry, that two dopamine systems exist in the cephalopod retina, one associated with the efferents from optic lobe, and the other with the photoreceptor cells. Gleadall *et al.* (1993) also investigated the effects of dopamine in the cephalopod retina and found that it affects the screening pigment migration and early receptor potential in *Octopus*. Suzuki & Tasaki (1983) showed that dopamine perfusion increased the size of the ERG, and that this effect was specific to the cell body layer. They implied that dopamine mimicked the effect of efferent stimulation in the retina, as both of these treatments reduced the receptive field of nerve bundles. Closing gap junctions also reduces the receptive field of cells (Werblin, 1991), so it is possible that dopamine achieves its reduction in receptive field size by affecting gap junctions. However in this chapter, dopamine was found to increase and decrease the amplitude of the ERG. One large difference between this study and that of Suzuki and Tasaki was that they removed all intrinsic dopamine from the eye using injections of reserpine in the days before experiments, but this was not done here. Perhaps when no dopamine was present, the addition of dopamine, simply increased the ERG amplitude, but when dopamine was already present, adding more dopamine might initially have had this effect, but there then might have been an excess of this chemical present, so the ERG reduced in size. It is likely that dopamine blocked the gap junctions present in the retina of *S. officinalis*, as it has been

shown to have that effect in many other species (e.g. Table 5.1), but that the other effects it has within the cephalopod retina complicated the change in the ERG response amplitude found in this study (Figure 5.5).

Heptanol, octanol, and dopamine have been shown to block gap junctions in a variety of preparations (Table 5.1), so they were expected to have similar effects on the response amplitude of the ERG of *S. officinalis*. This was not entirely the case here. Heptanol and octanol simply decreased the size of the ERG (Figure 5.3 and Figure 5.4), while dopamine showed an initial increase, followed by a decrease (Figure 5.5). An increase in the ERG amplitude was thought to be indicative of an increase in resistance in the retina due to blocked gap junctions. This implied that heptanol and octanol did not appear to block the gap junctions in the retina of *S. officinalis*. It might be that heptanol and octanol affected both light-sensitive and voltage sensitive sodium channels as well as gap junctions in the retina of *S. officinalis*. The application of TTX showed that the effects of heptanol and octanol were not due to their effects on voltage sensitive sodium channels. But if these alcohols also blocked the light-sensitive voltage channels in the retina, this would explain the decrease in the maximum response amplitude of the ERG. The perfusion of dopamine over the retina showed an increase, but also had other effects on the ERG amplitude, that reflected the many ways in which dopamine influences the cephalopod retina. The effects of these three chemicals had on the response amplitude of the ERG were removed by perfusing the retinal slices in normal ASW, implying their effects were reversible, as would be expected of by the closing and reopening of gap junctions. As the majority of gap junction blockers, currently used are non-specific (Rozental *et al.*, 2001), it was difficult to predict their precise effect on the response amplitude of the ERG of *S. officinalis*. This chapter showed that certain gap junction blockers had a large impact on the amplitude of the ERG of *S. officinalis*, but it cannot be said for certain that their impact was on gap junctions alone. They were all likely to block

the gap junctions present in the cephalopod retina, but their influences on other processes made their impact on gap junctions alone difficult to determine.

When these experiments were undertaken, it was thought that closing the gap junctions would result in a significant increase in the size of the ERG. This, however, might not have been the case. The ERG is a measure of the field potential in the retina (Ziedins & Meyer-Rochow, 1990) and as such records the sum of the responses in all the cells. If all the cells are exactly the same and stimulated to the same degree, then the ERG should not change, even if the presence of functioning gap junctions changes. If there are differences within the retina, either in cell type or illumination, then the ERG will change, as cells would be differentially stimulated, which would result in a disparity of the current flow from cell to cell through gap junctions. Two papers, which used invertebrates as their test species, found that changes in functioning gap junctions resulted in a change in the ERG (Suzuki & Tasaki, 1983; Curtin *et al.*, 2002). For this reason, examining the response amplitude of the ERG of *S. officinalis*, as the retina was perfused with different gap junction blockers, was considered a worthwhile experiment.

5.4.2 Attempt to measure dye coupling

Two main techniques were used to fill the photoreceptors of *S. officinalis* in an attempt to measure the presence of functional gap junctions within its retina. Using intracellular electrodes to inject dyes into individual cells did not work as predicted, as the cells failed to fill in the manner expected. The photoreceptor cells of cephalopods are long and thin (Messenger, 1981). When slices injected with Lucifer yellow and neurobiotin, were examined using the fluorescent wavelength to excite Lucifer yellow, large collections of the dye were seen in the inner segment layer. As the Lucifer yellow never stained the cells in the expected manner, the slices were not processed up for staining with CY3. One of the possible reasons this technique did not work was the nature of the technique itself. Intracellular recordings are known to affect the cells used (Nasi & Gomez, 1992; Takagi,

1994). They can depolarise the cell, which would cause the gap junctions to close (Gho, 1994). Intracellular electrodes can also cause leakage from the cell (Takagi, 1994), which is likely to have happened here, so that the Lucifer yellow, instead of spreading upwards into the outer segment, leaked out of the cell. Lucifer yellow is known to pass through some gap junctions (Spray & Bennett, 1985), but unpublished work by Dr. A. Chrachri (University of Plymouth, UK), who has used the voltage patch clamp method on cephalopod photoreceptors, has shown that this dye remains within one photoreceptor cell, and does not diffuse out of it through gap junctions.

The second technique used was a less invasive one. It involved soaking a tiny sliver of GelFoam in a FITC-dextran/neurobiotin mix, and applying this to the cut ends of the optic nerves from the retina of *S. officinalis*. This technique proved partially successful. Several attempts were made to obtain the expected level of performance by changing different variables of the technique, but none proved entirely satisfactory (section 5.3.3). The theory behind this technique was that FITC-dextran and neurobiotin should enter the same cells, and then the neurobiotin would diffuse to others that were linked to the original cells via gap junctions. FITC-dextran, with a molecular weight of 3,000, is too large to diffuse through the small gap junctions of 1.5 nm pore size, known only to allow the passage of molecules less than 1,000 molecular weight (Hardie, 1991a). The molecular weight of neurobiotin is 323, which means it should pass through gap junctions. The staining pattern of the two dyes within the retina was never as predicted.

The main problem with the technique of scrape-loading, modified several times, was that the neurobiotin-CY3 staining did not change under several different protocols designed to alter the presence of functioning gap junctions, i.e. different adaptation protocols (Figure 5.7; Pottek & Weiler, 2003) and application of chemical gap junction blockers (Figure 5.8; Rozental *et al.*, 2001). The most likely reason for diffuse neurobiotin-CY3 staining throughout the retina of most preparations was the solubility of neurobiotin. Neurobiotin has a high solubility in water (Vector Laboratories Catalogue)

and, as it is smaller than FITC-dextran, it should also be more mobile than this chemical. It was possible, therefore, that not only did the neurobiotin do as it was supposed to, but that it also diffused out of the GelFoam in every other direction, and hence stained many more cells than was predicted. This would account for the faint background staining present in most of the photographs taken (Figure 5.9). It would also mean that this background was probably too faint to see in the whole mounts. One possible way to improve this technique would be to remove the eye with the optic lobe still attached, to cut a small proportion of the nerves, and to put the GelFoam on these only. This should limit the amount of neurobiotin that diffused into all cells in the retina. Similar techniques based on the scrape-loading method have worked in other preparations, e.g. cell culture of hepatoma cells (Li *et al.*, 1996) and rat glial cells (El-Fouly *et al.*, 1987), which is why it was attempted with the retina of *S. officinalis* in this chapter.

5.4.3 Summary

This chapter showed that certain gap junction blockers, namely dopamine, heptanol and octanol, affected the response amplitude of the evoked ERG of *Sepia officinalis*, but no direct morphological evidence of dye coupling between the photoreceptors of its retina was found. Various reasons were proposed for this, but none of these excluded the presence of functioning gap junctions. Gap junctions have been shown to exist in cephalopod retina through electron microscopy studies, but no physiological studies have been done on any changes in their operation under different lighting conditions. If the scrape-loading technique tried here had worked successfully, it would have been combined with the different chemicals used to perfuse the ERG, to test their effect on gap junctions in the retina of *S. officinalis*. This would have offered a simple way of checking this, and other dynamic changes in the gap junctions, rather than using the more traditional electron microscopy methods.

Chapter 6 Intracellular Recordings from *Sepia officinalis* Retinal Photoreceptors

6.1 Introduction

Photoreceptors are neurons that respond with an electrical change when stimulated by light. They contain rhodopsin, which when activated by light, changes configuration, allowing the activation of second messengers and eventually leading to the opening of ion channels in the cell membranes of invertebrates (see Chapter 1). The opening and closing of different ion channels results in changes in the cell membrane potential due to the movement of ions into and out of the cell through these channels (Johnston & Wu, 1995). Impaling a photoreceptor with an intracellular electrode, and then stimulating it with light flashes, allows the change in its membrane potential due to this process to be recorded. To investigate different characteristics of a cell, the light used to stimulate it can be varied, as can the solution in which the cell is bathed. There have been many such studies in invertebrate species, but there have been few reports that used these techniques in cephalopods, and so far no published account of intracellular recordings from the retinal photoreceptors of *Sepia officinalis*. It remains unknown how similar the characteristics of individual *S. officinalis* photoreceptors are to other cephalopod and invertebrate species.

Most invertebrate photoreceptors depolarise in response to light stimulation, but vertebrate photoreceptors hyperpolarise (see Rayer *et al.*, 1990 for a review). There is only one known type of invertebrate photoreceptor that hyperpolarises to light, that of the photoreceptor in the distal retina of scallops (Fuortes & O'Bryan, 1972), but these animals also possess photoreceptors that depolarise to light. Duncan & Pynsent (1979a) applied a model for vertebrate photoreceptors written by Baylor *et al.* (1974) to data they recorded from those of *Sepiola atlantica*, a cephalopod, and found that the model fit their data very well with one exception. In the model of Baylor *et al.*, it was assumed that the final step of the visual process blocked the membrane channels, so causing the cell to hyperpolarize. If

it is assumed that the final step in cephalopods was to unblock the same type of channels, then the vertebrate model fits the cephalopod data very well (Duncan & Pynsent, 1979a). In vertebrates, the phototransduction cascade has been used to study the chemicals and enzymes involved in an entire functioning intracellular signalling pathway (see Jindrova, 1998 for a review). By developing the techniques used to detect intracellular changes in cephalopods, the similarities and differences in the phototransduction cascades of these two very different animal groups could be studied further.

Intracellular recordings have been used to measure different characteristics of photoreceptors. The sensitivities of individual photoreceptors of many species have been investigated by varying the intensity of the stimulus applied to the cells (e.g. Lange *et al.*, 1979; Ziedins & Meyer-Rochow, 1990). These types of measurements show how well an animal can cope with the light intensities it normally encounters in its natural habitat. For example, Chase (1974) characterised the photoresponse of *Tritonia diomedia* (a nudibranch mollusc) and found that it would respond to 1.8 $\mu W/cm^2$, which is slightly higher than the minimum light intensity in its habitat. To measure the sensitivity of a cell, the maximum amplitude of the intracellular response is important, but Millecchia & Mauro (1969) characterised the intracellular response of *Limulus polyphemus* (horseshoe crab) ventral photoreceptors and showed that the latency, as well as the amplitude of the response, changed according to the stimulus used. The shape of most invertebrate photoreceptor intracellular responses is that of an initial peak when the light is switched on, followed by a plateau until the light is switched off (Fuortes & O'Bryan, 1972). The decline from peak to plateau is thought to be an effect of adaptation (Millecchia & Mauro, 1969). By examining the effect of different flash durations on this part of the response, some conclusions can be made about adaptation in individual photoreceptors. The interval between the stimuli applied to the retina can give some indication of the nature of the photoresponse. If the stimulus interval is varied, and the response amplitude monitored, the recovery rate of the phototransduction cascade can be estimated. By varying the flash

intensity, duration and interval, various characteristics of the photoreceptors of *S. officinalis* were determined.

Cephalopod photoreceptors share most of their traits, determined in only eleven published papers, with other invertebrate rhabdomeric photoreceptor cells. In general, they depolarise in response to light, and at high intensities, their electrical response shapes consist of a transient peak followed by a plateau until the light ceases (Tomita, 1968; Pinto & Brown, 1977; Cobb & Williamson, 1998a, b, 1999). The sensitivities of some octopus and squid species have been measured using intracellular recordings (Tomita, 1965; Tomita, 1968; Pinto & Brown, 1977). These previously published papers on various squid and octopus species and *Sepiola atlantica*, however are few in number and, they concentrated on the effects of chemicals on the response, rather than on those of the stimulus characteristics. In this chapter, intracellular recordings from the photoreceptors of *S. officinalis* were used to determine the sensitivity of a single cell, and then to further characterise some of the properties of these cells, as there is no published account of this type of experiment in this species.

The success of some previously published studies in obtaining intracellular responses from narrow cephalopod photoreceptors showed that this technique might provide useful data on the characteristics of individual *S. officinalis* retinal photoreceptors.

The main aims of this chapter were:

- to characterise the intracellular light response of individual photoreceptor cells of *S. officinalis*, including measuring their sensitivity,
- to compare their characteristics with those of photoreceptor cells of other cephalopod, invertebrate, and vertebrate species, and
- to test the suitability of using intracellular recordings for future experiments into the nature of the cephalopod photoresponse.

6.2 Materials and Methods

6.2.1 *Animals*

Two size cohorts of *S. officinalis* were used in this study, one ranging from 13-21 cm in mantle length (ML) (13 animals), and the other from 2-6 cm ML (27 animals). An animal was dark-adapted for thirty minutes before beginning experiments (Clark & Duncan, 1978). This was done between 08:30 and 09:30, which was in the light phase of the animal's circadian cycle. It was anaesthetised by immersion in 2% ethanol in seawater, humanely killed by decapitation, and then the eyes excised (Cobb & Williamson, 1998a). The anterior portion of the eye and the sclera were removed and pieces of retina, approximately 1 cm^2, dissected free. One of these was pinned receptor side down onto Sylgard (Farnell, UK) in a recording dish, and perfused with chilled artificial seawater (ASW: see Chapter 3 for recipe). The other pieces of retina were kept on ice, bubbled with atmospheric air and used the following day, because there was a limited number of animals available for experiments.

6.2.2 *Electrophysiological set-up and apparatus*

The electrophysiological set-up used to make intracellular recordings was the same as that detailed in Chapter 3 for extracellular recordings (see Section 3.2.2), with the following differences. Intracellular recordings were obtained using electrodes of 80-150 MΩ, filled with 4 M potassium acetate. An electrode was advanced into the retina, from the optic nerve side, until the noise level recorded by the electrode increased. A piezo-electric stepper (MPM-20, WPI Ltd, UK) was then used to penetrate the cells. Successful penetration of a photoreceptor was indicated by a sudden drop in resting potential (to more negative than –30 mV) and a light response significantly larger than the electroretinogram (ERG) of the surrounding tissue. The stimulating light, a yellow LED (RadioSpares, UK),

was positioned underneath the recording dish to allow direct stimulation of the photoreceptors (see Figure 4.1). Where necessary, the light intensity was attenuated using neutral density (ND) filters (Edmund Optics, UK) (Table 6.1). The irradiance of the stimulus light was measured at the end of each day using an optical power meter (TQ8210, Advantest, Japan).

ND filter	LI ($\mu W/cm^2$)
None	20.0
0.15	8.0
0.3	5.0
0.4	4.5
0.6	3.1

ND filter	LI ($\mu W/cm^2$)
0.7	3.0
0.9	2.5
1.2	1.2
1.5	0.4
2.5	0.2

Table 6.1: The light intensity (LI) passed by each of the neutral density (ND) filters used, when used in conjunction with a yellow LED[1].

6.2.3 *Stimulus protocols*

Once a cell was successfully impaled, its light response was monitored, using test stimulus flashes of 50 ms, at the maximum light intensity of 20 $\mu W/cm^2$, and separated by 30 s. Usually, the maximum response amplitude to a test flash of light increased, and the resting potential became more negative, within the first few minutes after penetrating a cell (Clark & Duncan, 1978). Once the amplitude of the response levelled out, the stimulus characteristics were modified. Flash duration and interval were changed using a pulse generator (D4030, Digitimer, UK), while flash intensity was changed using ND filters (Table 6.1).

Initially, the light characteristics were varied, using ranges in previously published papers as guidelines, to estimate the optimal ranges to use (e.g. Tomita, 1968; Pinto &

[1] These light intensities were different for those used in chapters 3 and 4, as the LED was in a different position.

Brown, 1977; Duncan & Pynsent, 1979a). Using this information, protocols were established to vary each of these (the stimulus interval, duration and intensity) in a repeatable way.

1) Flash Interval: To investigate if flash interval affected the intracellular response of *S. officinalis* photoreceptors, the following inter-flash intervals were tested: 10, 20, 30, 40, 50 and 60 s. The tissue was flashed with a 50 ms flash of maximum intensity, using each of these intervals for a total of 5 min. This meant that if an interval of 60 s was used, then 6 flashes were applied to the tissue in total, but if 30 s was the interval used, then 11 flashes were given. The responses to the first and last flash of each run were used in the analysis.

2) Flash Duration: To examine how flash duration affected the intracellular response, the flash interval was held constant at 30 s and the flash intensity was held at its maximum, while the flash duration was increased through the following steps: 1, 2, 5, 10, 15, 20, 30, 40, 50, 75, 100, 150, 200, 350, and 500 ms. Sometimes the series was stopped at 200 ms, to allow more experiments to be completed on one cell.

3) Flash Intensity: To measure the changes in the intracellular response to different flash intensities, the flash interval and duration were held constant, while flash intensity was varied. The protocol was to give flashes of 50 ms separated by 30 s, increasing the light intensity with each flash (see Table 6.1 for relevant intensities).

If an intracellular recording was maintained for long enough to complete one of the above protocols, 3 flashes of 50 ms at maximal intensity separated by 30 s were applied to the tissue. The response size and shape to these standard test flashes were compared to those recorded before the protocol of altering a stimulus characteristic was applied to the tissue. If the size and shape of the intracellular response had not changed, then another of the three variables was investigated. Once the cell began to deteriorate, i.e. its response amplitude to the standard flash decreased and/or its resting potential increased, the electrode was removed from the cell until the potential returned close to zero. This

potential reading was subtracted from the most negative potential recorded when the electrode was inside the cell to calculate the true resting potential of the cell.

6.2.4 *Histological study of the retina to examine any morphological changes over the time scale of an experiment*

To ascertain if keeping the excised retina overnight affected its morphology, a histological study was undertaken of pieces of retina at different times after dissection. Freshly dissected tissue and tissue 24 and 30 h after dissection were fixed in 4% paraformaldehyde, dehydrated, put into wax, and sectioned. Some slices of the retina were rehydrated, stained overnight in Toluidine blue (Sigma-Aldrich, UK), and then dehydrated again. The sections of retina were viewed to determine if there were any obvious morphological differences between them.

6.2.5 *Analysis*

For a preliminary analysis of the basic characteristics of the retinal photoreceptors of *S. officinalis*, all intracellular recordings that were maintained for longer than 5 minutes were used. Intracellular recordings from cells that lasted longer than 20 minutes were checked for stability, and analysed further if they proved to be stable. The stability of a recording was checked by comparing responses to standard test flashes applied to the retina at different times within the overall protocol. If the responses to the standard flashes were similar in size and shape, the response was analysed further using Signal (Version 2.03, CED, UK) and SigmaPlot 2001 for Windows (version 7.0; SPSS Inc, USA). While this greatly reduced the number of cells analysed, it meant that greater confidence could be placed in the results found (Duncan & Pynsent, 1979a).

The effects of flash interval on the photoresponse were briefly analysed, by overlaying the intracellular response traces to see if the shapes were the same before and after a given amount of stimulation and by measuring the maximum amplitude. Flash

duration and intensity effects were examined more thoroughly using four variables: maximum amplitude, latency, decay constant(s) and number of spikes. The latency of the response was defined as the time from the start of the stimulus to the start of the response. Decay constants were measured using the curve fit tool in Signal. Large spikes were easy to count, but spikes smaller than 1 mV in size were difficult to count, so the numbers given were an estimate.

SPSS (Version 11.0.1; SPSS Inc, USA) was used to carry out the linear regressions and analysis of variance (ANOVAs) when required. Statistical tests were not performed on the data collected when the flash interval and flash intensity was varied (Sections 6.3.2 and 6.3.4) due to the small number of cells recorded from successfully. If the same trends were generally seen in four or more cells, they were considered to represent what occurs in most other cells, and were thought unlikely to be anomalous.

6.3 Results

6.3.1 *General characteristics of the retinal photoreceptors of* Sepia officinalis

Individual photoreceptors had membrane resting potentials of –58 ± 14.1 mV (mean ± standard deviation (sd); n=42, two ML cohorts grouped) when dark-adapted. They responded to a controlled flash of light (50 ms duration, 20 $\mu W/cm^2$ intensity) with a depolarising response of up to 53 mV (30 ± 12 mV). The maximum response amplitude recorded from a cell was correlated significantly with its resting potential (r^2=0.3856, df=38, $p<0.05$; Figure 6.1). Intracellular recordings were maintained for different durations of time, varying from 20 to 210 min, with a mean of 46 ± 41 min. There was no relationship between the duration a cell was recorded from and either its resting potential, or its maximum amplitude.

As the retina was approached somewhat blindly, care had to be taken that the electrode entered a photoreceptor cell and not a supporting cell. Each time the resting potential dropped to a negative value indicating that a cell had been penetrated, the preparation was flashed with a control stimulus. A note was made of whether or not an intracellular light response was recorded. The supporting cells of the retina should not have had any intracellular light response. Of the 381 individual times the resting potential recorded by the electrode dropped below –30 mV, 165 had a light response bigger than the ERG of the surrounding tissue. Supporting cells were penetrated by an intracellular electrode significantly more frequently than photoreceptors (Chi-square=6.56, df=1, $p<0.05$).

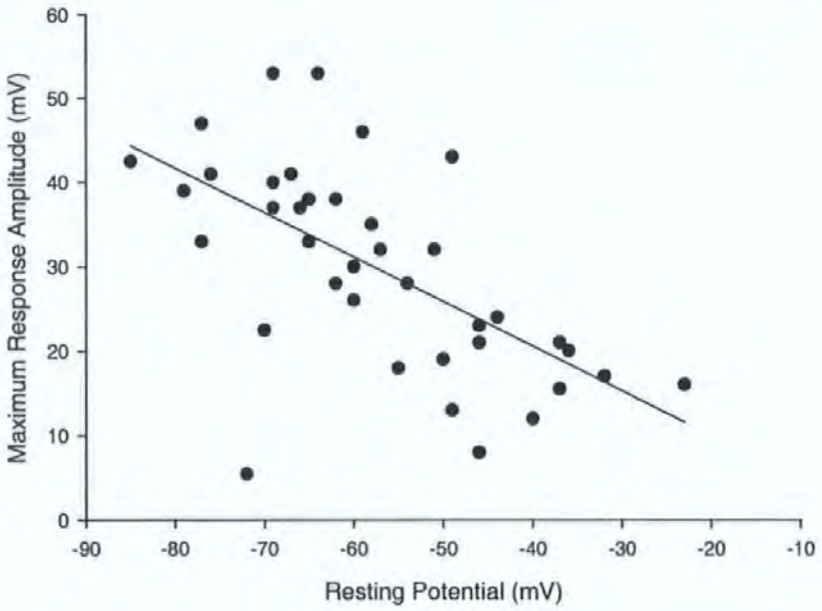


Figure 6.1: The relationship between membrane resting potential and maximum response amplitude of the light-evoked intracellular response recorded from different cells. The equation of the regression line is y = -0.53x – 0.58 (r^2 = 0.385, df = 38, p<0.05).

Clear bursts of action potentials, or spikes, in response to light stimulation were observed in 35% of the stable (for criteria see Section 6.2.5) intracellular recordings (see Figure 6.2 for example). The size of the spikes ranged from 0.67 to 20 mV (3.5 ± 5.7 mV, mean ± sd, n=12), but ones as large as 20 mV were rare. In general, cells with larger intracellular responses had smaller spikes, although this linear relationship was not statistically significant (r=0.31, df= 10, p>0.05) (Figure 6.3). Spike size was not correlated with the durations for which cells were held or the resting potentials of these cells. There were no differences in the number of times spikes were in fresh or day-old tissue. Sometimes the spike amplitude and frequency did not remain constant over the time a cell was monitored, which was thought to be an early indication of cell deterioration.

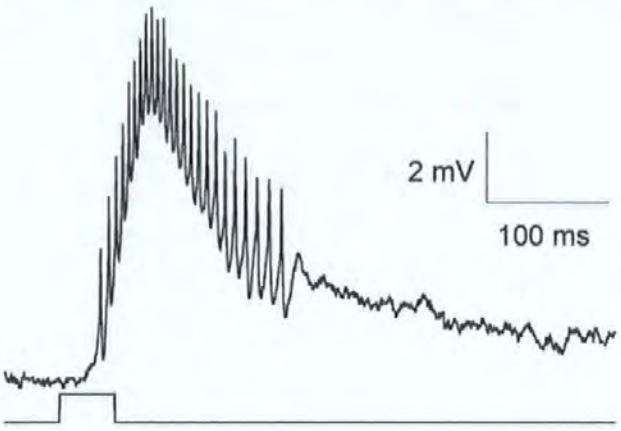


Figure 6.2: An example of the action potentials, or spikes, superimposed on the light response, observed in some intracellular recordings from photoreceptor cells. The lower trace represents the timing of the stimulus.

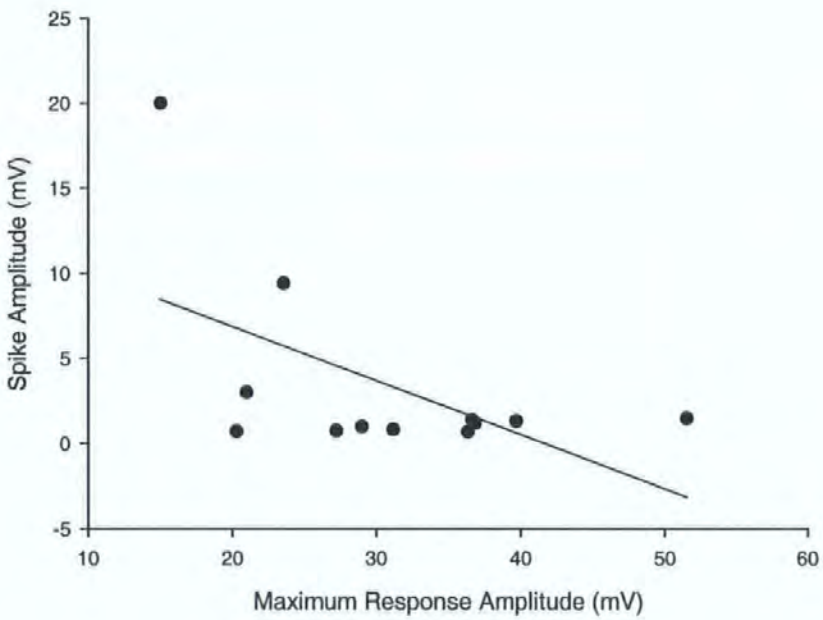


Figure 6.3: The relationship between spike amplitude and the maximum amplitude of the intracellular response. The equation of the linear regression line is y = -0.32x + 13.21 (r^2=0.31, df=10, p>0.05).

Of the 42 recordings used in the preliminary analysis above, only 17 were found to be stable using the criteria of section 6.2.5 (12 of which had spikes). All of these

recordings were obtained from the tissue of larger animals, so the success of recording an intracellular response seemed dependent on the ML of the animal (see Section 6.4.4). Two cohorts of animals, based on their ML, were used for these experiments. In the cohort of smaller sized animals (2-6 cm ML), an intracellular response was obtained in only 29% of the animals employed, and none of these proved to be stable. The larger cohort (13-21 cm ML) had a higher overall success rate in that intracellular recordings were obtained from 75% of animals used. Approximately half (8) of the 17 stable recordings were obtained from the tissue on the day after the animal was dissected.

The response of these 17 stable cells to the test flash was examined in detail. There was an unexpected difference in that the light induced response of ten of the cells had a single decay constant, but in the responses of seven cells the decay constant was best fitted using two time constants (Figure 6.4). This difference was not related to the time interval between the dissection of the animal and the performance of an experiment on its tissue. In all cases where two decay constants were present, the first was always much shorter than the second. The first decay constant was usually less than 0.1 s^{-1}, but the second was always greater than 0.3 s^{-1}. If however there was only one decay constant to a response, its value was between 0.1 and 0.3 s^{-1}. The decay constant measured how quickly the response returns to baseline levels, so the presence of two indicated that this was a stepped process in some cells. The existence of two decay constants in an intracellular recording was not related to the presence of spikes in that recording (Fisher's exact probability test, $p>0.05$).

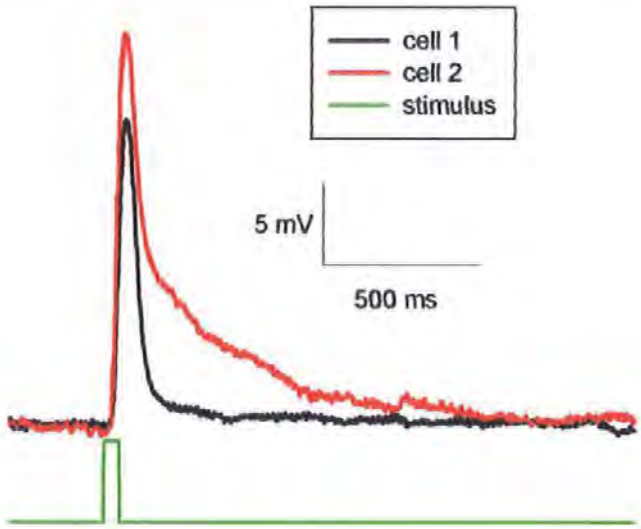


Figure 6.4: The intracellular response of two cells to a 50 ms flash. Cell 1 has a single decay constant, but cell 2 has two decay constants.

6.3.2 *The effect of flash interval on the intracellular response*

The effect of altering the stimulus interval used to elicit intracellular photoresponses was investigated in four stable cells. The inter-flash intervals tested ranged from 10 s to 60 s, and it was found that this has no effect on the intracellular response shape or amplitude (Figure 6.5). Statistical tests, such as ANOVAs, were not performed on these data, as only four cells were used, which was too low a number to allow any certainty in the outcome of such tests. None of these four cells showed a dramatic change in their maximum response amplitude with the change in flash interval tested (Figure 6.5). To examine this using a quantitative approach, best fit linear regressions were applied to the data from each cell recording presented in Figure 6.5. The slopes of these best fit lines were then compared to zero, as a slope of zero would indicate that there was no trend in the response amplitude caused by increasing the inter-flash interval (Table 6.2). Each of the slopes in Table 6.2 is extremely close to zero, indicating that increasing inter-flash interval did not cause a noticeable trend in the maximum amplitude of the response. These data also showed that the recovery time of these photoreceptors was <10 s, as even with 10 s

inter-flash interval the response always had the same amplitude. From then on, all test flashes were separated by 30 s, as it was mid-way through the intervals tested. If the intracellular recording was unstable, the flash interval was found to have an effect on the response.

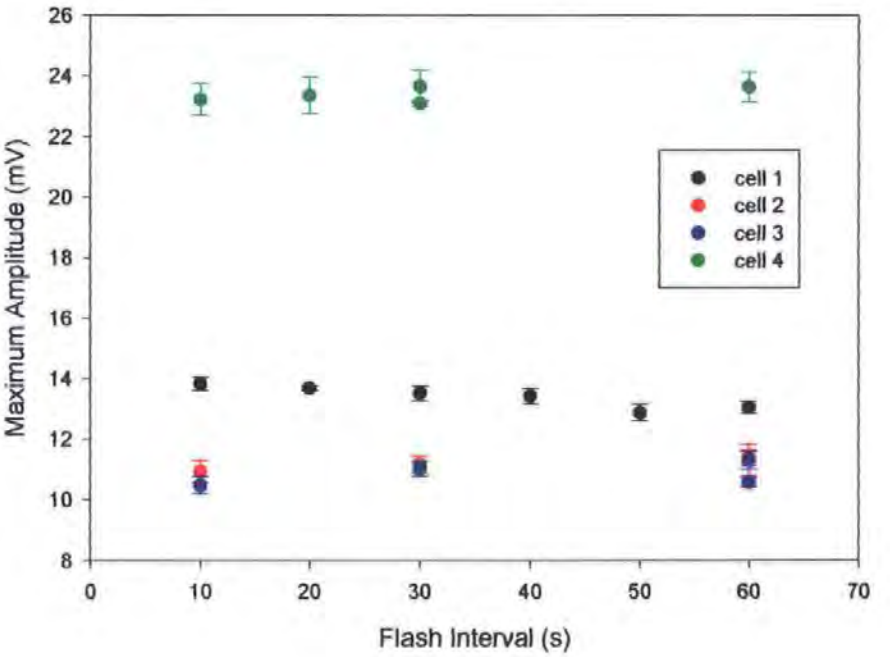


Figure 6.5: The influence of flash interval on the maximum amplitude of the intracellular response. Each colour represents a different cell. The error bars represent +/- one standard error. n depended on the number of flashes given in a 5 min period, i.e. 7 for 60 s interval, but 31 for 10 s interval.

Cell	Slope (mV/s)	r^2	df	P
1	-0.02	0.74	4	<0.05
2	8.12×10^{-3}	0.95	2	<0.05
3	7.88×10^{-3}	0.21	2	>0.05
4	7.17×10^{-3}	0.37	3	>0.05

Table 6.2: The slope of the best fit lines of the data from each cell shown in Figure 6.5.

6.3.3 *The effect of changing the flash duration on the intracellular response*

The effect of varying flash duration on the light response was investigated using twelve of the stable cells. In 66% of them, the response shape changed in the following way. With short stimulus flashes, the response was simply a small positive deflection in the resting membrane potential. As the flash duration increased, the size of this deflection grew, but it remained a simple response. Once a critical flash duration was reached, the response shape changed to have a transient peak followed by a plateau. For even longer flashes, the amplitude of the peak remained the same, but the duration of the plateau lengthened (Figure 6.6).

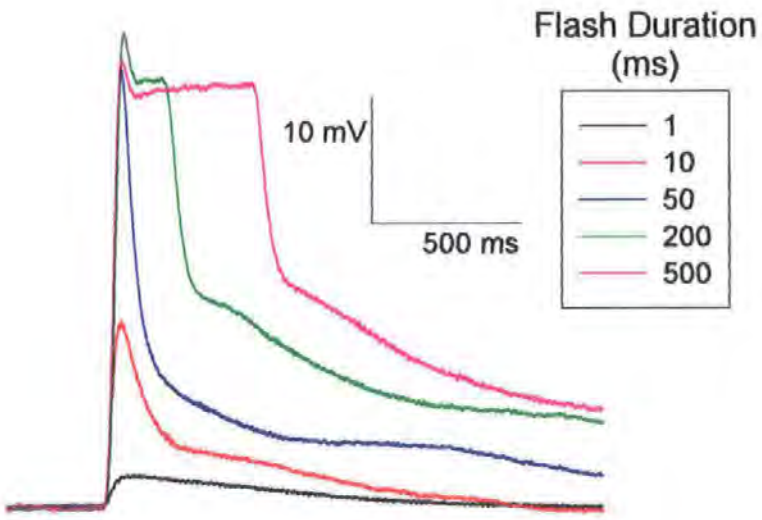


Figure 6.6: The change in the intracellular response shape with changing flash duration.

With five cells, the transient peak was much smaller, and the plateau was not a true plateau, as the response continued to increase to a greater amplitude than the initial peak (Figure 6.7). This different shape was only recorded from cells in tissue that had been dissected the day before experiments were undertaken.

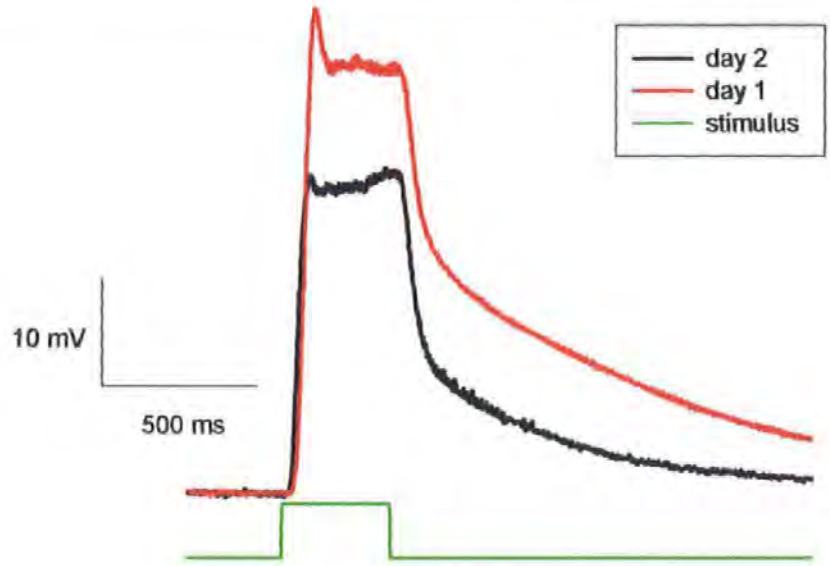


Figure 6.7: Representative traces from two cells, one recorded on the day of dissection (day 1) and one recorded on the day after (day 2) to illustrate the difference in response shape to a 350 ms flash.

All twelve intracellular recordings used to investigate the effect of varying the stimulus duration on the intracellular response showed an increase in maximum response amplitude with an increase in flash duration until a flash duration of 45 ± 13 ms (mean ± sd) was reached. When flashes of this duration and longer were used, the maximum amplitude plateaued, despite increasing flash duration (Figure 6.8). This corresponded to the change in response shape. The flash duration at which the plateau in maximum amplitude began varied from cell to cell, but lay within a range of 30-75 ms (45 ± 13 ms). As 50 ms lies in the middle of these two values, this was subsequently used as the standard test flash duration. A repeated measures ANOVA was performed (as the data came from the same cells) on the maximum response amplitude values obtained using flash durations of 1, 10, 20, 50, 100, and 200 ms. The maximum response amplitude increased by a significant amount as the flash duration increased (Wilks' Lambda = 0.1, $F_{(5,13)} = 16.4$, $p < 0.001$, Eta squared = 0.9). Table 6.3 shows the relevant means of maximum amplitude and

standard deviations obtained using these stimulus durations, and also showed that the maximum amplitude of all cells levelled out with flash durations of 50 ms and greater.

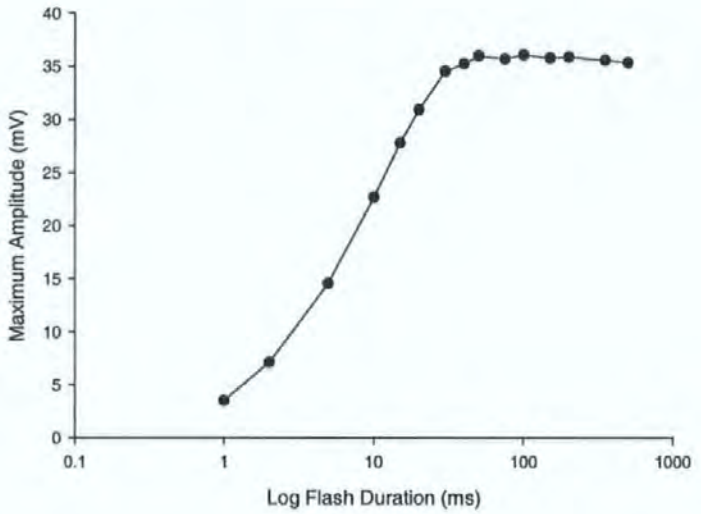


Figure 6.8: The relationship between the logarithm of the flash duration and maximum amplitude of an intracellular response (one cell shown as an example).

Flash Duration (ms)	Mean	sd	n
1	2.0550	1.13816	14
10	13.9616	9.08688	14
20	21.0833	10.54642	14
50	26.4709	12.21213	14
100	25.3427	12.13269	14
200	25.9450	12.12656	14

Table 6.3: The mean maximum response amplitudes and standard deviation (sd) of the intracellular responses elicited with different flash durations.

Four of the 12 recordings were stable enough to allow the flash duration protocol to be completed more than once. Of these four cells, three showed responses, which were very close matches between the first and second runs of the flash duration protocol. For the fourth intracellular recording, the amplitude of the transient peak increased between the

two times this protocol was applied to the tissue, but the flash duration at which the amplitude reached its plateau (i.e. when the transient peak appeared) remained the same.

The latency of the evoked response ranged from 16 to 45 ms, when measured using stimulus flash durations of 1 - 200 ms. Flash duration significantly affected the latency of the evoked intracellular response (Repeated measures ANOVA: Wilks' Lambda = 0.15, $F_{(5,10)}$=5.65, p=0.03). The response latency decreased to a plateau with increasing flash durations (Figure 6.9). The response latency reached a plateau with shorter flash durations (10 – 20 ms) when compared with the flash durations that elicited the plateau with the maximum amplitude of the response. Again in cells where the response to stimulus duration was measured more than once, there was agreement between the repeats.

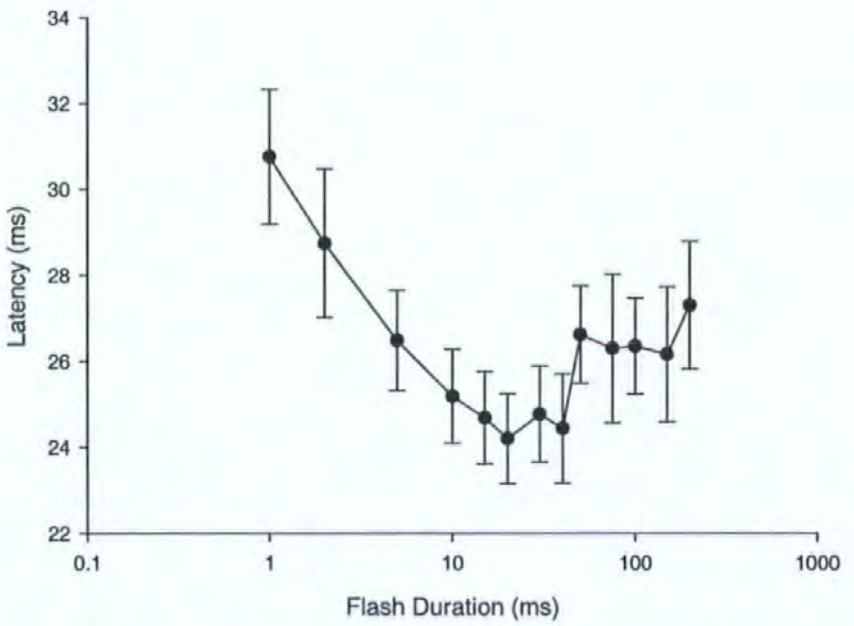


Figure 6.9: The effect of increasing flash duration on the latency of the intracellular response (n = 12 cells; error bars represent +/- one standard error).

The effect of increasing the stimulus duration on the decay constant of the intracellular responses was analysed carefully, due to the presence of two decay constants in some recordings. When two decay constants were present in an intracellular response, the first one was compared with the single decay constant of other responses, as these two

types of decay constant were most similar. When these decay constants were compared with changes in flash duration, they showed a typical pattern of decreasing with increasing flash duration until it reached a plateau (Figure 6.10). The first decay constant levelled out at a maximum of 0.1 s^{-1} (or smaller). It reached its plateau at approximately the same flash duration as did the maximum amplitude, i.e. 30 – 50 ms. The second decay constant was more variable, and it showed no pattern with increasing stimulus duration.

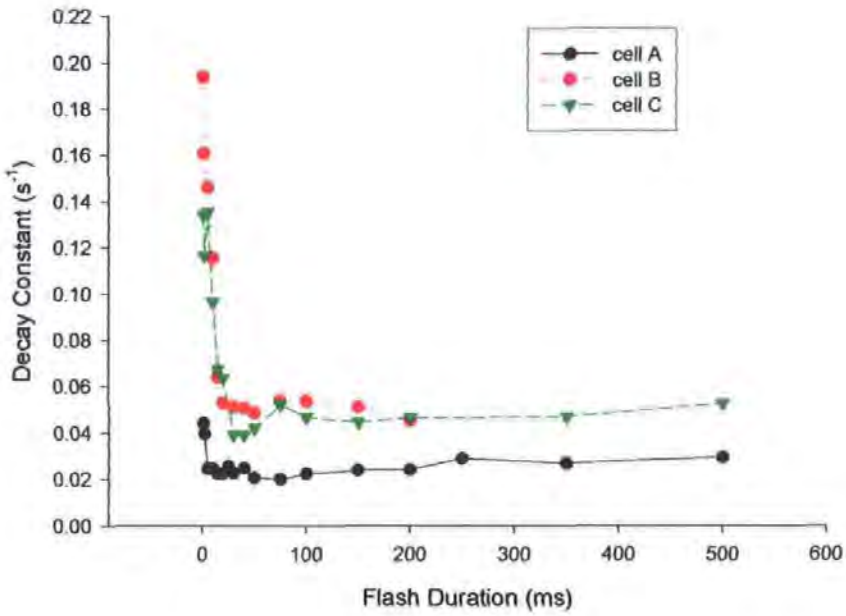


Figure 6.10: The effect of increasing flash duration on the decay constant of three representative cells.

Spikes were present in eight of the twelve stable recordings from photoreceptors and these were used to examine the effects of flash duration. In all but two of these the number of spikes increased as the flash duration increased. For some cells, the rate of increase was somewhat faster for shorter flashes than longer ones, so the pattern they exhibited was partially logarithmic, as it never reached a plateau. When the data from all the cells were considered together, a linear regression line was found to fit the data well (r^2= 0.63, df=120, $p<0.01$) (Figure 6.11).

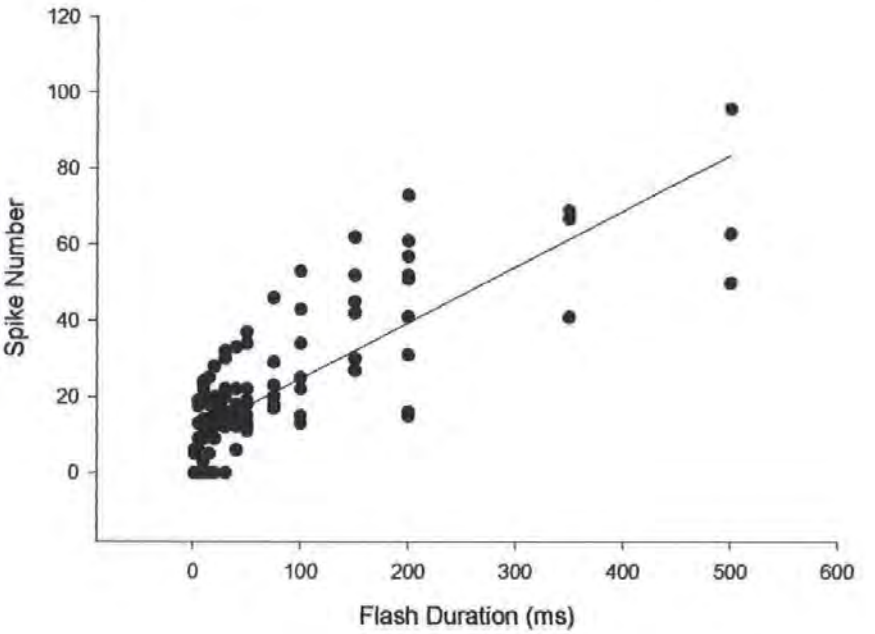


Figure 6.11: The change in spike number with an increase in stimulus flash duration. The equation of the best fit line was y = 0.15x + 10.03, r^2 = 0.63, df = 120, p <0.01.

6.3.4 *The effect of changing flash intensity on the intracellular response*

Since each experiment examining the relationship between the intensity of the stimulus flash and the photoreceptor response used seven different intensity levels, with each intensity repeated three times, it took 10.5 min to complete, so five of the cells recorded from were held for the complete series. The overall shape of the evoked response did not change dramatically with increasing light intensity, but became larger and faster (Figure 6.12).

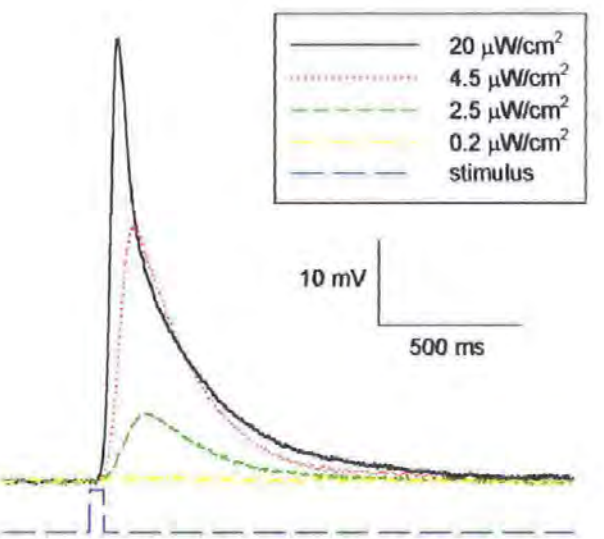


Figure 6.12: The change in the intracellular response shape to a change in stimulus intensity. The stimulus duration was 50 ms.

The maximum amplitude of the intracellular response increased as the light intensity increased. This relationship was sigmoid (Figure 6.13). Three cells reached a plateau with the light intensities used. This allowed the absolute sensitivity to be calculated according to the widely-used definition in scientific literature[2] (see also Section 3.1). The mean absolute sensitivity was 0.35 ± 0.03 cm^2/μW (mean ± sd), but using the definition of absolute sensitivity employed in chapter 3, it was 3.70 ± 1.41 cm^2/μW. The mean absolute threshold of visual sensitivity of the five cells was 1.66 ± 1.2 μW/cm^2. The cells responded to light intensities ranging within 2 log units. Their dynamic range, i.e. the range of intensities over which the maximum response amplitude changed, was slightly less than this at 1.3 log units (Figure 6.13).

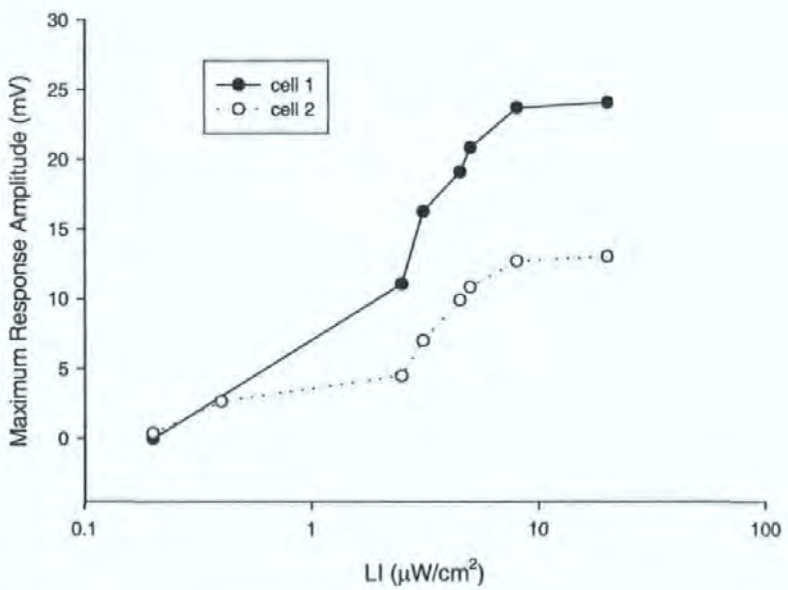


Figure 6.13: The effect of increasing light intensity (LI) on the maximum response amplitude of intracellular recordings from two representative cells.

The activation kinetics of the intracellular response, as measured using the response latency, changed with increasing stimulus intensity. The response latency varied from 20

[2] The definition most common in the scientific literature is the inverse of the light intensity that caused the response to be half of the maximum (Autrum, 1981). This was altered in chapter 3 to be the inverse of the light intensity that caused the response to be 0.4 mV in amplitude.

to 50 ms with changes in the light intensity of the stimulus. It decreased with increasing light intensity in all five cells examined (Figure 6.14), and never reached a plateau within the range of light intensities tested. When the data were plotted on semi-logarithmic axes, a linear regression was found to describe the data well (r^2=0.38, df=54, p<0.01).

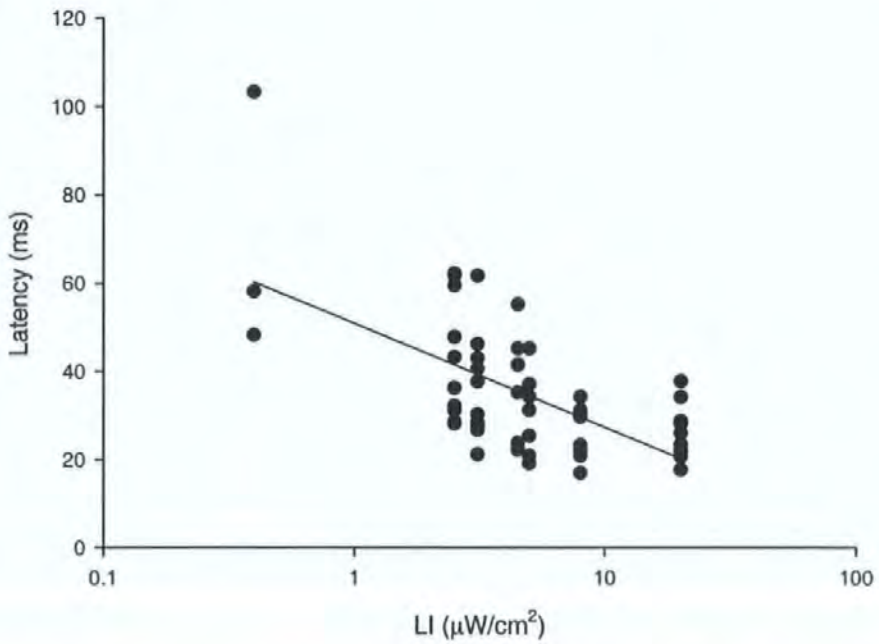


Figure 6.14: The effect of increasing stimulus light intensity (LI) on the response latency of the intracellular response. The equation of the best fit line was y = -23.58x + 50.92 (r^2 = 0.38, df = 54, p<0.01).

Four of the recordings, used to investigate the influence of light intensity on the intracellular photoresponse, had a single decay constant. For the other recording, the decay phase of its response was best fitted using two decay time constants, of which only the first decay constant was used in this analysis. All cells showed a decrease in decay constant with an increase in light intensity (Figure 6.15). Unlike the pattern of the continuous decrease in response latency with increasing stimulus intensity (Figure 6.14), the decay constant of most cells reached a plateau within the light intensities used. This plateau began at approximately 5 μW/cm^2.

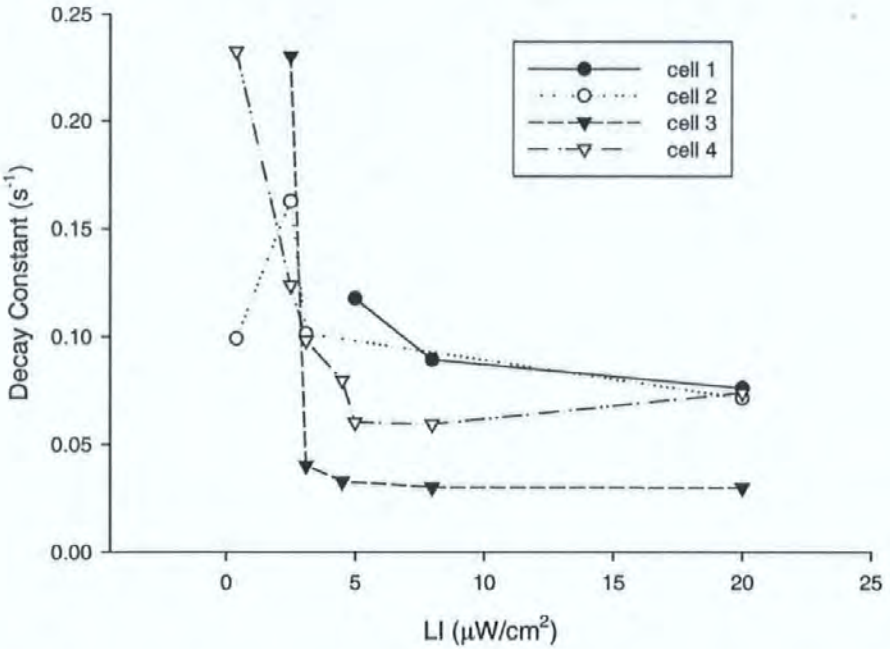


Figure 6.15: The effect of light intensity (LI) on the decay constant of the intracellular response recorded from four individual cells.

Only three of the five cells tested with different intensities had spikes, and their number did not change with light intensity in a consistent manner (results not shown). The lack of a pattern cannot be considered a conclusive result because of the low number of stable cells found with spikes.

6.3.5 *The morphology of the retina after dissection of the animal*

Sections taken of tissue fixed on the day of dissection and on the day after dissection showed no apparent differences in gross morphology between these two types (Figure 6.16). One slight way in which the tissue varied was the degree of attachment of the limiting membrane. The limiting membrane separates the photoreceptors from the vitreous humor of the eye (Messenger, 1981), and is thought to be secreted by the supporting cells (Young, 1962; Messenger, 1981). Thirty hours after dissection, this component of the retina was not as well connected to the outer segment layer as it was in freshly dissected

tissue. Further studies with an electron microscope would show if there were any changes at sub-cellular level.

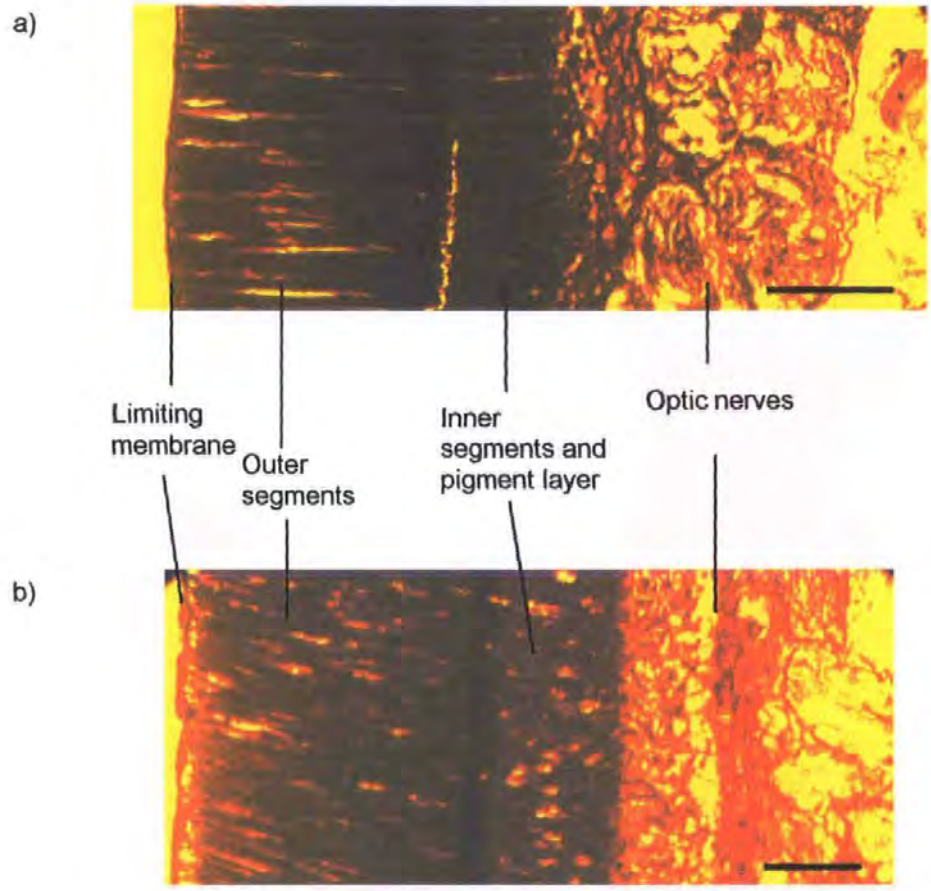


Figure 6.16: Sections of the retina taken at two different times after dissection: a) freshly dissected tissue, b) tissue 30 h after dissection (scale bars = 100 μm).

6.4 Discussion

6.4.1 *General findings*

The resting potential of *S. officinalis* retinal photoreceptors ranged from –58 ± 14 mV (mean ± sd), with a maximum depolarising response to light of 30 ± 12 mV. The resting potential was similar to that found for other cephalopod species, but the maximum amplitude of the response was somewhat smaller (e.g. Tomita, 1968; Pinto & Brown, 1977; Duncan & Pynsent, 1979a), as previous papers reported light-evoked responses that

reduced the resting potential to zero; this did not occur here. This difference was probably due to the maximum intensity of stimulus used here, which was relatively low at 20 $\mu W/cm^2$, compared to the maximum light intensity of 700 $\mu W/cm^2$ used by Pinto & Brown (1977). The response shape was typical of other invertebrate photoresponses, with a transient peak and plateau at high light intensities (Fuortes & O'Bryan, 1972). Again, there were slight differences between this and other reports, probably due to the differences in the stimulus intensity used. The maximum response amplitude depended on the resting potential of the cell (Figure 6.1). This was probably because both the resting potential and the size of the response are indicators of cell health (Tomita, 1968). An undamaged cell, therefore, was more likely to have a more negative resting potential and a larger photoresponse. Significantly more non-light responsive cells were recorded from than light responsive ones (see Section 6.3.1), which was probably related to the side of the retina approached by the electrode, i.e. the optic nerve side. The electrode first went through the plexiform layer, to the inner segment layer, where the cell bodies of both the photoreceptors and supporting cells lie. Within the plexiform layer are the cell bodies of epithelial cells, which are not responsive to light, and so these were encountered before any light-responsive cells, creating a higher proportion of non-light responsive recordings.

An unexpected discovery was that some intracellular recordings had two decay constants, while others had the expected single decay constant (Figure 6.4). The decay constant is related to the rate at which the shut down mechanism (or deactivation) of the photoresponse operates (Fein & Szuts, 1984). Two decay constants within a single light response, therefore, indicated that some change occurred in the shut down process. The second decay constant was either present or absent in a cell and when present, it was obvious at the majority of light intensities used. Other studies that used cephalopod retinal photoreceptors also found two decay constants to their responses. Duncan and Pynsent (Pynsent & Duncan, 1977; Duncan & Pynsent, 1979a, b) and Nasi & Gomez (1992) found that a second, slower decay constant became apparent at high light intensities. The second

decay phase measured in these four reports had a time constant greater than 10 s, which was much longer than those measured here. The second decay constants from this chapter and these other papers are probably not equivalent, as the light intensity used here never reached the super-saturating intensities that Nasi & Gomez (1992) showed were necessary to record their second decay constant.

One possible reason for the two stepped decay phase of some of the recordings reported in this chapter might be the location of the recording electrode in the cell. The inner and outer segment of the cephalopod photoreceptor are quite different in structure and composition (Messenger, 1981). The outer segment contains all the rhodopsin and light-activated channels, while the inner segment contains mainly voltage-sensitive channels (Duncan & Pynsent, 1979a). These different types of channels have different properties, including opening times and repolarisation rates. If the electrode was in the outer segment, away from the cell body, then the light response should only have had one decay constant, that reflecting the light-sensitive channels. This was the recording location used by people in the past and they reported only one decay constant on their recordings (Tomita, 1968; Pinto & Brown, 1977). If, however the electrode was in the cell body, the light response could be affected by both of these channel types, perhaps creating a stepped decay to the response. Much more specific studies need to be completed on these different channel types in cephalopod photoreceptors before this could be ascertained for certain. It could also be that the second decay constant measured in some cells here was an indicator of cell damage, somehow causing the ion channels or gap junctions to function abnormally.

Action potentials, or spikes, were recorded in 68% of the impaled, stable cells. There was a near significant relationship between the amplitude of spikes and the size of the intracellular response (Figure 6.3) (similar to that seen by Tomita, 1956). The generator potential (i.e. the large, graded intracellular response) is produced in the outer segment, but the spikes are initiated on the other side of the cell body at the beginning of

the axon in an area termed the action potential initiating zone (Fuortes & O'Bryan, 1972). The size of both of these therefore depended somewhat on the location of the electrode in the cell. If the electrode was closer to the action potential initiating zone then the action potentials would be apparently larger and the generator potential smaller. The opposite should be true when the electrode was close to, or in, the outer segment, which would explain Figure 6.3. It, therefore, verified that the electrode was, as proposed in the previous paragraph, in the cell body of the photoreceptor, close to the action potential initiating zone on some occasions, which was a reasonable supposition as the retina was approached from the optic nerve side. Not all previous papers have recorded spikes, but the likely reason for this was that they approached the retina from the outer segment side (Tomita, 1968; Pinto & Brown, 1977), and so probably impaled them in an outer segment, far away from the action potential initiating zone. There was no significant correlation between the occurrence of spikes and two decay constants within a single intracellular recording (see Section 6.3.1). This does not invalidate the theories that both of these factors depend on the location of the electrode within the cell, because the sample sizes were small, but shows that further work is needed in this area.

Quantum bumps were not seen in any of the intracellular recordings made from the photoreceptors of *S. officinalis*. Quantum bumps are responses to individual photons and are commonly recorded from some species, e.g. *Locusta migratoria* (Shaw, 1968) and *Calliphora vicina* (Laughlin, 1989). Mauro & Baumann (1968) have made the only report of recording of quantum bumps in a cephalopod species, but this was in extraocular, not retinal, photoreceptors. In many preparations, these are quite difficult to distinguish from the background noise, particularly when the tissue is not fully dark-adapted (Laughlin, 1989), so although quantum bumps were not seen in *S. officinalis*, they may still be present, but were simply not measurable under the conditions used in the experiments reported in this chapter.

6.4.2 *The effect of stimulus duration on the intracellular response*

The maximum amplitude of the evoked intracellular responses varied logarithmically with flash duration, rising to a plateau with flashes of approximately 50 ms and longer in duration (Figure 6.8). This plateau in the maximum amplitude of the response coincided with a switch in the response shape from increasing in size to increasing in duration, and was marked by the appearance of a transient peak (Figure 6.6). It is unclear exactly what the transient peak represents (Naka, 1961), but it is known to depend on the calcium concentration of the extracellular solution (Pinto & Brown, 1977). It is highly probable that the plateau represents some type of adaptation to long, bright flashes (Autrum, 1981). Once the phototransduction cascade is initiated, negative feedback chemicals are produced, which then limit the size of the response, resulting in the plateau (Rayer *et al.*, 1990). Within the first few milliseconds of the response, there are none of these chemicals present, so the response continues to grow, resulting in the transient peak.

The latency of the response decreased to a plateau with increasing flash duration (Figure 6.9). The switch between these two states occurred with a flash of approximately 15 ms duration, and the plateau was at a response latency of approximately 26 ms. A repeated measures ANOVA showed that the response latency changed significantly with flash duration (see Section 6.3.3). The photoreceptor cell, probably, did not receive enough light within a 1-10 ms flash to initiate a response of the appropriate size to have the short latency of the plateau. Once a critical flash duration was applied to the tissue, it received enough light within the first 15 ms of the latency to ensure a short average latency of 26 ms. Any flash duration longer than the critical one of 15 ms could not reduce the latency further, as the activation kinetics were already working at their maximum speed.

The decay constants of the intracellular responses were more complicated to analyse because the decay phase of some recordings were better fitted by two decay constants. The first decay constant probably represented the activation of voltage-sensitive

potassium channels in the inner segment, which is why it was relatively fast (Duncan & Pynsent, 1979a). It decreased with increasing flash duration until it reached a plateau. The switch between a larger, more variable decay constant to the plateau level occurred with a flash duration between 30 ms and 50 ms (Figure 6.10). The decrease in the first decay constant implied that for some reason the phototransduction cascade shut down faster with longer flashes of light. It might be possible that within the plateau phase of longer flashes, the chemicals controlling the phototransduction cascade (i.e. those activating it, and those deactivating it) were finely balanced. Once the stimulus stopped, no more activation chemicals, e.g. activated meta-rhodopsin, were produced, but there was far more of the negative feedback chemicals, e.g. calcium, present than a shorter flash would ever produce, so the decay constant was shorter with longer stimuli. Chase (1974) found no change in the decay constant of responses to flashes ranging in duration from 67 ms to 1 s, which agrees with the results presented here.

The maximum amplitude of the intracellular response and its decay constant reached their respective plateaux at approximately the same flash duration, which implied that these two variables may be linked (compare Figure 6.8 and Figure 6.10). This flash duration also corresponded to the appearance of the transient peak. If the transient peak and plateau in the shape of the response actually represented different aspects of the phototransduction cascade (in that the peak was initially unlimited by feedback chemicals, but the plateau showed a finely balanced interaction between negative feedback chemicals and those initiating the phototransduction cascade) (Autrum, 1981), then the above observation could make sense. Before the transient peak had reached a maximum, the amount of feedback chemicals produced probably depended on the duration of the flash. The amount of these feedback chemicals also determined the decay constant. Once the transient peak and plateau were evident, it implied that there was always the same amount of feedback chemicals, therefore the decay constant did not vary. This implied the cell was

in a dynamic equilibrium, so neither the maximum amplitude nor the decay constant changed.

The number of spikes increased with the duration of the stimulus flash (Figure 6.11). Chase (1974) also examined the effect of flash duration on the response shape, but in *Tritonia diomedia* (nudibranch), and found that increasing the flash duration did not lead to the appearance of the transient peak, but did elicit more spikes. Every nerve cell that produces action potentials has a stimulus threshold, which must be reached before the cell will produce one (Matthews, 2001). So once the duration of the flash reached over 2 ms, there was a large enough photoresponse to produce some spikes in the photoreceptors of *S. officinalis*. The longer the stimulus was, the greater the number of spikes that were generated (Figure 6.11).

6.4.3 *The effect of stimulus intensity on the intracellular response*

The maximum amplitude of a response increased with increasing light intensity (Figure 6.13), following a similar pattern to that found in squid (Pinto & Brown, 1977). These data allowed the sensitivity of a single cell to light with a peak emission wavelength of 590 nm to be measured. The mean absolute threshold of sensitivity (see Chapter 3 for definition) was 1.66 ± 1.2 $\mu W/cm^2$ (mean ± sd, n = 5). This lies towards the lower end of the range of light intensities that *S. officinalis* would encounter in their natural habitat (Clarke & Denton, 1962). It was impossible to compare the absolute sensitivities of *S. officinalis* with numerous different animals as different measurements of light intensity were used in most cases. Cobb & Williamson (1998a), who also used irradiance, found that the extraocular photoreceptors of *Eledone cirrhosa* (the lesser octopus) responded to light intensities under 1 $\mu W/cm^2$. This was slightly lower than that found for the retinal photoreceptors of *S. officinalis*. The reason for this disparity was probably due to the wavelength of the stimulating light used here. The LED was used here had a peak

wavelength emission of 590 nm. Brown & Brown (1958) found that the peak absorption of cephalopod rhodopsin was approximately 490 nm, so it is likely that *S. officinalis* would be less sensitive to the stimulus used here than to the white light used by Cobb and Williamson (1998a).

There was a sigmoid relationship between light intensity and the amplitude of the intracellular response (Figure 6.13). This approximated to the self-shunting model first used by Lipetz (1971), showing that the intracellular photoresponse from *S. officinalis* was similar to those described in other animals (e.g. Matic & Laughlin, 1981; Eguchi & Horikoshi, 1984). Although the dynamic range of the photoreceptors of *S. officinalis* was relatively small, at 1.3 log units, when compared to some other invertebrates, it lay within the range found for other cephalopods from 1 log unit in the extraocular photoreceptors of *Eledone cirrhosa* (Cobb and Williamson, 1998a) to 1.8 log units for *Sepiola atlantica* (Duncan and Pynsent, 1979a).

The latency of the intracellular response decreased as the light intensity of the stimulus increased (similar pattern to that found by Millecchia & Mauro, 1969; Cobb and Williamson, 1998a), but it never reached a plateau (Figure 6.14). This decrease was probably due to the reduction in amplification needed with a stronger stimulus (Saibil, 1986). Lower intensity flashes require longer times to produce a response, as the chemicals in the phototransduction cascade must diffuse across greater distances, due to their smaller numbers, to initiate the next step in the cascade. The latencies recorded here were smaller than those reported by Cobb and Williamson, indicating that the extraocular photoreceptors of *Eledone cirrhosa* react more slowly to light than the retinal photoreceptors of *S. officinalis*. The decay constant of the intracellular response decreased with increasing light intensity, and in most cases reached a plateau (Figure 6.15). With higher intensities, more of the chemicals that deactivate the phototransduction cascade could be produced, so causing the cascade to switch off faster than with lower intensities.

Spikes were seen in too few cells to allow any conclusions to be made about the effect of changing light intensity on their numbers or frequency.

6.4.4 *Success of technique*

In spite of the small diameter of *S. officinalis* retinal photoreceptors, which was estimated to be 2 – 12 μm (S. Farley, unpublished data), several long-lasting and stable intracellular recordings were achieved. On average, recordings from 0.9 cells per animal were obtained. Stable recordings were obtained only from cells in the larger sized of animals, and then the success rate changed to 1.2 cells per animal. Although this was a low proportion, particularly considering that each animal has millions of photoreceptors, it still made this method of recording a feasible one, particularly as each large animal was likely to provide useable results. It must be remembered that attempts to obtain intracellular recordings were generally made on freshly dissected tissue, and also on tissue the day after dissection. So when the success rate was viewed in terms of days, it fell to 0.6 cells per day, making it very time consuming. Of all the intracellular recordings obtained, 40% were stable and therefore analysed further. Duncan & Pynsent (1979a) found that 30% of the intracellular recording they made from *Sepiola atlantica* remained stable. The reason for the difference in success rate between small and large animals was unknown. Sophia Farley (unpublished results; University of Plymouth, UK) found that cell length, but not diameter changed with the ML of the animal. Cell length, however, should not have influenced the success rate of obtaining an intracellular recording.

Within the experimental protocol used, the eye was dissected on one day, and some tissue kept for the following day. Young (1962) showed that the photoreceptor cells did not deteriorate when the optic nerve was severed 9 days previous to examination of the cells. Although Young did this *in situ*, it showed that the retina could survive damage to its nerves for some time. Others, working on vertebrate retina, kept their preparations up to 48 hrs after dissection (e.g. Schneeweis & Green, 1995). The number of *S. officinalis*

cells penetrated, for which the intracellular recordings remained stable, on the day after dissection (9 cells) did not vary much from those obtained on the day of dissection (8 cells). The majority of variables examined were the same on the second day, only the response shape changed, in that the transient peak was much reduced in size (also found by Naka, 1961). Pinto & Brown (1977) found that squid photoreceptors were very sensitive to hypoxia. Attempts were made to keep the tissue as healthy as possible by bubbling it with air overnight and keeping it at a low temperature, but these might not have been as successful as initially thought. With all of these factors considered, it seemed a viable option to keep tissue until the next day, and this also decreased the number of animals required for experiments.

Although the success rate of penetrating a photoreceptor seemed promising, there were other problems to address with intracellular recordings. As a tool to perform preliminary investigations into the light response of photoreceptors, this technique was satisfactory. Duncan and Pynsent (Pynsent & Duncan, 1977; Duncan & Pynsent, 1979a, b) highlighted the fact that intracellular recordings did not give true readings of the membrane potential, a concern shared by Takagi (1994). To gain an accurate picture of the changes in membrane potential under certain conditions, Duncan and Pynsent used extracellular recordings to validate the intracellular recordings they made from *Sepiola atlantica* retinal photoreceptors. Any future studies that require the precise measurement of membrane potentials would have to ensure that the precautions taken by Duncan and Pynsent were followed.

6.4.5 *Summary*

The characteristics of *S. officinalis* retinal photoreceptors, determined from intracellular recordings, proved that they were similar in many ways to other invertebrate photoreceptors, with respect to their response shape, and the influence of flash duration and flash intensity on their light response. The intracellular recordings highlighted some

interesting trends in the decay constants of these cells, and in the maximum amplitude of the response attained with a stimulus of a given duration, implying that there could be a switch between two different phases of adaptation with longer flashes. Overall, recording from the retinal photoreceptors of *S. officinalis* proved feasible, but not entirely satisfactory. The technique was not entirely acceptable due to the small number of stable cells compared to the number of animals used, and the time taken to obtain a stable recording. This implied that it would only be a practical technique to develop further for investigation of changes in membrane potentials, if the success rate of the technique was first improved.

Chapter 7 General Discussion

7.1 Introduction

The principal aim of this thesis was to combine behavioural and physiological techniques to characterise the performance of the cephalopod visual system, using *Sepia officinalis* as the study species. The approach taken was to use a behavioural technique to measure the spatial resolution and visual sensitivity and then to use physiological methods to measure the visual sensitivity of the isolated retina and individual photoreceptor cells. In this way, the visual sensitivity of this species was studied systematically at three different physiological levels. By measuring the same parameter at the level of the whole animal, of the isolated retina and of the photoreceptor cell, the processing of visual information that occurred between these three levels was investigated. The spatial resolution, i.e. the minimum separable angle (MSA), and the visual sensitivity of *S. officinalis* have not been measured in any previously published work. By studying these parameters in this thesis, knowledge of cephalopod vision was increased. This chapter summarised each of the results sections to show how their individual objectives were fulfilled and then compared selected data from these sections to determine how similar the visual performance of *S. officinalis* was to those of other species.

7.2 Fulfilment of objectives

7.2.1 Behavioural measurements of spatial resolution and visual sensitivity

Chapter 2 used the optomotor apparatus to measure the spatial resolution and visual sensitivity of different sized *S. officinalis*. The objective was to determine the influence of mantle length (ML) and light intensity on the MSA of this species. The MSA of 8 cm animals, measured with 15 $\mu W/cm^2$ ambient light intensity, was calculated to be 42' of arc. This MSA value is comparable to that of *Octopus vulgaris* (27'; Packard, 1969) but larger

than that found for *Sepia esculenta* (3'; Watanuki *et al.*, 2000). As the mantle length (ML) of *S. officinalis* increased, the MSA decreased (Figure 2.4), revealing that larger animals see more detail than smaller animals. With all sizes of *S. officinalis*, a decrease in the ambient light intensity reduced the MSA. Figure 2.3 showed that all sizes of animal tested responded to some stripe width from the lowest to the highest light intensity used (4 $\mu W/cm^2$ - 15 $\mu W/cm^2$), which indicated that the behavioural visual sensitivity of this species was less than 4 $\mu W/cm^2$, and probably less than 1 $\mu W/cm^2$ for 8 cm animals if the graph was extrapolated. The changes in MSA with ML and light intensity found here were similar to those found from studies on fish and other species (Table 2.8), with larger animals able to distinguish finer lines at all light intensities, and all animal sizes able to differentiate more detail at higher light intensities. It was concluded that logarithmic growth within the visual system was not solely responsible for the improvement in behavioural MSA with increasing ML and that all sizes of cephalopods adapted to changes in light intensity with the same efficiency.

7.2.2 Extracellular measurements of visual sensitivity

The objective of Chapter 3 was to investigate the effect of size (ML), stimulus light wavelength and stimulus duration on the visual sensitivity of *S. officinalis*, using the electroretinogram (ERG). The absolute sensitivity of *S. officinalis* was estimated to be 1.35 $cm^2/\mu W$ (Figure 3.6) and, from the same data set, the absolute threshold of the excised retina was estimated to be 0.67 $\mu W/cm^2$, which corresponded to the low end of the range of light intensities these animals would encounter in their natural habitat (Clarke & Denton, 1962). The sensitivity of the retina decreased as the ML of the animal increased (Figure 3.1). This was an unexpected result, as the longer photoreceptors of larger animals were predicted to have greater sensitivity (Land, 1981). Physiological processes, such as dark noise and changes in cell resistance, were probably responsible for the change seen in

absolute sensitivity with increasing ML. These processes do not form part of the formula used to predict sensitivity (Land, 1981). The dynamic range of the ERG fell within the limits previously recorded from other cephalopod species (Duncan & Pynsent, 1979a; Cobb & Williamson, 1998a). The retina of *S. officinalis* proved to be more sensitive to blue light than to yellow light (Figure 3.6), which agreed qualitatively with the absorption spectrum of the rhodopsin of *S. officinalis* published by Brown & Brown (1958). Decreasing the flash duration decreased the slope of the V/log I curve (Figure 3.5) in similar manner to that previously seen in invertebrates (Matic & Laughlin, 1981).

7.2.3 Extracellular measurements of light adaptation and the effect of calcium

The objective of Chapter 4 was to examine how the sensitivity of the retina of *S. officinalis* changed when the retina was adapted to light, and any alterations to this process of adaptation with changes in the calcium concentration of the external solution perfusing the preparation. The sensitivity of the retina decreased when exposed to a constant level of background light, and with a constant light of 5.5 $\mu W/cm^2$ intensity, the response amplitude of the evoked ERG had reduced to almost zero (Figure 4.2). The calcium concentration of the perfusing solution had a large impact on the sensitivity and light-adaptation capabilities of the retina (Table 4.6). Applying a constant background light to the preparation caused response compression in the V/log I curve of *S. officinalis* (Figure 4.3), similar to that seen in *Necturus maculosus* (Normann & Werblin, 1974) and *Gekko gekko* (Kleinschmidt & Dowling, 1975). When the calcium concentration was increased, the V/log I curve was altered less than by the application of a background light alone, but decreasing the calcium concentration increased the effect of the background light on the tissue (Figure 4.11). Some of the effects caused by altering the external calcium concentration of the perfusion solution and illuminating the preparation with a background light were unexpected, for it appeared that lowering the calcium concentration mimicked

the effect of light adaptation, which is contrary to results obtained for some other species, e.g. *Apis mellifera* (Bader *et al.*, 1976).

7.2.4 *Physiological evidence for functioning gap junctions in the retina of* Sepia officinalis

The objective of Chapter 5 was to determine, using physiological techniques, whether functioning gap junctions exist in the retina of *S. officinalis*. Three different techniques were used, but none gave conclusive results as to the presence or absence of functioning gap junctions. The response amplitude of the light-evoked ERG of *S. officinalis* was affected by three chemicals known to be gap junction blockers. Heptanol and octanol, both blocked the ERG and showed a dose-dependent effect on the amplitude of the ERG (Figure 5.3 and Figure 5.4). Dopamine, on the other hand, increased the ERG amplitude, and then decreased it (Figure 5.5). The difference in effects, which the alcohols and dopamine, had on the amplitude of the ERG was probably related to the other effects these chemicals can have on cells, e.g. heptanol and octanol block sodium channels (Haydon & Urban, 1983; Guan *et al.*, 1997), while dopamine influences the movement of screening pigment in the retina (Gleadall *et al.*, 1993). Dye injection using intracellular electrodes did not work as expected, as photoreceptors did not fill, but the dye simply collected in the inner segment layer of the retina. Applying dyes to the cut ends of the photoreceptor axons using GelFoam proved a more successful way of filling photoreceptor cells. FITC-dextran filled only some cells in the retina, but neurobiotin seemed to be in every photoreceptor. Changes made to this technique were to slice the retina after fixation, and to ensure the tissue was fully dark-adapted to enable better visualisation of the fluorescence when viewing a whole mount of the retina. Several attempts were made to close the gap junctions, using both light stimuli and gap junction blockers, but no change was observed in the patterns of dye staining in the retina under the different treatments.

7.2.5 Intracellular measurements of visual sensitivity

The objective of Chapter 6 was to characterise the intracellular response of the retinal photoreceptor cells of *S. officinalis* to light stimulation. The absolute sensitivity of an individual cell was calculated to be 3.7 $cm^2/\mu W$, and the absolute threshold was estimated to be 1.66 $\mu W/cm^2$ (Figure 6.13). This was from animals with an average ML of 17 cm. Intracellular recordings were attempted from other animals of smaller ML, but no recordings from such animals could be maintained for longer than the necessary 20 min (see Section 6.2.5). By varying the flash interval, it was found that the photoreceptors of *S. officinalis* need less than 10 s to recover from the stimulus light intensities used here (Figure 6.5). When the flash duration was lengthened, two phases of the light response were seen, a transient peak where there was no inhibition by feedback chemicals, and a plateau where there was an equilibrium between the initiation of the cascade and this feedback (Figure 5.6). This was similar to results found in other invertebrate light responses (Fuortes & O'Bryan, 1972). Action potentials were seen in 35% of the recordings made from the retinal photoreceptors of *S. officinalis*, which probably indicated that the electrode was within the cell body area of the photoreceptor (Figure 5.2 and 5.3). Overall the characteristics of the retinal photoreceptors of *S. officinalis* proved to be similar to those of other cephalopod photoreceptors (Pinto & Brown, 1977; Duncan & Pynsent, 1979a; Cobb & Williamson, 1998a), and to those of other invertebrates (Fuortes & O'Bryan, 1972), and even in some ways to those of vertebrates (Baylor *et al.*, 1974; Duncan & Pynsent, 1979a).

7.3 A comparison of the measurements of visual sensitivity and adaptation

The estimates of absolute threshold of visual sensitivity measured from animals (as close as possible to 8 cm ML), using behavioural and physiological techniques, were collated to form Table 7.1.

Method	Estimated Absolute Threshold ($\mu W/cm^2$)	ML (cm)	Light Used	Preparation Used	Relevant Section of Thesis
Behaviour	<1	8	White	Entire animal	2.3.2
ERG	0.1	7	Blue	Excised retina	3.3.4
	10	7	Yellow	Excised retina	3.3.4
Intracellular	1.66	17	Yellow	Photoreceptor	6.3.4

Table 7.1: The estimated minimum light intensities required to elicit a response in *S. officinalis* when measured using different methods (ML = Mantle Length).

As can be seen from Table 7.1, the estimates of absolute threshold of visual sensitivity obtained from behavioural, intracellular and extracellular (with a blue wavelength stimulus) techniques were within a similar range, of 0.1 - 1.66 $\mu W/cm^2$, which correlated well with the lower end of the range of light intensities they would encounter in their natural habitat (Clarke & Denton, 1962). The slightly higher result from the intracellular recordings could be explained by the use of a yellow light source for stimulation. If a blue LED had been used, the absolute threshold of individual photoreceptors would most likely have been less than 1 $\mu W/cm^2$, as the rhodopsin of *S. officinalis* is more sensitive to blue wavelengths of light (Brown & Brown, 1958).

There was a discrepancy between the absolute threshold measured from the ERG and that from an individual photoreceptor obtained using the same stimulus type. The

retina, when examined as a whole, was 6 times less sensitive than individual photoreceptors. When one cell was recorded from, the response measured was predominantly influenced by the reactions of that cell alone and to a lesser extent by any cells coupled to it via gap junctions. But when the response of the retina was measured, using the ERG, there were many interactions to consider, as it recorded the events within the entire extracellular space (Johnston & Wu, 1995; McIlwain, 1996). These interactions, and that the electrode was separated from the direct effect of the intracellular reactions by cell membrane, meant that the ERG was smaller in amplitude than the intracellular response (Duncan & Pynsent, 1979a). As the ERG was smaller, it had a less extensive range and the signal-to-noise ration was poorer (signal amplitude/noise amplitude, 82.5 for intracellular recordings and 14 for ERG), and so could not produce a measurable response to the same intensities as an intracellular recording. Thus, the difference in recording technique was probably responsible for the apparent difference in the sensitivity of individual cells and the retina.

The behavioural sensitivity matched the physiological sensitivity measured using intracellular recordings, implying that all the information individual photoreceptors collected was used by the animal. It would be an inefficient system if the animal could not respond to all the information the eye collected, because of filtering or some other processing step. Throughout the scientific literature, there have been few studies that measured both the physiological and behavioural sensitivity of the same species. But Hecht *et al.* (1942) proved that humans could detect a flash of light equivalent to one photon being absorbed by a rod. Later Fain (1975) showed that the rhodopsin in toad rods was responsive to one photon of light using physiological techniques. Since then it has been assumed that most animals are physiologically responsive to low light levels and that their responses in behavioural tests would match these, if the correct behavioural test is chosen. For example, *Carassius auratus* (gold fish) responded to light levels approximately 15% of the human threshold (Northmore, 1977); the physiological

sensitivity of *Spermophilus beecheyi* (Californian ground squirrel) matched its behaviourally measured sensitivity (Jacobs, 1980). The problem with many of the behavioural tests used in non-human species is that they are not responsive enough to show this level of sensitivity, due to their in-built limitations, e.g. inability of scientist to communicate with test subjects and motivation of the animals (Muntz, 1974; Douglas & Hawryshyn, 1990). Although both behavioural and physiological tests showed the absolute threshold of *Sepia officinalis* to be less than 1 $\mu W/cm^2$ (equivalent to 2.515×10^{12} photons/s[1], or 5×10^5 photons/s/photoreceptor, taking the average diameter of a photoreceptor to be 5 μm), this species can probably respond to much lower light intensity levels.

In Chapters 2 and 3, the visual sensitivity of *S. officinalis* was measured for a range of different sized animals, using both behavioural and physiological techniques. The previous paragraph showed that the absolute thresholds of visual sensitivity of animals 8 cm in ML measured using each technique were similar, but this changed when the trends with increasing mantle length were investigated. From the behavioural experiments, the visual sensitivity of the animals increased as size (ML) increased (Figure 2.3), but this was the reversal of to the trend in absolute sensitivity, measured using the ERG (Figure 3.1). Other animals ranging from *Lepeophtheirus salmonis* (parasitic salmon louse) (Flamarique *et al.*, 2000) to *Clupea harengus* (herring) (Blaxter, 1968) show an improvement in behavioural visual sensitivity with increasing animal size. In section 3.4.2, Land's (1981) formula for sensitivity was used to predict that sensitivity should improve as the length of the photoreceptors increased, and hence with increasing mantle length. It was surprising therefore that the behaviour results fit the predicted, theoretical trend, but the physiological results did not, especially when the calculated values used the parameters that were believed to determine the physiological sensitivity.

[1] 1 μW at 500 nm wavelength is equivalent to 2.515×10^{12} photons/s, using a formula published in the Oriel Instruments catalogue (http://www.oriel.com/down/pdf/01002.pdf, 2003)

The probable reason for the disparity between the physiological and behavioural results was the lack of efferent input to the excised retina, which has been shown to be necessary for some of the physiological mechanisms operating in the cephalopod eye by Gleadall *et al.* (1993). *Sepia officinalis* was able to react to higher light intensities (up to 15 $\mu W/cm^2$) (Figure 2.3) when tested in the optomotor apparatus, than when its isolated retina alone was examined (5 $\mu W/cm^2$ of background light was found to reduce response amplitude of the ERG amplitude to almost zero; Figure 4.2). Another reason for the disparity might have been the lack of the pupil. This is the first line of adaptation for the cephalopod retina and without it, the mechanisms intrinsic to the retina might have received too much light, and so caused them to function abnormally. The formula used by Land (1981) to estimate visual sensitivity includes the aperture dimensions, which would also be important to behavioural measurements, but was not a part of the sensitivity of the ERG of the excised retina. Hemmi & Mark (1998) also found a discrepancy between the physiological and behavioural visual performance of *Macropus eugenii* (the tammar wallaby). They considered that the number of ganglion cells in the retina involved in a given task to be important in determining this discrepancy. The more cells involved, the closer the two variables should be. As the isolated cephalopod retina is removed from its initial processing layer, i.e. the optic lobe, this might have increased the difference between the two measurements.

The amount of adaptation that occurs in *S. officinalis* of different sizes was measured in Chapter 2 using behavioural techniques and in Chapter 4 using the physiological technique of the ERG, and it was found that there were differences. In Chapter 2 (section 2.3.2.3), the adapting capabilities between smaller and larger *S. officinalis* were investigated using the measurements of MSA obtained from the optomotor behavioural experiments. Initially, there appeared to be a difference in the measured MSA between the animals of different sizes. This was because a change in light intensity

reduced the absolute MSA of smaller animals by a larger amount than the larger animals, but the percentage difference in the MSA did not change with ML (Table 2.6), so it was assumed that the adaptational capabilities of all sizes of *S. officinalis* were similar. An experiment in Chapter 4 examined adaptation in *S. officinalis* of different ML at the physiological level using the ERG, by investigating the light adaptation of two different sizes of animal. The retinas of the larger animals (7 cm ML) adapted better to changes in the light intensity than those of the smaller animals (1 cm ML) (Figure 4.6). It was probable, in this case, that the length of the photoreceptors made a difference. Cephalopod species can change their photoreceptor length by 20 –35% in response to background light (Young, 1963). It might be that this capability was removed when the eye was excised, so the cell length of the animals, determined by their ML, was probably the biggest difference between the retinas of the two groups. In the intact animals used for the behavioural experiments, the retina would still have had efferent input allowing all mechanisms to function correctly. Another difference was that the ERG was used to investigate light adaptation, but the optomotor apparatus tested dark adaptation. These two processes have different controlling mechanisms (Meyer-Rochow, 2001) and so both results highlighted in this paragraph might be true, in that the ML of *S. officinalis* does not affect dark adaptation, but does affect light adaptation.

The adaptational capabilities of *S. officinalis* are limited when compared to those of other animals, in particular its retina was only found capable of adapting to 2 log units of background light (Figure 4.2). Other animals can adapt to much larger ranges of light intensity, e.g. *Necturus maculosus* (mudpuppy) up to 5.5 log unit range (Normann & Werblin, 1974), *Locusta migratoria* (locust) at least a 3 log unit range (Matic & Laughlin, 1981), and *Pagothenia borchgrevinki* (Antarctic fish) at least a 3 log unit range (Morita *et al.*, 1997). A full range of light intensities was not tested with the optomotor apparatus due to limitations in the equipment and the retinal adaptation was probably less than other

animals for the reasons already outlined. It is likely that *S. officinalis* can adapt to greater ranges of light intensities, but further experiments are necessary to prove this.

7.4 A comparison of histological and behavioural measurements of minimum separable angle

The MSA of *Sepia officinalis* was determined to be 42' of arc, using the optomotor apparatus (Figure 2.3). This study measured how the MSA changed with increasing ML. As no measurement of spatial resolution using physiological techniques was made in this thesis, the behavioural measurements obtained here were compared with results from published papers, and with histological experiments completed by Sophia Farley (unpublished results; University of Plymouth, UK).

There was a large absolute difference found between the histological MSA and behavioural MSA of *S. officinalis*, which is similar to the results obtained from other species (Table 7.2).

Species	MSA		Behavioural Technique	Reference
	Histological	Behavioural		
Sepia officinalis (c)	1.7'	42'	Optomotor	Farley (unpublished); and Chapter 2
Sepia esculenta (c)	1.12'	2.78'	Reactive distance	Watanuki *et al.* (2000)
Octopus vulgaris (c)	1.3'*	27'	Optomotor	Land (1981); Packard (1969)
Pagrus auratus (f)	52'	488'	Optomotor	Pankhurst (1994)
Pleuronectes platessa (f)	40'	11'	Optomotor	Neave (1984)
Scophthalmus maximus (f)	20'	11'	Optomotor	Neave (1984)
Lepomis macroshirus (f)	7'	12'	Reactive distance	Hairston & Li (1982)

Table 7.2: Histological and behavioural estimates of MSA measured in the same study. (In studies where there was more than one measurement made, the smallest angle is included here.) (* this value was calculated by Land, 1981 from previously published data; c=cephalopod, f=fish.)

A disparity between histological and behavioural MSA was measured when both the reactive distance technique and optomotor apparatus were used to estimate MSA (Table 7.2). This illustrated that the difference between histology and behaviour was not unique to the optomotor apparatus. Possible reasons for the discrepancies between histological and behavioural estimations of MSA are varied, but can be classified into two main groups: environmental changes and physiological processes (Watanuki *et al.*, 2000). The histological MSA gives the visual acuity of an eye under optimal conditions (Muntz, 1974). But rarely in the natural world does the eye function under those ideal conditions to achieve that MSA. Environmental variations disrupt the optimum, for example by changing ambient light intensity, temperature, and turbulence etc (Watanuki *et al.*, 2000), of which only the ambient light intensity applied to the conditions under which the animals were tested in the optomotor apparatus used in Chapter 2. Once the ideal situation is deviated from, physiological processes may become more important than the spacing of the photoreceptors. For example, some neighbouring photoreceptor cells may have receptive fields that overlap, causing detail to blur, as Messenger (1981) said "spatial resolution of fine lines may depend more on the width of the receptive fields than on the size of the rhabdomes". Other physiological processes that can affect visual acuity are the dilation of the pupil, the length of the photoreceptors, the presence of gap junctions and other photoreceptor interconnections in the retina and neural processing (Land, 1981). The relative importance of these different processes is unknown, but all could play a part in the difference between histological and behavioural MSA estimates.

7.4.1 Changes in histological and behavioural MSA with mantle length

The changes in histological MSA of *S. officinalis* with increasing ML were plotted on the same graph as changes in the behavioural MSA and as changes in the lens

diameters[2] with ML (Figure 7.1). Lens measurements were taken as estimates of the eye size, to show the change in magnification of the eye with increasing ML. The behavioural MSA decreased more rapidly than the histological MSA with increasing ML (Figure 7.1).

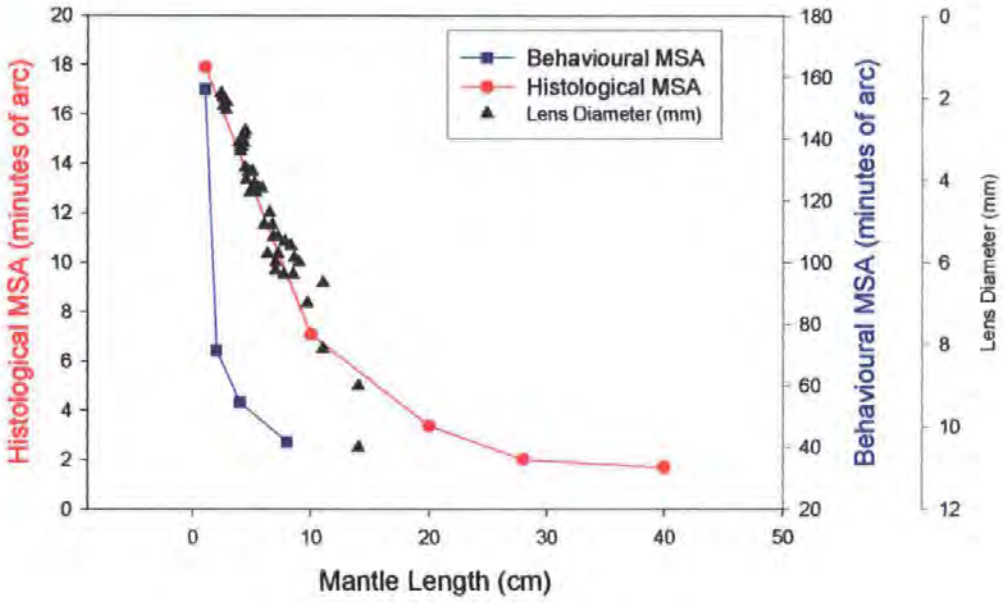


Figure 7.1: A comparison between the behavioural results and lens diameters from this study, and the histological results from S. Farley (unpublished data), and the variation in both with the size of the animal.

The magnification of the eye was more closely related to the histological than the behavioural MSA (Figure 7.1). This implied that there was at least one other factor, apart from eye size and magnification, that improved rapidly with ML, allowing the behavioural MSA to improve faster than the changes in histology of the visual system allowed. The behavioural MSA was shown in section 2.3.2.2 to decrease with increasing ML, not by a logarithmic curve, but by one of three other possibilities, an inverse function, an S-power function and a power function (Table 2.6). This implied that some factor other than the logarthimic growth within the retina, e.g. the area of the retina or another unknown factor, was responsible for the increase in behavioural MSA with ML. Rahmann *et al.* (1979),

[2] Lens diameters were measured, using digital callipers (DigiMax, Camlab Ltd, UK), from some of the animals killed for electrophysiology experiments.

Neave (1984) and Pankhurst *et al.* (1993) found a similar discrepancy in the improvement of behavioural and histological MSA with increasing animal size in the fish species they studied. They attributed it to changes in the optic tectum and inter-neuronal connections as the animals increased in size. These changes probably also played a role in decreasing the behavioural MSA of *S. officinalis* faster than the histological MSA with increasing animal size.

The improvement in behavioural sensitivity of *S. officinalis* matched the predicted, theoretical sensitivity, but this was not the case for MSA (sections 7.3 and 7.4). Although sensitivity and acuity are both related to the eye and photoreceptor size (Land, 1981), they probably involve different amounts of processing. The visual sensitivity of an animal simply requires the animal to ascertain if there was a reaction in a photoreceptor. For acuity, an animal has to assess if photoreceptors were differentially excited, which requires some level of processing of the visual information it receives (Pankhurst *et al.*, 1993). This difference in processing may be why the theoretical values of visual sensitivity more closely approximated that measured using behavioural and physiological methods, but the theoretical and practical measurements of spatial resolution did not match as closely. This difference illustrates the importance in measuring the same variable using different techniques, as it can elucidate the degree of processing between certain levels of the visual system (Douglas & Hawryshyn, 1990).

7.5 Comparing the visual performance of *Sepia officinalis* to those of other species

The individual results sections of this thesis measured different characteristics of the visual system of *S. officinalis*, thereby increasing the information known about cephalopod vision. This information was used to gauge how comparable the visual

performance of *S. officinalis* was to those of other species, including both vertebrates and invertebrates.

Sepia officinalis followed many of the same trends as shown by several other species:

- Behavioural MSA decreased with increasing ML of the animal (Figure 2.4), as is also found in for example cyprinids (Wanzenbock & Schiemer, 1989), *Pagrus auratus* (Pankhurst, 1994) and *Pleuronectes platessa* and *Scophthalmus maximus* (Neave, 1984).
- Behavioural MSA decreased with increasing ambient light intensity (Figure 2.3), as is also found in for example *Macropus eugenii* (Hemmi & Mark, 1998), *Peromyscus* spp. (Rahmann *et al.*, 1968), and *Clupea harengus, Pleuronectes platessa*, and *Solea solea* (Blaxter, 1968, 1969).
- The retina of *S. officinalis* proved to be more sensitive to blue wavelengths of light than to yellow wavelengths (Figure 3.6), which correlates well with the wavelengths of maximal transmission in water, i.e. 480-500 nm, to which most marine fish are also sensitive (Packard, 1972).
- Exposing the retina to a constant background light reduced the sensitivity of the animal (Figure 4.3), which is similar to the results obtained from *Necturus maculosus* (Normann & Werblin, 1974), *Gekko gekko* (Kleinschmidt & Dowling, 1975), and *Carassius auratus* (Northmore, 1977).
- Functional gap junctions are likely to exist in the cephalopod retina (Chapter 5), which is similar to the retina of *Carassius auratus* (Schmitz & Wolburg, 1991), *Cyprinus carpio* (Teranishi *et al.*, 1983), and *Necturus maculosus* (Dong & McReynolds, 1991).
- When the flash stimulus duration increased, the intracellular response changed in shape from a simple curve to a response with an initial transient peak followed by a plateau phase (Figure 6.6), which is also found in many

invertebrates (Fuortes & O'Bryan, 1972), and in some vertebrates; see Duncan & Pynsent (1979a) for a comparison between the cephalopod and vertebrate intracellular photoreceptor responses.

These points illustrate how similar the visual system of *S. officinalis* is to those of other animals. It is only in intracellular mechanisms that cephalopods prove to be extremely different to vertebrates. This is because vertebrate photoreceptors hyperpolarize in response to stimulation by light, but those of cephalopods depolarise (Rayer *et al.*, 1990). In many aspects, highlighted in this thesis, cephalopods proved to be very similar to vertebrates, in particular to fish, and so could provide not only a good model for visual processing studies of invertebrate species, but also for vertebrate species. In one particular way, *S. officinalis* and other cephalopods offer a unique model. Their retina is composed mainly of photoreceptors. There are no other neuronal cell types within the cephalopod retina (Figure 1.3; Messenger, 1981), so the ERG is only influenced by the visual processing that occurs between the photoreceptors alone, and not that between any other cells as is the case in vertebrate species.

The characteristics of the visual system of *S. officinalis* did not always match those of other invertebrates, which illustrates the limitations of generalisations. In one respect, the visual performance of *S. officinalis* proved to be more similar to those of vertebrates, than those of other invertebrate species. This was because the change in the slope of the V/log I curve with different background light intensities (Figure 4.3) was more similar to the changes found in vertebrates than in invertebrates (Matic & Laughlin, 1981; Eguchi & Horikoshi, 1984). This is probably related to intracellular changes associated with the shut down mechanism of the photoresponse. The mechanisms used by photoreceptors to adapt to different light intensities depend on the chemicals and enzymes used by the cascade and the membrane properties of the cell (Perlman & Normann, 1998), and these vary slightly between different groups of animals. This similarity of the visual system of *S. officinalis* with those of vertebrates should be interpreted with caution. Not enough similar data on

the visual systems of non-insect invertebrates has been published to judge whether, or not, it is unique to *S. officinalis*.

In three ways, *S. officinalis* showed differences to data previously published on all other species in relation to the cellular mechanisms of phototransduction. Firstly, increasing the background light shining on the retina, increased the latency of the evoked ERG (Figure 4.4). Secondly, increasing the calcium concentration of the solution perfusing the retina, increased the response amplitude of the ERG at most of the stimulus light intensities tested (Figure 4.8). And finally, there were two relatively fast decay constants present in some intracellular responses (Figure 6.4), which were unlike the fast and long decay constants recorded in responses elicited with super-saturating light intensities by Duncan and Pynsent (Pynsent & Duncan, 1977; Duncan & Pynsent, 1979a, b) and Nasi and Gomez (1992). These differences showed that there were species specific mechanisms within the cells of *S. officinalis*. So although this species proved to be quite similar to invertebrates, sweeping generalisations cannot be made. It is likely that *S. officinalis* would prove to be a useful model in terms of extracellular and behavioural work, but not on an intracellular level.

7.6 Ideas for future work

To extend this systematic study of the visual performance of *S. officinalis*, the contrast sensitivity function (CSF) of these animals could be measured. The CSF is obtained by recording either the brain waves, or the ERG of an animal, as the visual stimulus shown to it changes in contrast and grating width until the animal no longer responds to the difference (McIlwain, 1996). This would provide a physiological measurement of MSA, i.e. an intermediate measurement between the behavioural measurements from this study and the previously completed histological measurements of

MSA. The CSF would also provide data about intermediate steps to the visual sensitivity results presented here, as it monitors the ERG of the intact animal, which would allow the influence of the efferent inputs to the retina to be determined. This technique has been successfully used with some species (e.g. Northmore, 1977; Jacobs, 1980; Hemmi & Mark, 1998).

A second area of research that would complement this study would be to measure the receptive fields of individual photoreceptors, under different levels of background stimulation. Messenger (1981) said that the receptive fields of photoreceptors might be more important in determining MSA than receptor spacing. For this the responses of one cell would be monitored as a spot of light approximately 2 μm in diameter was moved across the retina. To enable this to work efficiently, the success rate of obtaining an intracellular recording would first have to be improved. Receptive fields are also directly linked to sensitivity, as the larger the receptive field, the more photoreceptors that are pooling information, so the lower the sensitivity (Land, 1981; Werblin, 1991). Therefore by studying the receptive fields of the photoreceptors a link between visual acuity and sensitivity could be quantified. The receptive fields of other animals have been studied in depth and it is now known that cells of the visual pathway in the brains of cats are selectively sensitive to movement of stimuli in one particular direction (advances in this field are reviewed by Hubel (1995).

7.7 Overall conclusions

The principal aim of this thesis was to combine behavioural and physiological experiments to characterise the cephalopod visual system, using *Sepia officinalis* as the study species. This was fulfilled by measuring the visual sensitivity of this species using

behavioural, extracellular and intracellular techniques, and was complemented with related studies. The conclusions reached on completion of this study were:

- the ability of *Sepia officinalis* "to collect and transmit information about the visual world" (Land, 1981), as determined by the optomotor apparatus, increased with increasing mantle length;
- the behavioural MSA of *S. officinalis* depended on at least one factor, other than those considered in the calculation of histological MSA, which increased at a faster rate than normal logarithmic growth, e.g. the area of the retina, processes in the brain;
- the change in visual sensitivity with animal size, as determined using the optomotor apparatus, matched the predicted (theoretical) change, but not that measured using the isolated retina, which showed that the efferent inputs to the retina were necessary for it to function correctly;
- *Sepia officinalis* provided an adequate model of behavioural and extracellular data, but not of intracellular photoreceptor changes, as not all parameters measured using the ERG and intracellular recordings agreed with previously published data on invertebrate photoreceptors.

By measuring the visual sensitivity and acuity of *S. officinalis*, a significant contribution was also made to the field of cephalopod biology. Visual sensitivity was studied at different physiological levels of the animal to discover how it systematically changed with different amounts of processing. In doing this, the principal aim of this thesis was fulfilled, and our knowledge of the visual system of *Sepia officinalis* significantly advanced.

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APPENDIX

Stripe Width (cm)	ML (cm)	4 μW/cm^2	6 μW/cm^2	10 μW/cm^2	15 μW/cm^2
control	1	0/20 = 0%	0/20 = 0%	0/20 = 0%	1/20 = 5%
	2	0/20 = 0%	0/20 = 0%	0/20 = 0%	0/20 = 0%
	4	0/20 = 0%	0/20 = 0%	0/20 = 0%	0/20 = 0%
	8	1/11 = 9%	2/11 = 18%	3/11 = 27%	6/11 = 55%
0.08	8			1/3 = 33%	2/2 = 100%
0.1	1				
	2				0/10 = 0%
	4				
	8		0/1 = 0%	2/7 = 29%	4/6 = 67%
0.15	1		0/1 = 0%	0/2 = 0%	0/1 = 0%
	2			0/1 = 0%	3/20 = 15%
	4			0/20 = 0%	1/20 =5%
	8	0/9 = 0%	1/8 =13%	3/8 = 38%	2/5 = 40%
0.4	1		½ = 50%	2/11 = 18%	¼ = 25%
	2	0/10 = 0%	0/10 = 0%	1/20 = 5%	5/20 = 25%
	4	0/20 = 0%	0/20 = 0%	5/20 = 25%	4/20 = 20%
	8	4/9 = 44%	6/9 = 67%	4/9 = 44%	7/8 = 88%
0.6	1	0/1 = 0%	2/12 = 17%	5/20 = 25%	4/20 = 20%
	2	1/20 = 5%	0/20 = 0%	5/20 = 25%	8/20 = 40%
	4	5/20 = 25%	7/20 = 35%	9/20 = 45%	14/20 = 70%
	8	6/11 = 55%	5/9 = 56%	8/9 = 89%	½ = 50%
1.9	1	1/12 = 8%	7/20 = 35%	10/20 = 50%	8/20 = 40%
	2	5/20 = 25%	17/20 = 85%	13/20 = 65%	18/20 = 90
	4	15/20 = 75%	19/20 = 95%	17/20 = 85%	17/20 = 85%
	8	6/7 = 86%	4/5 = 80%	5/5 = 100%	2/2 = 100%
3.2	1	3/13 = 23%	12/20 = 60%	14/20 = 70%	11/20 = 55%
	2	11/20 = 55%	15/20 = 75%	19/20 = 95%	9/10 = 90%
	4	20/20 = 100%	8/10 = 80%	2/3 = 67%	1/1 = 100%
	8	0/2 = 0%	3/3 = 100%		
4.5	1	8/20 = 40%	15/20 = 75%	9/11 = 82%	14/20 = 70%
	2	9/20 = 45%		7/10 = 70%	
	4			0/1 = 0%	
	8	½ = 50%			
5.6	1	14/20 = 70%	5/11 = 45%		½ = 50%
	2	3/3 = 100%			
7.0	1	13/20 = 65%	1/1 = 100%		
8.1	1	0/1 = 0%			

Table A.1: The number of positive responses shown by animals divided by the number of animals tested and the percentages to which these equate. At the extreme stripe widths, only animals that either had responded, or had not responded to all other widths were tested. This is why group sizes are not consistent at each stripe width and why all group sizes were not tested with each one.

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