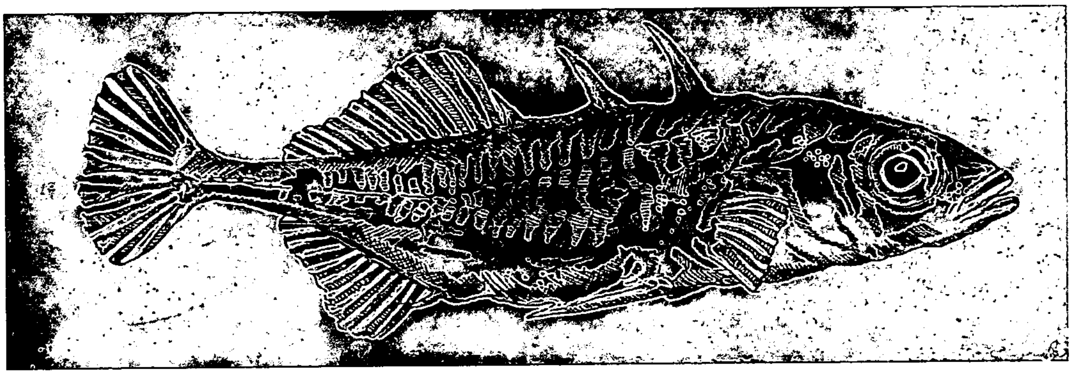
The visual ecology of the three-spined stickleback

Philip Boulcott



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Preface

This work has been composed by me, is the result of my own research and contains no work done in collaboration except where stated otherwise. The text does not exceed 70,000 words. No part of this thesis has been submitted to any other university in application for a higher degree.

Title page illustration: an etching of the three-spined stickleback, *Gasterosteus aculeatus*, by Hetty Haxworth

Abstract

For almost a century the three-spine stickleback, *Gasterosteus aculeatus*, has been a principle subject in the field of animal behaviour, and as a consequence, we know more about the behaviour and ecology of this fish than any other. Yet, despite our understanding, implicit in much of this study is the assumption that the three-spined stickleback views the world in much the same manner as humans do. Such an assumption is, however, critically flawed. An emerging body of work now suggests that shallow dwelling fish such as the three-spine are often found to be sensitive to ultraviolet light, and that this sensitivity is often conferred by the existence of a fourth, independent cone photoreceptor in the retina. Moreover, since colour has been found to be crucial in the mate choice decisions of the stickleback, with the potential for ultraviolet sensitivity arises the possibility that visual behaviour in this species may also have been misclassified. In fact, rather than being static, there is evidence that the visual system of the stickleback is plastic, and this could be responsible for the maintenance of secondary sexual characteristics in the stickleback.

Using various spectrophotometric, behavioural, and electrophysiological techniques, I have investigated the visual capabilities of the three-spined stickleback. Scans of the ocular media of the stickleback revealed a visual system compatible with ultraviolet photoreception, where the optics of the eye permitted wavelengths as low as 350nm to reach the retina. Electroretinograms obtained from the stickleback confirmed that the photoreceptors within the retina are capable of ultraviolet photoreception, and using chromatic isolation, it was found that this sensitivity was mediated by the presence of a fourth, independent cone photoreceptor. A survey of the habitat from which subjects were collected confirmed that functional levels of ultraviolet wavelengths were present in the photic environment.

The presence of an ultraviolet photoreceptor in the retina does not automatically imply its input to the highest level of the visual system. A simple two choice test, run under conditions similar to ambient, confirmed that the three-spined stickleback is able to respond behaviourally to ultraviolet light. In view of this, mate choice in the sticklebacks was re-examined under conditions where the ultraviolet component of the male's nuptial signal could be altered. Females were able to recognise individuals not displaying in the ultraviolet region of the spectrum as conspecifics but were, nevertheless, found to prefer males displaying across the ultraviolet and human visible region of the spectrum during trials.

The issue of seasonal plasticity in the visual sensitivity of the stickleback was re-examined using modern techniques. Contrary to a previous study where only females were found to undergo a change in sensitivity, behavioural techniques investigating seasonal plasticity in spectral sensitivity revealed that both males and females became increasingly sensitive to red light during summer. This shift was found to accompany a summer shift towards red light in the ambient spectra. The mechanisms that might cause such a shift are discussed.

Acknowledgements

I have received an enormous amount of help in the production of this BBSRC funded thesis, for which I am truly grateful. Although almost too numerous to mention, I would like to mention a few people who have helped me find my way.

Firstly, I would like to thank my supervisor Victoria Braithwaite. I would like to apologise for all the trouble I have doubtlessly caused, though unintentional I swear! and thank her for seemingly unlimited patience, kindness, and know-how. Similarly, I would also like to thank Derek Cosens for his support and vital supply of technical equipment. Together, their thoughts and comments on the subjects touched by this thesis have proved invaluable. Both have a passion for their subject which can only be described as infectious.

I think I remember starting this thesis with a pen and a library card and would, therefore, like to thank Lloyd Mitchell, Graeme McKenzie and all the other boys in the workshop who helped me to build a lab. In this aspect I must also thank Michael Dye for the blind eye he directed my way when temporarily misappropriating equipment, Will Hossack for the optical equipment, and Mike Gentle who provided me with the necessary hardware and expertise (along with Lynne Sneddon) to complete the electrophysiology.

ICAPB provided me with an excellent environment in which to study and I would like to thank all those who made it so. Many of you have helped me out with a few problems – Nick Colgrave always springs to mind – and I can say with certainty that all have helped to keep me smiling in times of utter despair. Thank you Jono, Catherine, Martin, Antonis, Helen, Lucy, Carolyn, Culum, Felicity, Becky C, Stu, Becky W, Becky T, Dave, Alex, Stu, Sue, Graham, Robert, Barbara, Kirk, Zoë, Kate, Tom L, Tom Mc, Julie, Tam, and Peter.

I have been fortunate enough to work with two excellent undergraduate students who helped in the production of this thesis. Ellie Leadbetter collected a preliminary data set used in chapter 4, and Keith Walton helped collect the behavioural data set analysed in chapter 5. Thank you, I wish you both luck.

I would also like to thank my fellow participants in the fields of visual ecology and stickleback ecology– Jim Bowmaker, Julian Partridge, Ron Douglas, Andrew Bennett, Craig McDonald, Liz White, Charlie Baube, Ellis Loew and Jeff McKinnon – all of whom were kind enough to answer questions throughout the period of my study.

Thanks to Sporting ICAPB FC who taught me to lose gracefully, and unfortunately, rather regularly also. I will always remember the two goals I scored with them, and the fact I was substituted at half-time despite my pursuit of a hat-trick. Like snow in July, goals have been scarce since.

Thanks must also go to my father, Michael, for helping me translate the numerous technical texts written in German (thank you also Barbara). Without his help, and that of my mother, Margaret, I would not have been able to complete this thesis.

Finally, to my dear wife Hetty, thank you for all your unerring love, support, and the title page etching – again, this thesis couldn't have been done without you.

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Chapter 1

The visual ecology of the three-spined stickleback *Gasterosteus aculeatus*: An introduction

1.1 Introduction

With a history of scientific enquiry spanning three centuries, the three-spined stickleback, *Gasterosteus aculeatus*, is perhaps the most studied and best understood of all fish. Inhabiting a wide range of freshwater and marine waters of the Northern Hemisphere (Fig. 1.1), the three-spined stickleback is one of the few species of fish for which we have some idea of its evolutionary past (Bell & Foster, 1994). In fact, the considerable intra- and inter-population phenotypic variation exhibited by the three-spined stickleback has established it as a model system in evolutionary study. What is of particular interest to the visual ecologist, however, is that vision and visual cues have been found to underpin much of the behavioural and life history characteristics of this small fish.

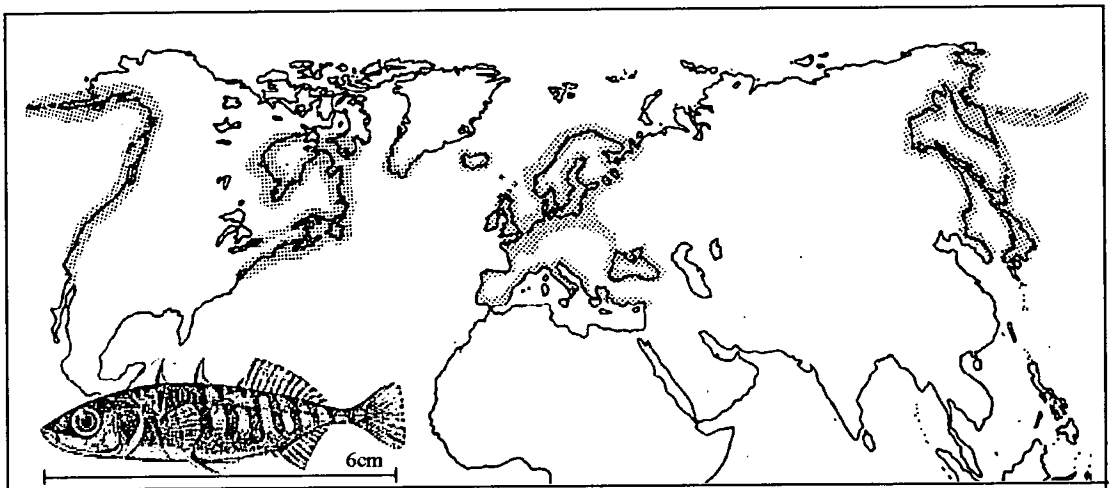


Figure 1.1 Shaded areas depict the approximate global distribution of the *Gasterosteus aculeatus* species complex, adapted from Wootton, 1984.

1.1.1 Vision and visually mediated behaviour in <u>G. aculeatus</u>

The pre-eminence of vision in the ecology of the three-spined stickleback is reflected in the relative size of its retinal surface, approximately 3.5% of the entire body surface, in comparison to the 0.5% of the olfactory epithelium (Beukema, 1968).

Further evidence suggesting the significance of vision in this species is demonstrated by in the considerable changes in male colouring apparent during spring; a period when the previously cryptic colour pattern of the male is compromised by the adoption of a bright red patch around the throat and an iridescent blue tint to the iris. That these changes occur in conjunction with the onset of breeding condition (Tinbergen, 1951), and that this colour change is costly in terms of metabolic investment (Barber *et al.*, 2000) and predation pressure (Baerends, 1976), emphasises the relevance of visual communication in this species. With such a considerable metabolic investment associated with a sensory structure such as the eye (Walls, 1942), one might expect to find evidence of biologically significant visual tasks upon which both reproductive success and survival depend - and this has, indeed, proved to be the case.

Sexual selection

Since the classic work of Niko Tinbergen and co-workers in the late nineteen thirties and forties, it has become apparent that the visual system of the three-spined stickleback plays a pivotal role in intra-sexual selection (ter Pelkwijk and Tinbergen, 1937; Tinbergen 1951). In winter, male and female sticklebacks, in common with many other fish, are countershaded against the down-welling light with olive green dorsal striations overlying their sliver flanks. During the spring, however, reproductively active males exhibit considerable sexual dimorphism, developing blue-green eyes and an intensely red throat and belly. Tinbergen's work established that this vivid colour pattern was essential for advertising the sexual readiness of the male and, in turn, attracting potential mates. Further investigation has revealed that the degree of expression of the red throat signal affects female preference, with females showing a strong preference for redder males (McLennan and McPhail, 1990; Bakker and Mundwiler, 1994; Braithwaite and Barber, 2000). It is also suggested that the male's ability to express an intensely red throat is an honest evolutionary signal (Zahavi, 1975), positively correlated to male quality in terms of resistance to parasites, physical condition (Milinksi and Bakker, 1990; Barber *et al.*, 2001; Candolin and Voigt, 2001), and energy investment in paternity (Frischknecht, 1993).

Tinbergen also recognised that fighting between male three-spined sticklebacks during the breeding season is particularly directed at those males with nuptial markings (Tinbergen, 1948). He postulated that the red throat colour acted as a sign-stimulus, essential in release of the male's aggressive response. Later studies suggest that the relative intensity of the red mosaic pattern may act as a badge of status during the aggressive defence of breeding territories (Rohwer & Rowher, 1978), where the intensity of the red breeding colouration is positively correlated with dominance (Peeke and Veno, 1973; Bakker and Svenster, 1983; Rowland, 1984; Baube, 1997).

As with any signal receiver relationship in visual ecology the effectiveness of a signal will vary according to the transmissive medium, and, as such, signal design will be largely determined by the sensory perception of the signal receiver (Endler, 1992). The existence of black-throated variants, often in dystrophic waters, suggests that preference for an epigamic optical signal is likely to be on its efficacy in generating visual contrast rather than its intrinsic spectral characteristics (McDonald *et al.,* 1995).

Prey detection and predator avoidance

The lifetime reproductive success of any organism is contingent upon its survival until sexual maturity. In this, the ability of an animal to successfully detect prey items whilst, at the same time avoiding being predated upon, is paramount. The three-spined stickleback has been found to be primarily a visual predator, utilising cues such as colour, colour contrast (Beukema, 1968), shape, size (Eggers, 1977) and movement (Meesters, 1940) in the detection and capture of prey. Visual cues are also used in the selection of prey and so play an important role in foraging efficiency (Hughes *et al.*, 1992). In a similar manner, the three-spined stickleback has been found to rely heavily upon visual cues in developing a strong escape response from predators (Giles, 1987; Huntingford and Wright, 1989). Indeed, the nature of the light absorbing visual pigments found within the stickleback's photoreceptors suggests that the stickleback eye is particularly suited to detect decrements of light

(McDonald and Hawryshyn, 1995). Such a mechanism is particularly suited to the detection of shadows caused by predators moving overhead (Northmore, 1973, cited in McDonald and Hawryshyn 1995).

Spatial behaviour and orientation

Fish also use visual information during spatial behaviour: allowing them to keep station in flowing water, find food, locate refuges, or define territories (Douglas, 1996). Evidence from the goldfish, *Carassius auratus*, suggests that visual angle – calculated from the retinal size of the visual image - is important in completing such tasks (Douglas, 1996). The use of visual landmarks by the three-spined stickleback has also been found to be important in learning and spatial memory (Girvan and Braithwaite, 1998; Odling-Smee and Braithwaite, 2003). Rather intriguingly, however, the importance of visual information in solving spatial problems was found to vary between different stickleback populations. Fish were found to employ a hierarchy of different spatial strategies, including both visual and turn-sequence information. It is possible that such differences in cue use during these spatial tasks relate to the nature of the habitats from which each population originated. Furthermore, the emergence of evidence indicating that fish are able to respond to polarised light (Waterman, 1975; Loew and McFarland, 1990) opens the possibility that light may serve as a compass during orientation. This ability appears to be linked to the possession of different cone classes, particularly the UV photoreceptor, when arranged in a well defined square cone mosaic pattern (Hawryshyn and McFarland, 1987; Coughlin and Hawryshyn, 1995). It is thought that the underlying biophysical mechanism is based on the selective reflection of polarized light by the presence of a partitioning membrane formed along the contact zone between the members of the double cones and the neighbouring ultraviolet-sensitive cones (Hawryshyn 2000). The resultant ability to discern *e*-vector information from polarised light has been found to be employed in orientation in the rainbow trout, *Oncorhynchus mykiss*, (Hawryshyn and Bolger, 1990), and could potentially exist in the three-spined stickleback.

1.1.2 Assessing and misclassifying colour

No two species have the same sensory capacity. Each animal operates in its own *Merkwelt* (perceptual world) that differs from our perception of the environment (Tinbergen, 1951). This is undoubtedly true in the case of vision, as colour is context dependent, contingent both on the constituents of illuminating light and the psychophysical properties of the animal's visual system (Endler, 1990). Hence, without a true understanding of both the visual cues to which the stickleback is responding and its own visual capability, any inference made about visually mediated behaviour will be flawed. Such sentiments are not new. Lord Avery, Sir John Lubbock, at the end of the nineteenth century called on science to ensure it did not mistakenly adopt anthropomorphic assumptions when examining the visual system of animals. Whilst this sentiment is one that is eminently reasonable, it is in the design of experiments that we still risk misclassifying colour. By misclassifying colour, hypotheses that attempt to predict colour trends, commonly applied in both behavioural and evolutionary biology, will be unsound (Bennett *et al.*, 1994).

By way of an example, in an attempt to classify the red ventral patch of breeding males, there are numerous instances in the stickleback literature where rank-scores generated by a naïve human observer have been used to quantify male ornamentation (Rowland, 1984; Baube, 1997). Such an approach is invariably unsafe, as subjectivity, observer variation, and discrepancies caused by variation in illumination will introduce error (Endler, 1990). To remedy this, several authors have automated the assessment of colouration in the stickleback by using photographic imaging techniques (Frischknecht 1993; Bakker and Mundwiler, 1994, Barber and Braithwaite 2000). Whilst removing experimental subjectivity, the use of such techniques is flawed, as they express colour in a three co-ordinate colour space derived from the tri-chromatic properties of the human visual system. It has been argued that such systems are suitable since the stickleback possess a trichromatic system similar to our own (Künzler and Bakker, 2001); however, there is evidence to

suggest that sticklebacks are sensitive to ultraviolet light, and may even possess an independent ultraviolet photoreceptor (Merker, 1939). If this is true, then trichromatic visual systems and computer programs based on trichromatic models would provide an inappropriate measure of colour (Wyszecki and Stiles, 1982). For example, if ultraviolet sensitivity is brought about by a fourth, spectrally distinct photoreceptor, it is possible that an organism has the potential for tetrachromacy, or higher (Thompson *et al.,* 1992), and may then perceive many more hues than humans (Burkhart, 1989, Goldsmith, 1990, King-Smith, 1991).
In order to gain a truer picture of any colour patch, it is necessary measure the reflectance spectra of that patch (Endler, 1990). Yet, whilst it is possible to quantify reflectance spectra, that are independent of the visual properties of the observer, we are still unable to make any inference about the perceived colour. To do so requires a detailed understanding of the visual system, the ambient light regime, and the transmissive properties of the medium (Bennett *et al.*, 1994; Grill and Rush, 2000).

Whilst a considerable body of work has been devoted to the visually mediated behaviour of the stickleback, sadly our understanding of the visual capabilities of this important species is limited (McDonald and Hawryshyn, 1995). Considerable disagreement still exists regarding the visual capabilities of this fish. Without a clear picture of its visual capabilities, we will be unable to discern which properties of the external world influence behaviour and, of equal importance, which do not.

1.1.3 The visual ecology of the three-spined stickleback

Much of what we understand about the evolution of the visual system comes from the correlational study of marine and freshwater teleosts (Lythgoe, 1979; Crescitelli *et al.,* 1985). By examining the match between an organism's phenotype and its natural environment, a picture has emerged of how natural selection has driven different species to possess the diversity of visual systems that are evident today. From this, it is clear that numerous adaptations have evolved in many aspects of the visual system including: ocular anatomy, visual physiology, biochemistry, behaviour, diet and life history (Partridge and Cummings, 1999). Nevertheless, whilst the comparative method is an effective way to reveal patterns (Huey and Bennett, 1986), it is limited in its ability to demonstrate causal relationships (Gould and Lewontin, 1979). For this reason, there has been a re-emphasis in visual ecology towards the identification of visual tasks that demonstrably affect fitness; that is, those tasks which are essential for survival and reproduction (Endler, 1991; Partridge and Cummings, 1999). If such an experimental approach is to be successful, it will most likely be achieved by an in-depth study of a single species. Indeed, the three-spined stickleback may prove to play a pivotal role in this approach as significant developmental and behavioural changes in the life history of the species have been documented. Moreover, the multitude of photic environments in which its populations have been found to reside – rivers, lakes, estuaries and coastal waters - offer an unusually accessible occasion to examine visual evolution and function. Such diverse habitats impose divergent selection pressures on the morphology and behaviour of individuals, providing an opportunity to compare populations within a species, and to identify causal links with their ecology. The use of divergent phenotypes in addressing evolutionary aspects of this species has been applied successfully in elucidating matters in morphology, life history, and behaviour (Reimchen, 1980; Huntingford and Wright, 1989; Bakker, 1994; Foster, 1994; Girvan and Braithwaite, 1998).

1.2 The visual environment

The visual capability of any organism is ultimately determined by its own physiology. However, knowledge of the light environment, that forms the backdrop to visually mediated behaviour, is essential if we are to fully understand visual performance. This is especially true if one considers that the habitats in which three-spined sticklebacks have been found to reside – rivers, lakes, estuaries, and coastal waters - often vary greatly in terms of brightness, colour, and turbidity. This diversity in photic regime is due to the fact that the optical properties characteristic to each water body affects the transmission of radiant energy from the sun differentially (Fig. 1.2), altering radiant light in terms of both quantity and distribution in the aquatic habitat (Duntley, 1963).

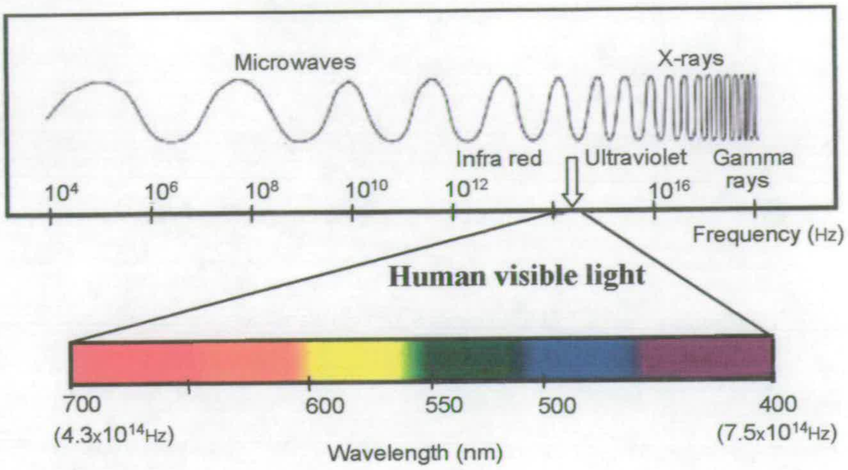


Figure 1. 2 The spectrum of electromagnetic radiation

In terms of trying to classify the photic characteristics of these water bodies, much can be explained by the degree of scatter and absorption inherent in the medium (Loew and McFarland, 1990). Crucially, absorption and scatter depend on depth and wavelength - the greater the path-length for a photon of light, the greater the attenuation - with some wavelengths being more attenuated than others. In general, it is the short wavelengths that are attenuated most due to scatter, and this is certainly true when one considers the clear blue skies of earth's atmosphere. However, in the aquatic mediums, radiant flux, whilst affected by the same optical processes apparent in the air, is usually affected more acutely.

For example, even in clear oligotrophic waters, whether marine or freshwater, water will act as a monochromator, attenuating both short and long wavelength light, with a maximum transmission around 470nm (Tyler, 1959; Tyler and Smith, 1970). However, where there is a high degree of suspended particles and optical contaminants, the spectral composition of ambient light may be very different (Yentsch, 1962). Dissolved organic matter and decayed products of photosynthesis (Gelbstoff) so often found in freshwater systems, absorb strongly both short and long wavelengths of light and, as a direct consequence, the water will transmit maximally in the green region of the spectrum (Kalle, 1966). Accordingly, freshwater can transmit maximally in the green, yellow-green, or yellow region of the spectrum. But in some freshwaters systems, such as peat stained lakes, the tea-coloured Gelbstoff is found in such high concentrations that red wavelengths of light transmit furthest (Jerlov, 1968; Levine and MacNichol, 1979; Baker and Smith, 1982). In addition to these characteristics of underwater light, for shallow dwelling fish such as the three-spined stickleback other factors such as wave conditions, solar elevation, and reflection from the substrate will be of importance (Loew and McFarland, 1990).

1.2.1 Plasticity in the light environment

It should be remembered that the photic environment is not static, and especially in land locked freshwater systems the light environment will vary considerably. At the most fundamental level, atmospheric daylight will alter continuously in quantity and composition according to cloud cover and the position of the sun (Sastri and Das, 1968). Such shifts in light quality and quantity can prove to be crucial if biologically significant visual tasks, such as feeding or reproduction, take place during a certain period of the day (McFarland and Munz, 1975a, 1975b; Endler, 1987, 1991). On a larger temporal scale, the seasonal breakdown of organic material so apparent in freshwater lakes and ponds will also have to be taken into account when classifying the photic environment (Muntz and Wainright, 1978). Changes in ambient light conditions may also be contingent on behaviour, where fish actively move from one photic environment to another during the course of their lifespan (Beatty, 1966; Muntz and Mouat, 1984).

1.3 Investigating the visual ecology of the three-spined stickleback

It is against the backdrop of diverse photic environments that the visual system of the three-spined stickleback must necessarily be assessed. If we are to consider this diversity in the context of visual performance, it is not unreasonable to suppose that the visual system of a given species such as the stickleback will be adapted to the light environment in which it has evolved (Walls, 1942). Whilst it may be somewhat unusual to talk of the adaptive radiation of the an organ such as the eye, with the exception of the brain, it is difficult to imagine an organ so harmonious in its construction, or subject to such high selective pressure (Walls, 1942; Endler, 1991). A consequence of the close matching of an organism's phenotype to its natural environment means that through studying the design of the eye we have been able to infer much about the visual ecology of many organisms (Lythgoe, 1979).

1.3.1 Structure and function of the vertebrate eye

The ultimate purpose of the eye's optical components - lens, cornea, and humour – is to transmit and focus photons of light onto a light sensitive retina (Fig. 1.3) and in this respect the structure of the stickleback's eye, which follows the general vertebrate plan, is no different. Typically, the cornea is a flat structure, and is covered in the three-spined stickleback by an outer protective layer, or secondary spectacle (Walls, 1942). The lens is spherically shaped and has high dioptric power, compensating for the lack of refractive strength at the corneal surface. Light that is able to pass through the cornea and lens is focused onto a light sensitive retina, which is responsible for the transduction of photons of light into the electrical nerve signals that are ultimately transmitted to the brain.

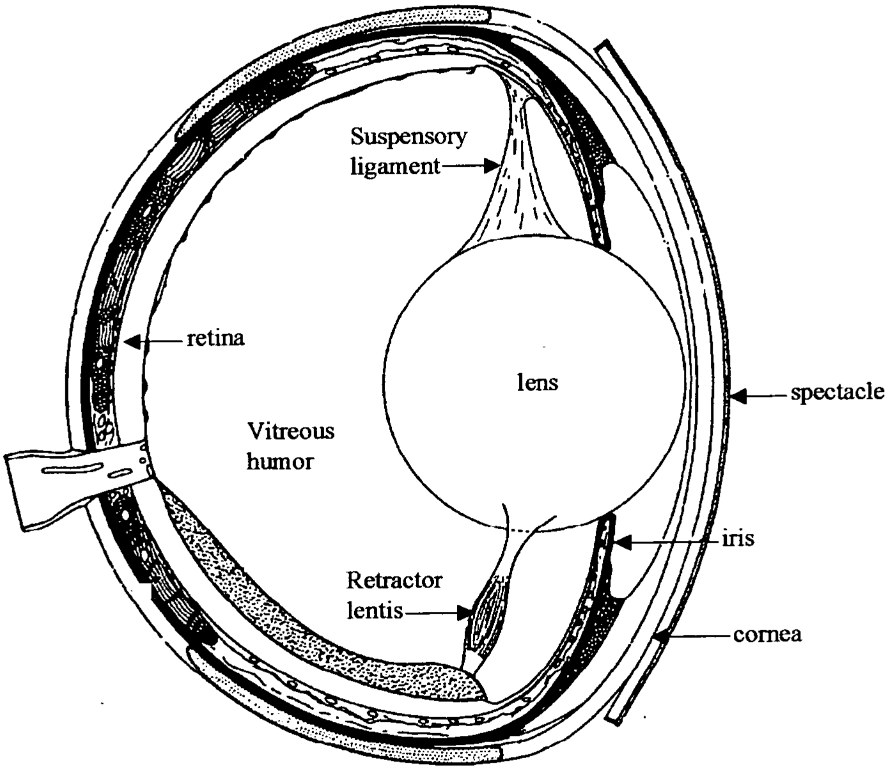


FIGURE 1. 3 Vertical section of a generalised teleost eye (adapted from Walls, 1942).

The sensitivity of the fish eye to light in true absolute terms, recorded in the goldfish, is remarkably high, revealing a sensitivity threshold to light 85% lower than that of humans (Northmore, 1977). The efficiency of the teleost eye in detecting photons could be accounted for by two mechanisms: the relatively low f-number (the ratio of the focal length of a lens to the effective diameter of its aperture) of the optics in the eye, and the greater transitivity of ocular media, producing a brighter image on the retina than in humans (Douglas and Hawryshyn, 1990). Furthermore, the fish eye may be more effective at operating at lower light levels as the low body temperature of ectotherms allows the retina to function at lower signal to noise ratios (Aho *et al.*, 1988, Douglas and Hawryshyn, 1990). In terms of resolving power the fish eye is very good, and only at very small sizes does the resolution of the lens tend towards the limit imposed by the retina (Tamura, 1957). This limit is governed by the degree of separation of the photoreceptors in the retina, and the specific architecture of postreceptoral processing (Loew and McFarland 1990). Although poorer than humans, behavioural tests of acuity in the three-spined stickleback by Moore and Moore (1979) have revealed that foraging individuals are able to detect a 10mm long *Gammarus* at a distance of 440mm: 1.75° subtended at the eye.

1.3.2 The retina

The retina of teleosts display a degree of complexity at least as high as those of higher vertebrates - with three or more photoreceptor types, two bipolar cell types, three kinds of horizontal cell, and six or more types of ganglion cell being typical (Ali and Anctil; 1976; Wagner, 1978) – and forms three distinct layers (Fig. 1.4). The exact layout of the retina will differ from species to species, and this will depend on the nature of the visual tasks performed by the animal. In this, the visual layout of the retina in the three-spined stickleback is no exception as is it characteristic of the photic regime that its shallow-dwelling diurnal life style necessitates (Lythgoe, 1979).

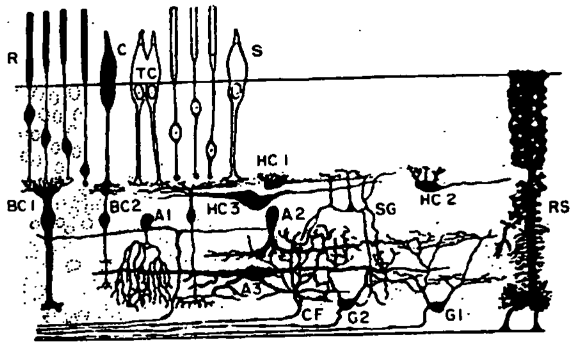


FIGURE 1.4

Diagrammatic vertical section of a teleost retina.

A1-5, amacrine cells, BC1-5, bipolar cells, C, Cone, G1-3, ganglion cells, HC1-3 and HS1-2 horizontal cells, R, rod, TC, double cones.

S - the outer section of the photoreceptor where the visual pigment is contained.

[taken from Tomita, 1971]

1.3.3 Two classes of photoreceptor

The retina of the three-spined stickleback is duplex, containing both rod and cone photoreceptors (Munk, 1966; Ali and Anctil, 1976). In addition, the cone receptors found in the stickleback retina can be further divided into two further categories: double and single cones. The outer segment of the photoreceptor, or sclerad, contains light sensitive pigments that are embedded into in folded membranes and formed into a series of discs (Piantanida, 1991). In this respect, the double cones are particularly noteworthy as they may contain either the same photopigment, or two that absorb light of differing wavelengths (Loew and Lythgoe, 1978). In general, rods and cones have different functions in vision – rods functioning at low light levels, whilst cones function at higher levels of radiance. The relative number of these two photoreceptors in the retina is related to whether the organism is most active during the day or night (Lythgoe, 1979). To complicate matters further, fish have complex mechanisms of adaptation to different irradiances, including diurnal retino-motor movements of the rods and cones to bring the active photoreceptors into play. Such photomechanical movements serve to equate the sensitivity of the visual system to the level of environmental irradiance.

1.3.4 The visual pigments

Irrespective of the photic environment, the ultimate limit to visual sensitivity must lie with the photoreceptors and the visual pigments contained therein. Visual pigments are membrane-bound proteins composed of a transmembrane protein - opsin - that has embedded within it a chromophore (Hargrave *et al.*, 1984). The chromophore, which is an aldehyde of vitamin A, retinal, exists in two forms: A_1 and A_2 (Knowles and Dartnall, 1977). This dual-form generates two large families of pigments; the rhodopsin based on $retinal_1$, and the porphyropsins based on $retinal_2$ or 3-dehydoretinal. The spectral location of the absorption maximum of a visual pigment is governed by two criteria, the nature of the chromophore, whether retinal or 3-dehydroretinal, and the genetically determined amino-acid sequence of the opsin (Piantanida, 1991). Thus for any opsin there can be two spectrally distinct visual pigments: a rhodopsin (A_1) and a “paired” porphyropsin (A_2) that has an absorption maximum shifted towards longer wavelengths. As a general rule, visual pigments of freshwater fish are dominated by porphyropsins, whereas in marine species rhodopsins dominate, but many species contain mixtures of the two kinds of pigments (Partridge, 1990). Indeed, an A_1/A_2 mixture (paired pigment) of a particular opsin may be present within a single photoreceptor. In such a case, the spectral sensitivity of the receptor may vary: the exact position of the absorption maximum being contingent upon the ratio of rhodopsin and porphyropsin present in the mixture. The retina of the three-spined stickleback has been found to contain such a paired pigment mixture (Ali and Wagner, 1975).

1.4 Visual performance

The importance of vision in the behaviour of the stickleback suggests that strong evolutionary forces have shaped the present form of the eye. Since visual pigments differ in their ability to absorb certain photons of light, it is likely that they too have been selected on the basis of their visual performance – a fact that is borne out by the occurrence of similar pigments in similar environments irrespective of phylogeny

(Lythgoe, 1979; Crescitelli *et al.*, 1985). However, the identity of the selected pigment will depend upon the nature of selection. In light of this, two theories were proposed to identify the crucial elements of visual performance that may directly affect the fitness of the individual: the sensitivity hypothesis and the contrast sensitivity hypothesis.

1.4.1 The sensitivity hypothesis

At its simplest level, the sensitivity hypothesis supposes that the spectral sensitivity of the visual pigments will be tuned in such a manner as to maximise the capture of photons from the photic environment (Lythgoe, 1979). Such a relationship was proposed for the rod pigments of deep-sea fish as early as 1936 by Bayliss, Lythgoe and Tansley (1936), and by Clarke (1936). The authors independently argued that, since light at depth in the ocean is reduced to a narrow peak at 470nm, deep-sea fish, in order to be maximally sensitive to down-welling light, should have pure rod retinae and a visual pigment with an absorption maximum corresponding with the 470nm peak of maximum transmission. Owing to the intervention of the Second World War, the idea was not put to the test until Denton and Warren (1957) and Munz (1957), again working independently, examined the rod pigments of deep-sea fish. Their results showed that not only were the visual pigments of the deep-sea fishes shifted in sensitivity to shorter wavelengths, but that the maxima of the visual pigments coincided almost exactly with the wavelength of light associated with maximum water transparency. This result has proved to be particularly robust and has been replicated by studies using other deep-water fish (Lythgoe, 1972; Partridge *et al.*, 1988).

The idea that the rod pigments were positioned in such a manner as to confer maximum sensitivity was soon extended to waters other than clear blue oceans, but with mixed success (Dartnall, 1965; McFarland, 1971; Munz and McFarland, 1973). In contrast to the results from deep-sea fish, in coastal waters that have the transmission maximum shifted to longer wavelengths, the corresponding shift in the absorption maximum of the pigment is insufficient to explain the absorbing properties of the rods on sensitivity grounds alone (Dartnall, 1965; Lythgoe, 1968).

A further implication of the sensitivity hypothesis is that, if valid, one could expect that fishes from the same or similar photic environments should have similar visual pigments. Evidence to this effect is contrasting; work by Munz (1964, 1965) largely supports this view, whilst Dartnall and Lythgoe (1965) found that the absorption maxima of visual pigments from fishes from the Mediterranean and eastern Atlantic did not coincide with the maximum light transmission of the environment. If anything can be taken from these findings, it is the notion that whilst the sensitivity to ambient conditions may be a prominent factor in the selection of visual pigments, the selection of visual pigments is more complex than that suggested by sensitivity grounds alone.

1.4.2 The contrast sensitivity hypothesis

To account for the presence of pigments that were shifted away from the transmission maxima of the water body, John Lythgoe (1968) proposed a second hypothesis: The Contrast Sensitivity Hypothesis. Lythgoe suggested that in environments where light is abundant, selection would favour those pigments that maximised the contrast between an object and its background. This hypothesis considers the fact that an object (silver or white) when viewed against a lighted background (the sea) is most visible under conditions that increase the contrast between it and its background. Using a mathematical model based on the visual contrast equations of Duntley (1962, 1963), Lythgoe argued that if the background light is blue, and if the viewer's visual pigments have an absorption maximum offset towards longer (or possibly shorter) wavelengths, then this would enhance contrast between the bright object and its dark background. An interesting caveat to the contrast sensitivity hypothesis is that the offset pigment must not deviate too far away from the absorption maxima, as sensitivity must also be maintained; too great a deviation will render the object invisible. This is not the whole story as the identification of dark objects in the water column requires pigments that are maximally sensitive to the ambient spectrum of down-welling light.

Lythgoe's hypothesis implies something rather specific about scotopic (rod based) vision: that the strongest selective forces for those organisms with offset pigments are those associated with the identification of bright objects against a lighted background. Munz and McFarland (1973) were uneasy with Lythgoe's hypothesis, as vision under scotopic conditions is highly dependant upon sensitivity, regarding the offset visual pigments as a special case rather than a general case. In answer to the occurrence of many offset pigments, Munz and McFarland attempted to analyse the positions of the rod pigments within an ecological framework. They recognised that the crucial period for scotopic vision will be during the hours of twilight, and suggested that the apparent offset position of many pelagic and shore fishes in tropical waters could be explained in terms of their behaviour – for example, the downward migrations of parrotfish during the hours of twilight explains the predominance of blue shifted pigment - and by a corresponding shift in the spectral distribution of ambient light during the hours of twilight.

Whilst the work carried out by Munz and McFarland addresses the problem of offset pigments found in some shallow, clear waters, they did not attempt to explain the apparent disparity between the absorption maxima of fish living in green coastal waters and the transmission properties of these waters. In these waters, the relationship suggested by the sensitivity hypothesis appears to break down, as the absorption maximum of the rod pigments do not deviate further than 545nm despite a shift in transmission to 650nm (Lythgoe, 1984). One possible reason for this is that sensitivity of the rods at very low light levels is hampered greatly by noise from heat quanta (Barlow, 1962; Barlow *et al.* 1993). This point can be illustrated if one considers the sensitivity of the visual system in terms of the signal to noise ratio, S/N. In this case, any increase in signal strength (S) caused by spectral tuning will only be advantageous if the increase in noise (N) is relatively lower. At low light levels the noise, both from non-image forming light (photon noise) and from the visual system itself (dark noise), imposes limits on the S/N ratio and hence on visual performance. On thermodynamic grounds, visual pigments with shorter absorption maxima are less susceptible to spontaneous transduction due to thermal excitation (Barlow, 1957), and hence, there will be selection pressure for these pigments at low light levels due to their greater stability (Firsov and Govardovskii, 1990). Thus, it is quite possible that the evolution of the pigments for scotopic vision has been driven largely by the need to maximise S/N rather than sensitivity. If this is so, the conditions set under the sensitivity hypothesis cannot be met due to the constraints imposed by dark noise.

Despite the constraints associated with scotopic pigments, the sensitivity hypothesis still remains theoretically possible in those systems that are not constrained by dark noise. Furthermore, in such systems where irradiance levels (S) are high – that is, in daylight conditions associated with photopic vision – the increase of dark noise associated with the selection of certain pigments will be negligible in terms of S/N. The development of microspectrophotometry (MSP) in the latter part of the 1960's provided strong evidence that the spectral locations of the cone pigments are correlated with the spectral composition of the photic environment (Loew and Lythgoe, 1978; Levine and MacNichole, 1979; Lythgoe, *et al.,* 1994). The data produced by microspectrophotometry suggests that some classes of cone, in particular the double cones of surface dwelling fish, show a surprisingly close match to the quality of underwater light (Levine and MacNichol, 1979; Lythgoe, 1984). Two main correlations have also emerged from this data: firstly, the spread of the cone absorption maxima seems to depend on the bandwidth of ambient light, thereby allowing the eye to utilise efficiently all available wavelengths; and secondly, red-sensitive cones containing 620nm - 625nm porphyropsins have only been found in fish living in waters that are fairly rich in red light (Lythgoe, 1984).

Interestingly, subsequent accumulation of data relating to the absorption maxima of the cone pigments has also provided strong support for the contrast sensitivity hypothesis (Munz and McFarland, 1975; McFarland and Munz, 1975a, 1975b). This is due to the fact that if light levels are appreciably high - such as that experienced under photopic conditions - the photo-contrast of bright and dark objects viewed simultaneously against a bright background would be maximised by a retina containing both matching and offset pigments.

1.4.3 Spectral clustering of visual pigments

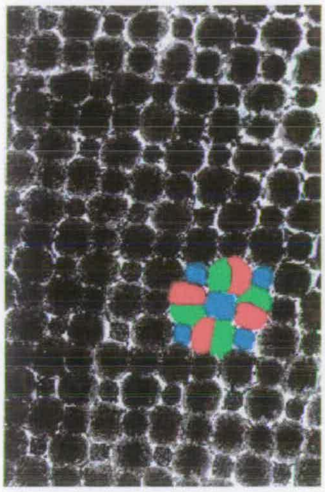
The discussion so far has assumed that the choice of a visual pigment is contingent upon the selective forces acting upon the organism. However, if constraints are put upon the range of spectral positions possible, the selected pigment may be offset from that which is ideal (Bridges, 1964a). Dartnall and Lythgoe (1965), following a survey of visual pigment extracts from some 83 species of teleosts, suggested that the absorption maxima (λ_{max}) of both rhodopsins and porphyropsins were not uniformly distributed throughout the spectrum, but tended to cluster at specific locations separated by approximately 8nm. They suggested that secondary interactions with opsin take place at a limited number of points along the polyene chain of the chromophore group, thus giving rise to a restricted number of λ_{max} values. Such clustering was still apparent when looking at the distribution of the λ_{max} of all rhodopsins reported up to 1975 (Knowles and Dartnall, 1977). More recently, from an extensive survey of 52 species of deep-sea fish, Partridge *et al.* (1989) have also suggested that the λ_{max} of their rhodospins clustered at intervals of between 6-10nm. As knowledge of molecular biology has increased, an explanation for the empirical observation of spectral clustering of λ_{max} values has emerged, and is thought to be due to the conformational properties of particular amino acids within the opsin molecule. More specifically, it is believed that only a limited number of amino acid positions within the opsin molecule can affect the transduction process involved in the absorption of photons as others are simply too isolated from the chromophore to have a significant influence on a visual pigment's absorption spectrum. It is also likely that there are other molecular constraints on the spectral location of a visual pigment's λ_{max}. For example, the structure of the visual pigments must also be compatible with other requirements: they must be structurally coherent, stable (that is, only become activated upon the absorption of a photon), and they need to interact efficiently with other proteins involved in transduction (Piantanida, 1991).

1.5 The visual pigments of the three-spined stickleback

The spectral range of light found in a particular aquatic environment generally dictates the number of photoreceptor classes found in the retina of fish. For example, in the blue of deeper marine habitats (420nm to 500nm), wavelength discrimination is generally facilitated by the presence of two broadly overlapping cone pigments (McFarland and Munz 1975b; Loew and McFarland, 1990). However, in shallow freshwater systems, where the ambient spectrum is much broader, it is likely that three, or possibly four, broadband cone pigments may be employed in extracting visual information (Barlow, 1982).

FIGURE 1. 5

Electron micrograph of the cone mosaic in the stickleback *Gasterosteus aculeatus*; one mosaic has been colour coded. The colours loosely correspond to the absorption maxima of each receptor class. The blue cones represent the possible positions of the blue and UV cones (see text, section 1.5). (Taken from Lythgoe, 1979).



Established thought suggests that the three-spined stickleback contains three cone pigments that are maximally sensitive at 452 (blue), 529 (green) and 609nm (red) respectively (Lythgoe, 1979), and these are made up of a mixture of porphyropsin and rhodopsin (pers comm. C. Baube). Evidence also suggests that the medium and long wavelength photoreceptors numerically dominate the retina (McDonald and Hawryshyn, 1995), interacting neurally to provide a colour opponent mechanism (Baube *et al.* 1995). In common with other shallow dwelling fish, it is believed that the short wavelength pigment is housed in the single cones, while the medium and long wavelength pigments reside in each limb of the double cones (Lythgoe, 1979). It is also believed that the double cones form the sides of a mosaic pattern (Eigenmann and Schafter, 1900), with the blue cones situated at the corners (Fig. 1.5). The exact pattern of the mosaic will, however, vary across the retina, and care must be taken not to oversimplify matters. Photoreceptors may undergo a reorganisation during the course of development – a possibility that has been found to be true in the case of salmon (Lyall, 1957) – and can also be apparent following retinomotor migrations (Kunz, 1980). Interestingly, whilst the existence of double cones with non-identical pigments has been known for some years (Walls, 1942), the adaptive significance of these cones is not yet fully understood (Lythgoe *et al.*, 1994). With regard to the rod pigment of the three-spined stickleback, Ali and Wagner (1975) recorded the presence of a mixed pair of pigments with maxima at 501nm (A_1) and 522nm (A_2).

1.6 Colour and colour vision

Acting in isolation, each photoreceptor will essentially act as a photon counter, able to capture some wavelengths more than others (Lythgoe, 1979). A single class of photoreceptors will not, however, be able to distinguish between a dim light at a wavelength that it is sensitive and a bright light at a wavelength to which it is less sensitive to. To perceive colour, it is necessary to compare between two or more classes of photoreceptor (Goldsmith, 1991). Crucially, these photoreceptor classes overlap in their spectral sensitivity, but are able to behave in a spectrally distinct manner. The inputs from these two photoreceptor classes must then be integrated, via a colour opponent mechanism, before colour information can be extracted (King-Smith, 1991). Without such a system only achromatic information can be encoded.

1.6.1 Assessing colour vision in G. aculeatus

Evidence for colour vision in the three-spined stickleback, although largely qualitative, was established in the early part of the twentieth century by several German workers (for reviews see Herter, 1953). Despite the simplicity of the techniques employed, they were able to establish that the three-spined stickleback could differentiate between different spectral lights or reflective colour patches on the basis of wavelength. This work has been largely confirmed by more recent electrophysiological work that has revealed the existence of a green-red opponent colour mechanism (McDonald and Hawryshyn, 1995). In addition to this, both dichromatic and trichromatic models of colour vision have been found to fit behavioural data obtained during mate choice experiments (Baube *et al.*, 1995).

1.7 Ultraviolet sensitivity

The discovery that invertebrates can detect shorter wavelengths than humans dates back more than a hundred years (Goldsmith, 1994). However, it was almost a century later when the first indications that certain vertebrates possess an independent ultraviolet photoreceptor came from evidence obtained from the roach, *Rutilus rutilus* (Avery *et al.*, 1983) and in the Japanese dace (Hárosi and Hashimoto, 1983). Independent ultraviolet photoreceptors have since been found in many freshwater species such as the carp, *Cyprinus carpio* (Hawryshyn and Harosi, 1991); goldfish, *Carassius auratus* (Neumeyer, 1985); roach, *Rutilus rutilus* (Douglas, 1986); euryhaline species such as the juvenile salmonids (Novales Flamarique, 2000); and many coral reef fish (McFarland and Loew, 1994).

Of these fish, true colour tetrachromatic vision has been found to exist in the goldfish (Neumeyer, 1992), although the application of similar colour mixing studies would probably reveal this ability to be widespread. Nevertheless, where tetrachromatic systems do exist, there is the potential for non-spectral colours (Goldsmith, 1990). It is also possible that some retinas with four photoreceptors may be locally no more than trichromatic (Goldsmith, 1994). In addition, evidence from the goldfish suggests that the dimensionality of colour vision may be a function of intensity, with one or more receptors classes failing to participate at low ambient light levels (Neumeyer and Arnold, 1989).

1.7.1 Ultraviolet photoreception in the three-spined stickleback

Similar to other vertebrates, it has long since been assumed that the three-spined stickleback is only sensitive in the human visible range. Indeed, the close match of the three cones in the stickleback retina has led to the assumption that the stickleback perceives its environment in a manner similar to us (Künzler and Bakker, 2001). However, evidence to the contrary does exist. Rather regrettably, as the work is not published in English, it is often overlooked; especially in those fields not concerned with the exact mechanics of vision.

In a series of elegant experiments in the 1930's, Merker established that the three-spined stickleback is sensitive to ultraviolet wavelengths of light (Merker, 1932, 1934, 1937, 1939). He was able to demonstrate that the optical components – cornea, vitreous humour and lens – of the stickleback's eye, unlike our own, were transparent to ultraviolet wavelengths. He also showed that the stickleback was able to track a food reward illuminated only by ultraviolet light. Crucially, by examining visual angle, he was able to control for the possibility that such sensitivity was driven by fluorescence in the cornea.

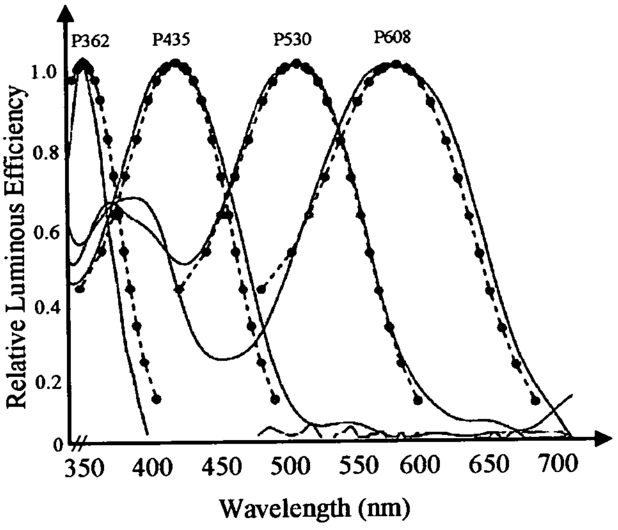
Merker's work suggests two possible mechanisms. Ultraviolet sensitivity could be due to the secondary absorption peak of the long-wavelength cone, corresponding to the cis-peak (or β band) of the rhodopsin pigment (Jacobs, 1992). Since photo pigments do not encode wavelength information, provided no other pigment is involved, absorption of ultraviolet wavelengths in this instance would not be qualitatively discernible from a purely red wavelength. Alternatively, sensitivity to ultraviolet wavelengths could arise from the existence of a fourth class of cone receptor- maximally sensitivity to wavelengths below 400nm.

From a theoretical standpoint, the occurrence of a fourth receptor pigment might be expected given that the spectral range of ambient light in shallow water is particularly broad (Barlow; 1982, Partridge, pers. comm.). In the case of a North American population of three-spined stickleback this has been found to be the case. Unpublished work in Crescitelli's lab (Ellis Loew, pers. comm.) has identified three cone types within the retina: a non-identical double cone, and two single cones. The absorption maxima of the two limbs in the double cone ranges from 575nm to 625nm and 519nm to 535nm, confirming the suspicion of a retenoid (i.e. A_1/A_2) mixture in this species (Ellis Loew, pers comm.). With regard to the two single cones, Crescitelli found one to be maximally sensitive in the blue region of 440nm-450nm and the other sensitive in the ultraviolet region of spectrum at 365nm (Fig. 1.6).

FIGURE 1. 6

Absorbance curve of the four cone pigments contained within the retina of a North American population of three-spined stickleback (*Gasterosteus aculeatus*). The dotted lines are fitted nomogram templates for each receptor class.

(Unpubl. data collected by F. Crescitteli, pers. comm. by E. Loew)



1.7.2 Colour vision and the role of ultraviolet light

The existence of an independent ultraviolet photoreceptor found by Crescitelli indicates nothing about its function. It is conceivable that it may not actually contribute to colour vision at all, supplying only achromatic information, or that its input is summed with another class of photoreceptor (Neumeyer, 1992). Indeed, the existence of tetrachromacy in the three-spined stickleback does not receive support in recent electrophysiological data (McDonald and Hawryshyn, 1995) and this has led to the advocation of a tri-chromatic system (Baube *et al,* 1995). Yet, whilst the green-red colour mechanism found by McDonald and Hawryshyn undoubtedly dominates colour processing in the stickleback, it is my opinion that none of these experiments conclusively rule out the possibility of tetrachromacy in the stickleback. Crucially, the behavioural experiments carried out in Baube *et al.* were performed in artificial light, entirely lacking ultraviolet wavelengths, and did not, therefore, test the visual system in the ultraviolet region of the spectrum. In addition, the experiments employed by McDonald and Hawryshyn (1995) were primarily directed at testing if visual pigments shift according to ambient light, and not at establishing tetrachromacy. The authors concede that their electrophysiological technique is insufficient to prove the absence of an ultraviolet photoreceptor in the retina. Furthermore, electrophysiological study of neuronal types in the retina will only reveal those visual processes that take place at the periphery of the visual system (Neumeyer, 1984). In order to determine the true dimensionality of colour vision, it would be preferable to perform colour-mixing experiments at the behavioural level (Neumeyer, 1991). Such a technique has only been carried out in the goldfish, *Carassius auratus,* (Neumeyer, 1992). However, recent behavioural evidence obtained from guppy mate-choice experiments, *Poecilia reticulata*, suggests that tetrachromacy exists also in this species (Smith *et al.*, 2002b).

1.8 Plasticity in the visual system

1.8.1 Tuning to environmental light

From a theoretical standpoint, fish living in highly variable photic environments will benefit from concomitant changes in the absorption maxima of their visual pigments. One mechanism that could underlie such a shift would be a change in the paired pigment ratio (A_1/A_2). In 1961, Dartnall *et al.* demonstrated that in the freshwater rudd, *Scardinius erythropthlmus*, the proportion of rhodopsin (A_1) and porphyropsins (A_2) in the rods was not fixed, but depended on the lighting conditions under which the fish are kept. The authors found that during long-day months the proportion of A_1 pigment increased, whereas in short-day months the opposite was true. Associated with this change in ratio is a change in the absorption maxima of the long-wave sensitive cones, shifting from approximately 565nm to 630nm. In addition to day-length, several other factors including, hormones, temperature, age, and retinal location sampled, can also be correlated with changes in the proportion of the two pigment types (Bridges, 1972a; Knowels and Dartnall, 1977; Muntz and Northmore, 1973).

Spectral shifts in sensitivity may also be initiated by the habitat transitions the animal makes in the course of its life history. An extreme example of this is evident in diadromous fishes, which at certain points in their life history will inhabit both marine and freshwater systems. The silver eel, *Anguilla anguilla,* is one such example. Spawning some 400m below the surface of the Western Atlantic the juvenile elvers make the arduous ascent up river, and after a second metamorphosis phase, return to the deep coastal waters from which they originated (Carlisle and Denton, 1959; Wood and Partridge, 1993; Archer *et al.*, 1995). The sequence of changes in the visual pigments that accompany this migration is no less remarkable. In common with the rudd, the silver eel possesses a paired pigment mixture (Carlisle and Denton, 1959). As one might expect from the previous discussion, this pigment ratio has also been found to vary with a change in habitat – the porphyropsin being entirely substituted by the rhodopsin. However, the rhodopsin itself also changes its absorption maximum via a modification in the opsin molecule (Bridges, 1972a). What makes this different from changes in spectral sensitivity brought about by a change in light regime, is that the migratory movements are anticipated, suggesting that visual pigment alterations are governed by the metamorphic process itself. Moreover, recent evidence suggests that changes in the opsin component of the visual pigment may be a common phenomenon in the development of many fish (Shand *et al.* 1998 & 2002).

Spectral tuning to environmental light in G. aculeatus

In common with the rudd and the silver eel, the photoreceptors in the three-spined stickleback have been found to contain a mixture of porphyropsin and rhodopsin (Ali and Wagner, 1975; Ellis Loew pers. comm.). Whilst such a mixture is capable of facilitating shifts in the absorption maxima of the photoreceptors, no such shift is evident in this species (Bowmaker, 1990). This evidence is supported by psychophysical data collected by Cronly-Dillon and Sharma (1968). Employing an optomotor technique, which exploits the animal's innate desire to keep station with bands of moving spectral light, the authors detected a seasonal change in the absolute sensitivity in the visual system of a population of *G. aculeatus*. They were unable, however, to find a shift in the position at which these maxima occurred, implying the maintenance of the A_1/A_2 ratio in the retina throughout the year.

Whilst seasonal shifts in the absorption maxima of stickleback pigments have not been found, McDonald and Hawryshyn (1995) were able to reveal substantial interspecific variation associated with the photic environment. They found that in fish from waters relatively transparent to red light, peak sensitivity in the both ON and OFF pathways was limited to long wavelength light. In contrast, the ON pathway of fish from waters relatively more transparent to blue light, exhibited sensitivity to both medium and long wavelengths; while the OFF pathway exhibited peak sensitivity to only medium wavelength light. What is of most interest, however, is that these results are consistent with spectral tuning acting independently upon the ON and OFF pathways. That this is so, can be explained by the OFF pathway's role in the in the detection of dark targets – a function that is suggested by the OFF pathways association with decrements of light intensity. According to the contrast sensitivity hypothesis (Lythgoe, 1968), by operating in such a role the OFF response will achieve optimum contrast, and therefore optimum signal strength, if the OFF response is maximally sensitive to ambient light. Hence, the OFF response in the visual circuit would be ideally suited as a shadow detector, a hypothesis which is supported by Northmore's (1973) finding that the escape response in the rudd is driven primarily by a red OFF stimulus (cited in McDonald and Hawryshyn, 1995).

1.8.2 Behaviour as a selective force

Behaviour will necessarily set the context in which the visual system has to operate. And if these behaviours are ecologically significant we might expect strong selective pressures on the eye taking precedence. One only has to look as far as some deep-sea fish, which have been found to possess rods with an absorption maximum ranging from 440nm to 540nm (Partridge, 1988, 1990) a figure in stark contrast to the waters narrow transmission peak of 470nm, to suggest that the photic environment is not the only force driving the evolution of the visual pigments. Indeed, the presence of red sensitive pigments combined with the presence of red photophores in some deep-sea fish intimates that behavioural adaptations - such as signalling or prey capture - may be paramount in the position of the absorption maxima of some visual pigments (Denton *et al*, 1985). An example of behaviour determining the spectral positions of cone pigments comes from a seasonal study of the brown trout, *Salmo trutta*, by Muntz and Mouat (1984). The retina of these fish contains an A_1/A_2 (paired pigment) mixture, the relative proportion of which is not fixed, but has been found to change in response to the level of seasonal illumination. What is of significance in this study is that a summer shift towards the pigment that is more receptive in the blue region (a rhodopsin) coincides with a change in the spectral characteristic of ambient light in the opposite direction. Taken at face value, this phenomenon is clearly at odds with the sensitivity hypothesis. However, a shift towards the rhodopsin could be interpreted in terms of the sensitivity hypothesis if such a shift coincided with a move towards the surface waters, which are relatively more abundant in blue light (Muntz and Northmore, 1971; Levine and MacNichol, 1979). The fact that trout tend to feed more on surface prey in summer than in winter is, indeed, consistent with such a shift (Hunt and Jones, 1972).

Reproductive behaviour and visual pigments

Using a behavioural assay, Cronly-Dillon and Sharma (1968) discovered a shift in the photopic spectral sensitivity of the three-spined stickleback. Of particular interest is that their results showed an increased sensitivity towards red light that occurred only in females. The authors noted that this change was only apparent during the breeding season when the males develop their characteristic red belly. The overall spectral sensitivity of the females tested during the summer did not, however, display a shift in wavelength, but differed in terms of a selective lowering of the optomotor threshold at which longer wavelengths were detected. The authors considered this change in sensitivity to be mediated by the selective sensitisation of the visual pathways, or the sequestration of more pigment in the retina, rather than any change to the pigment composition.

1.9 Summary

The three-spined stickleback has proved to be a useful subject for scientific enquiry. However, whilst much has been written about its visually mediated behaviour, a great deal remains to be investigated about the visual system of this species and, as such, it remains important to gain a clearer picture of the stickleback's visual capabilities. Without a comprehensive understanding their visual system, we risk misclassifying colour, and in so doing, risk misinterpreting much of the observed behaviour of this fish. The possibility that ultraviolet sensitivity exists in the stickleback necessitates further investigation. If such a capacity is widespread in this species, it will be important to assess its function in the performance of biologically significant behaviours that ultimately contribute to fitness. Furthermore, since the three-spined stickleback provides us with an ecologically diverse species complex with which to examine phylogentic and behavioural patterns, we are provided with an ideal opportunity to examine how the photic conditions of the environment, and the behaviour of the species itself, may shape the evolution of visual pigments.

1.10 Thesis aims

The aims of this thesis are two fold. First, it is necessary to obtain a clearer picture of the spectral sensitivity of the three-spined stickleback. Whilst limited data does exist for the spectral sensitivity of this species, much of this work was not concerned with ultraviolet sensitivity, and is therefore incomplete. In particular, given the discovery of ultraviolet sensitivity by Merker, and preliminary evidence indicating the existence of an independent photoreceptor, can we reasonably assume that ultraviolet sensitivity is universally found in the stickleback? Furthermore, as the existence of a photoreceptor indicates nothing about its function, it is necessary to establish if ultraviolet sensitivity is employed in visually mediated behaviour under ambient conditions, rather than the ultraviolet rich conditions used by Merker. Second, our understanding of the visual ecology of the stickleback would also benefit from a more holistic methodology: closely incorporating the spectral sensitivity, behaviour and photic environment of a single population. In so doing, I hope to isolate those factors, both abiotic and biotic, that are responsible for the adaptation of the eye.

1.11 Structure of the thesis

1.11.1 An examination of the eye

Fundamental to any understanding of the visual system of the three-spined stickleback is a detailed assessment of the eye, in terms of its optical properties, histology, and spectral sensitivity of its photopigments. Using an electrophysiological technique, chapter 2 examines the spectral sensitivity of a single population of sticklebacks across human visible and ultraviolet wavelengths of light. This study was carried out in a population of sticklebacks residing in shallow water, as it is likely that such waters are relatively abundant in ultraviolet wavelengths (Loew and McFarland, 1990). The ocular media of the eyes of individuals were also recorded in order to establish their degree of transparency, and thus, to determine the ultimate spectral composition of light impinging on the retina.

1.11.2 The nature of the photic environment

True understanding of the visual system can only be achieved with close reference to the transmissive medium in which vision must operate. In this respect, it is beneficial to classify the photic environment of the stickleback population examined in chapter 2. Of particular interest will be the wavelength composition of down-welling light and the relative abundance of ultraviolet photons. Chapter 3 documents irradiance and transmission data collected over the reproductive season. The timescale of this study is important in assessing concomitant shifts in the stickleback's spectral sensitivity.

1.11.3 Ultraviolet light and its role in visually mediated behaviour

Knowledge of both spectral sensitivity and the nature of the photic environment provide a strong base for the study of visually mediated behaviour in the three-spined stickleback. In light of the potential for ultraviolet photoreception in our study population, it remains important to establish if sticklebacks can utilise ultraviolet wavelengths under near-ambient conditions. As true visual capabilities can only be revealed at the behavioural level, chapter 4 uses a simple operant conditioning technique to establish ultraviolet sensitivity in this species. In contrast with Merker, the foraging task employed in this experiment is undertaken in conditions close to natural ambient conditions in order to establish if such behaviour is typical in the natural environment.

If the stickleback is, indeed, bestowed with ultraviolet sensitivity, and this sensitivity is part of the fish's normal visual capabilities, then this finding will also have relevance for other visually evoked behaviours in this species. To ignore this fact would be to fall foul of the general assumption that Lubbock warned against over one hundred years ago. Given the fact that ultraviolet light has been found to affect the mate choices of female guppies (Smith *et al.*, 2002b) it is necessary to examine whether ultraviolet sensitivity also is implicated in mate choice. This subject is dealt with in chapter 5, where spectral reflectances for males in breeding colouration are also given.

1.11.4 Spectral tuning of visual pigments

Cronly-Dillon and Sharma (1968) provide an intriguing result where the female becomes more sensitive to red during the breeding season, at a time when males are also developing their red nuptial colouration. The fact only females undergo this change in sensitivity lends considerable weight to the argument that such a shift is driven by colour change in sexually receptive males. This result is particularly surprising, and gives us one of the few examples where the female visual system has apparently been found to tune into the secondary sexual characteristics of the male. Yet despite the significance of this result, it has not been replicated, even though the detected effect found by Cronly-Dillon and Sharma was based on a small sample size. Chapter 6, in an attempt to discover the mechanism underlying this shift, re-analyses this result and examines whether such a shift is evident in the population of three-spined sticklebacks that have been the subject of previous chapters.

Chapter 2

A preliminary study of the visual system of the three-spined stickleback

EXPERIMENT 2a

EXPERIMENT 2b

EXPERIMENT 2c

All of the fish used in this chapter were treated in accordance with UK Home Office guidelines and out under Home Office Project Licence number PPL 60/2954 and Personal Licence number PIL 60/8697.

2. A preliminary study of the visual system of the three-spined stickleback

2.1 Introduction

Teleost eyes, in common with those of other vertebrates, are built upon one fundamental plan: each eye possessing a lens whose optical properties are such that it allows an image to form on a photosensitive retina (Walls, 1942). Within this basic plan the visual system has adapted to suit a wide range of light environments and behaviours, so that, even within a species, there is considerable potential for variation (Sivak *et al.*, 1999). Yet, despite the diversity displayed by the eye and its constituent parts, in all cases across the vertebrate group the function of the eye has been conserved. If we consider the anatomy of the vertebrate eye, it is particularly striking that there are few apparently superfluous structures that lie within the eye. Indeed, it is this functional balance that has proved useful in the study of the visual behaviour of fish, underpinning much of what we understand about their visual ecology. Hence, in studying the visual ecology of the three-spined stickleback, it is appropriate to start with an examination of the eye itself.

For example, as spectral information used by the organism depends ultimately upon the process of photoreception, it is the absorption characteristics of the particular photopigments residing within the different receptor cell-types that will dictate the bandwidth over which the visual system can operate (Bowmaker, 1990). Furthermore, as a minimum of two visual channels are necessary for wavelength discrimination, the presence of two or more classes of spectrally distinct photoreceptors presents a basis for colour vision (Lythgoe, 1979). These two concepts are not, however, mutually exclusive. The spectral sensitivity range of those fish which possess four cone types is perhaps the widest amongst the vertebrates, extending from 350nm to 800nm (Bowmaker, 1990), and in this there is potential for tetrachromacy; a phenomenon which is not unusual in vertebrates (Goldsmith, 1991). It should, nevertheless, be remembered that in determining the dimensionality of colour vision that not all retinal photoreceptors may contribute, and so the dimensionality of colour vision cannot merely be determined solely by the study of the retina (Neumeyer, 1992).

Thus, the visual performance of an organism's visual system is not governed by the photopigments alone but will also be contingent upon the exact nature of light impinging on the retina (Loew and McFarland, 1990). In instances where the spectral range of the environment is restricted, a situation typical of many aquatic habitats, visual information may easily be extracted with only two broad overlapping pigments (Barlow, 1982). In a similar manner, the bandwidth of spectral information will also depend on the extent to which electromagnetic radiation is modified by the optical components of the eye before reaching the retina (Lythgoe, 1991). This is not an insignificant point as, even in the absence of pigmentation, the physical nature of the biological tissue in the eye is such that little radiation below 310nm will pass through to the retina; the exact point of cut-off depending on the thickness of the ocular material in question (Douglas and Marshall, 1999). However, in many species, short-wavelength pigments in the lens and cornea limit the light reaching the retina. In such cases, the sensitivity at the shorter wavelengths will not be limited by the absorption characteristics of the photopigments but by the ocular media of the eye itself (Heinermann, 1984). Many diurnal fish have been found to possess lenses, and some instances corneas, that are opaque to ultraviolet radiation. In its most extreme form, the absorption of these pigments extend into the visible spectrum producing the conspicuous yellow pigmented "eye shades" found in many fish (Walls and Judd, 1933).

In those species where the ocular media does not act as a short-wavelength cut-off, there remains the potential for photoreception in the ultraviolet region of the spectrum (Jacobs, 1992). Indeed, evidence indicating that fish are able to perceive short-wavelength radiation across the ultraviolet region of the spectrum has existed since the 1920's. That fish were able to respond behaviourally to wavelengths down to 360nm was demonstrated in the minnow, *Phoxinus phoxinus*, by both Schiemenz (1924) and Wolff (1925) (cited in Walls, 1942). Whilst it is true that the results of these studies may have been driven largely by fluorescence in the ocular media (Douglas, 1986) rather than ultraviolet sensitivity *per se*, Merker, working on the three-spined stickleback in the 1930's, provided unequivocal evidence that an aquatic vertebrate was able to perceive ultraviolet illumination (Merker, 1937, 1939). Despite these findings, it was not until the 1980's that visual ecologists seriously considered the existence of an additional, independent photoreceptor that absorbed maximally in the ultraviolet region of the spectrum. The field only advanced when Douglas (1985) demonstrated ultraviolet sensitivity in the roach, *Rutilus rutilus*, using a two-choice appetitive training technique. By selectively bleaching all but the ultraviolet cones, they were able to establish that ultraviolet sensitivity in this species was due to a fourth class of cone receptor (with a maximum at 361-398nm) and not due to aberrant stimulation of the three other cones types. In the years that have followed, independent ultraviolet photoreceptors have been found in a number of freshwater fish such as: the Japanese dace, *Tribolodon hakonensis* (Harosi and Hashimoto, 1983), the brown trout, *Salmo trutta* (Bowmaker and Kunz, 1987), the goldfish, *Carassius auratus* (Hawryshyn and Beauchamp, 1985), the carp, *Cyprinus carpio* (Hawryshyn and Harosi, 1991), and the guppy, *Poecilia reticulata* (Smith *et al.*, 2002b).

Much is known about the mechanisms of colour processing in the three-spined stickleback in relation to the human visible region of the spectrum (Cronly-Dillon and Sharma, 1968; Ali and Wagner, 1975; Ali and Anctil, 1976; McDonald and Hawryshyn, 1995; Baube, 1997). Yet despite Meker's finding of sensitivity to ultraviolet radiation in one population of three-spined stickleback, little has been done to establish the mechanism for ultraviolet sensitivity in this species. This is of particular concern as many of the evolutionary hypotheses that involve this species assume colour vision and coloration to be appropriately described by the human visual system. Such an approach has been defended in the belief that the sticklebacks possess a trichromatic visual system sensitive across the same area of the electromagnetic spectrum as humans: 400nm to 700nm (Künzler and Bakker, 2001). However, if three-spined stickleback can perceive ultraviolet wavelengths, whether due to a tri-chromatic or tetrachromatic system, such an approach will be flawed as it arbitrarily omits a region of the electromagnetic spectrum to which the this species is sensitive.

This chapter examines the spectral sensitivity of a single population of sticklebacks across the visible and ultraviolet region of the electromagnetic spectrum. A particular aim of the work described here is to extend the study of Merker and

Crescitelli (Crescitelli pers. comm. E.Loew; Meker, 1932, 1934, 1937 & 1939) by assessing the potential for ultraviolet photoreception in sticklebacks. In the first instance, I examined the ocular media of the eye to determine whether appreciable amounts of ultraviolet radiation do reach the retina. Then, using an electrophysiological method, I derived a spectral sensitivity curve for the subject population. Previous electrophysiological studies examining the visual system of the three-spined stickleback have failed to demonstrate the existence of an independent ultraviolet photoreceptor (McDonald and Hawryshyn, 1995) despite Crescitelli's unpublished microspectrophotometric data confirming their existence in one North American population (pers. comm. E. Loew). The reasons for such conflicting evidence could be two fold: the cones identified by microspectrophotometric study may be limited to certain populations; or that the ultraviolet cones may be in such low numbers in the retina as to make their detection problematic.

Experiment 2a

2.2 Ocular media of the stickleback eye

Due to the absorption of characteristics of their constituent amino acids and protein structures, the optical components of the vertebrate eye are unable to transmit functionally significant amounts of electromagnetic radiation below 310nm to the retina (Douglas and Marshall, 1990). In addition to this physical limitation, fish, in common with most vertebrate species, often possess pigments in their corneas and /or lenses that selectively absorb ultraviolet radiation (Kennedy and Milkman, 1965; Moreland and Lythgoe, 1968; Muntz, 1973). Of all the ocular media, the lens is the optical element most usually associated with the filtration of spectral light and, with the exception of those species where the cornea contains a large amount of yellow pigment, is ultimately responsible for the spectral absorption of the whole eye (Douglas and Marshall, 1999). The situation is complicated further by the fact that as a vertebrate lens increases in size during the life history of the organism concomitant changes in path length will inevitably lead to an increase in opacity.

Such age related decreases in lens transmission have been recorded in several fish (Douglas, 1989). This effect may be even more pronounced in pigmented lenses where increased production of pigments may increase opacity further. However, such changes are not universal, and decreases in opacity with age are also found (Thorpe and Douglas, 1993).

The spectral transmission of the ocular media of the three-spined stickleback has been measured by Merker (1937). Using a photographic technique, Merker was able to demonstrate that, in one population of three-spined sticklebacks at least, the cornea, lens and humour allow the transmission of ultraviolet wavelengths. The aim of experiment 2a is to replicate these results in a second population of stickleback by using modern spectrophotometery techniques that allow us to quantify and examine the individual optical components of the visual system and to examine the opacity of the eye in its entirety.

2.3 Methods

2.3.1 Subjects

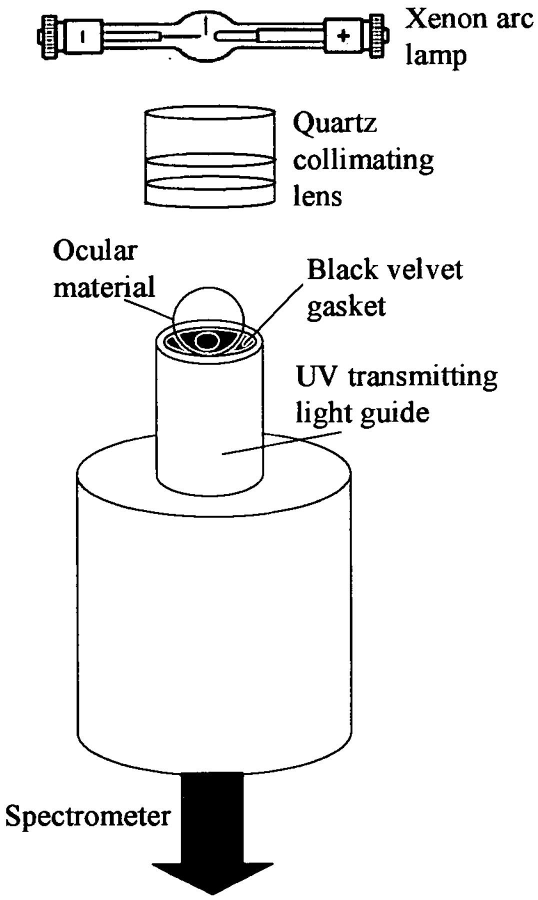
Ten adult, non-breeding three-spined sticklebacks were collected during February of 2002 from Balmaha Pond, Loch Lomond (chapter 3 provides a survey of this habitat). All fish were of a similar body length: 4-5cm. Since the spectral transmission of ocular media has been shown to decrease with age in some species (Douglas, 1989), only adult fish were used in the study as it was desirable to determine the lower threshold of transmission in this species. As the three-spined stickleback is typically short-lived, living only two years at the most, these fish are likely to be representative of the top age range of the population (Fitzgerald and Wootton, 1993). All fish were kept in holding tanks under a 10hr-light/14hr dark cycle at 12 °C for maximum duration of two weeks prior to experimentation. During this period, light was provided by two full spectrum fluorescent bulbs (Arcadia Ltd), each with a colour temperature of 5600°K, suspended 40cm above the holding tanks (see appendix 1). Fish were fed once daily on a diet of bloodworm.

2.3.2 Optical apparatus

The transmission properties of the eye - whole eye, cornea, lens and spectacle (Fig. 1.3) were measured using methods similar to those outlined in Siebeck and Marshall (2000). Here, spectral transmission curves were obtained using an Ocean Optics USB2000 portable spectrometer (Ocean Optics Inc. Florida) in conjunction with a 75W xenon arc lamp (XBO75W/2, Osram, Germany), and powered by a regulated voltage supply (Müller, Electronik Optik, TYP XH 100). Once prepared, biological tissue was placed in the optical axis of the recording fibre of the spectrometer and its transmission properties determined using a proprietary software package (OOIBase32, Ocean Optics). All samples were measured in air (Fig 2.1).

Figure 2.1

Apparatus used to determine the transmission of the ocular media. Light was transmitted to a portable spectrometer (USB2000, Ocean Optics) linked to a micro computer.



2.3.3 Procedure

Prior to examination fish were dark-adapted for 3hrs after which period they were anaesthetised in a solution of Tricane Methanosulphate (MS-222) and killed according to Schedule One. To avoid tissue degradation, enucleation and all subsequent manipulations of the eye were performed in teleost Ringer solution (Takahashi, 1992). This technique has been used successfully in previous studies and was not found to alter significantly the opacity of the experimental tissue within a time frame of 90 minutes (Douglas & McGuigan, 1989).

To measure the transmission properties of the whole eye, a small aperture was cut into the back of the eye, in line with the visual axis. Next, the prepared eye was then placed onto a black velvet gasket before being positioned over the recording fibre of the spectroradiometer. In an attempt to minimise the affect that peripheral pigmentation may have on the reading, all samples were measured through their centre (Douglas and McGuigan, 1989). All specimens were rinsed thoroughly in Ringer solution to remove remnants of blood and vitreous. After measuring the whole eye, lenses and corneas were dissected out and measured separately.

In accordance with Douglas and McGuigan (1989), transmission curves were normalised so that the absolute transmission of the graph at 700nm was set at 100%. This was done as absolute transmission figures are very much dependent on the position of the specimen on the spectrophotometer mount. Such a normalisation is acceptable, as the shape of the transmission curve remains unaltered.

2.4 Results

The absorption spectrum for the ocular media of the three-spined stickleback was examined in ten fish. Data for the relative spectral transmission of whole eye, spectacle, cornea and lens of the three-spined stickleback of three adult fish are presented in figure 2.2 as representative curves for all fish tested (curves obtained from the other test fish were found to be whole consistent with the data presented in figure 2.2). The data presented shows that the eye and all its constituent parts are transparent to ultraviolet radiation. In all instances tested, the relative spectral transmission curves of each ocular element conformed to the pattern displayed by these three individuals.

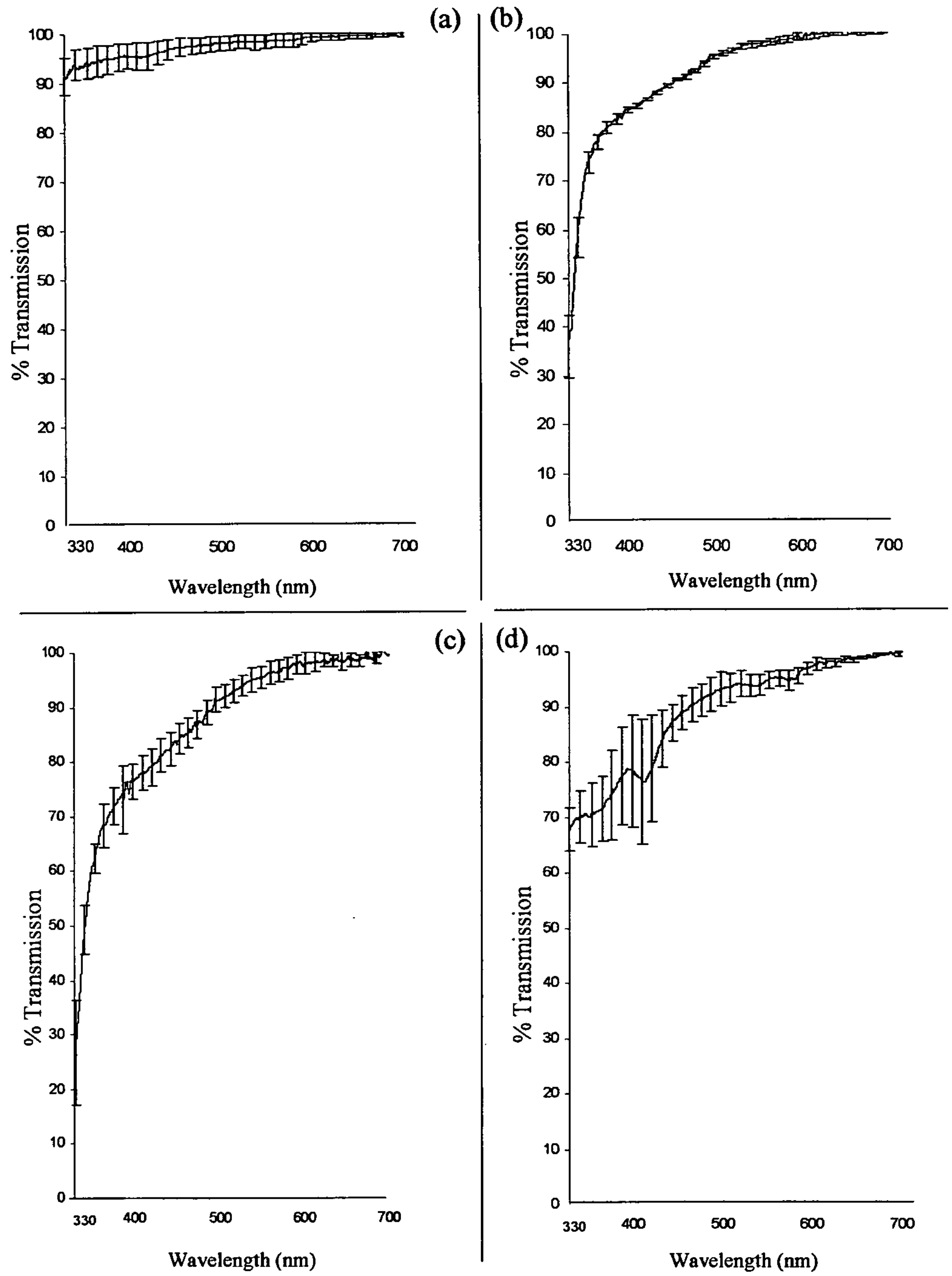


Figure 2.2 Average, relative spectral transmission of the structures forming the ocular media of the stickleback: (a) cornea (b) lens (c)whole eye(d) and spectacle for the three-spined stickleback (n=5). Error bars represent ±1 standard error of the mean.

2.5 Discussion

The results in figure 2.2 indicate that the ocular media of the three-spined stickleback are transparent to ultraviolet radiation and, as such, are in agreement with the findings of Merker (1937). Of the ocular media sampled, the lens was found to exert the greatest influence upon transmitted light. Indeed, among vertebrates the lens is often the most common ocular filter determining the absorption characteristics of the whole eye (Walls, 1942). All lenses examined in the experiment had smooth transmissions with a cut-off around 340nm – categorised by Douglas and McGuigan (1989) as a type one lens (315-354nm). Such lenses probably contain no specialised absorbing pigments and consequently can transmit far into the ultraviolet region of the spectrum. The transmission curves of the corneas did not exhibit such a strong short-wavelength cut-off as the lens; a difference which could relate to the shorter path length in this structure.

The absorbance characteristics of the vitreous humour were not measured in this study as, although theoretically possible, there is little evidence to support the occurrence of pigmented filters in the aqueous and vitreous humour of fish (McCandless *et al.*, 1969). Previous measurements of the vitreous humour of the three-spined stickleback support this assumption, revealing little absorption across the range of wavelengths tested in this study (Merker, 1932). This fact is borne out by the scan of the whole eye does seem to be accordance with the scans of its constituent parts, suggesting that the stickleback eye has the potential for ultraviolet photoreception.

In addition to the media measured by Merker, experiment 2a also produced spectral transmission curves for the spectacle which overlies the three-spined stickleback's eye (Fig. 2.2b). Of all the ocular media sampled, the transmission characteristics of the spectacle were found to be the most variable. Such results could be due to the optical flaws of the surface of the spectacle - a consequence of its protective function - or due to the presence of short-wavelength absorbing "skin" pigment. In either case, it should be remembered that the eye is able to respond over an intensity range of about 10 log units (pers comm. Ron Douglas), and therefore, a reduction in transmission from 80% to 60%, which represents a change of less than one log unit, is probably of little consequence.

2.5.1 Ocular media transmittance and vision in the stickleback

The transparency of the ocular media to electromagnetic radiation influences the passage of ultraviolet wavelengths to the surface of the retina. However, whilst the transmission of ultraviolet radiation to the retina is highly suggestive of an animal's ability to detect these wavelengths, it does not necessarily provide sufficient proof of their perception. Even if the stickleback does not possess an independent ultraviolet photoreceptor, it is possible that, given the absorption characteristics of the ocular media depicted in figure 2.2, they would be able to see into the ultraviolet region of the spectrum using the conventional blue absorbing pigment of their short-wavelength photoreceptor (Fig 1.6). An example of such sensitivity is illustrated in humans where the removal of the ultraviolet blocking lens during surgery allows the perception ultraviolet radiation to become possible (Stark, 1987).

The case for ultraviolet perception is made stronger if we consider that there are considerable adaptive advantages to be gained by a shallow dwelling fish such as the three-spined stickleback if ultraviolet radiation is prevented from reaching the retina (Judd and Walls, 1933; Douglas and McGuigan, 1989). Possible disadvantages associated with ocular media transparent to ultraviolet rays are: 1) a decrease in acuity due to an increase in chromatic aberration (Douglas and Marshall, 1999), 2) an increase in non image forming glare, 3) a decrease in acuity due to an increase in scattered light, 4) an increase in the potential for photo-oxidative damage to the retina (Freeman and Knox, 1964; Ham *et al.,* 1984; Chen *et al.*, 1999). Indeed, in those species where ultraviolet light is permitted to reach the retina, retinoic acid is implicated in the protection of the photoreceptors, playing an important role in limiting the oxidative damage caused by ultraviolet radiation (Chen *et al.*, 1999), and it is likely that it does so here. Whilst such a mechanism can confer protection to the retina, those species that are sensitive to ultraviolet light are likely to remain in waters where ultraviolet radiation is not too intense, avoiding prolonged exposure in surface waters or residing in fairly turbid waters, and this is the case in the stickleback. Furthermore, due to its propensity to scatter, ultraviolet wavelengths are probably only useful over short distances, and, as such, its effectiveness will be in part dictated by the visual tasks performed by the individual (Bowmaker, 1990).

Experiment 2b

2.6 Spectral sensitivity of the three-spined stickleback

While the wavelength composition of light reaching the retina provides some idea of the range of electromagnetic radiation to which the eye is sensitive, whether these wavelengths are utilised by the organism will be determined by the number of photoreceptor types, the bandwidth over which they operate, and the behaviour of the organism itself. One way to assess the sensitivity of the retina to electromagnetic radiation is to measure the transretinal voltages (electroretinograms) which are generated by the retina at photoreception. Specifically, the electroretinogram (ERG) is a complex extracellular waveform (Cosens and LeBlanc, 1980) which represents the summed electrical activity of neural and glial cells contained within the retina (Granit, 1933, Dowling, 1987). Hence, by recording light-elicited electroretinograms across the linear section of their response curve (Chen and Stark, 1994), and comparing this response to the known intensity of the stimulus wavelength, it is possible to construct a spectral sensitivity curve. Such a technique represents a level of recording intermediate between single units and the integrated behavioural response and may, therefore, be useful in relating physiological responses to behavioural measures of spectral sensitivity. Furthermore, since the reliability and stability of the ERG has proven to be good (Burkhardt. 1966; Chen and Stark, 1994), allowing sufficient time to present a detailed series of data, such a technique is appropriate to the study of the retinal sensitivity in the three-spined stickleback. To this effect, the ERG has been employed successfully in the production of spectral sensitivity curves in several other species such as the freshwater turtle, *Pseudemys scripta* (Deane *et al.*, 1958); the squirrel, *Sciurus vulgaris* (Tansley *et al.*, 1961); the goldfish, *Carassius auratus* (Burkhart, 1965); and the zebrafish, *Danio rerio* (Hughes *et. al*, 1998). Given this, it is the aim of experiments 2b and c to examine the cone contributions of the retina of the three-spined stickleback by constructing electrophysiologically derived spectral sensitivity curves and, hence, to determine the spectral range of photoreception in this species.

2.7 Methods

2.7.1 Subjects

Nine adult, non-reproductive three-spined sticklebacks were collected from Balmaha Pond, NS425905 (242775, 691050) GB Grid, in early September 2002. Subjects were held for a maximum period of two weeks in full spectrum holding conditions identical to those outlined in experiment 2a. Such lighting conditions in the holding tanks were necessary to avoid the effects of spectral deprivation which can result in retinal damage (Wagner and Kröger, 2000). To avoid circadian sensitivity effects, all data were recorded from 1:00pm to 7:00pm, with the daylight period commencing at 8:00am (Powers *et al.*, 1990). Upon the completion of the experiment, each individual was dissected and its sex determined.

2.7.2 Optical Apparatus

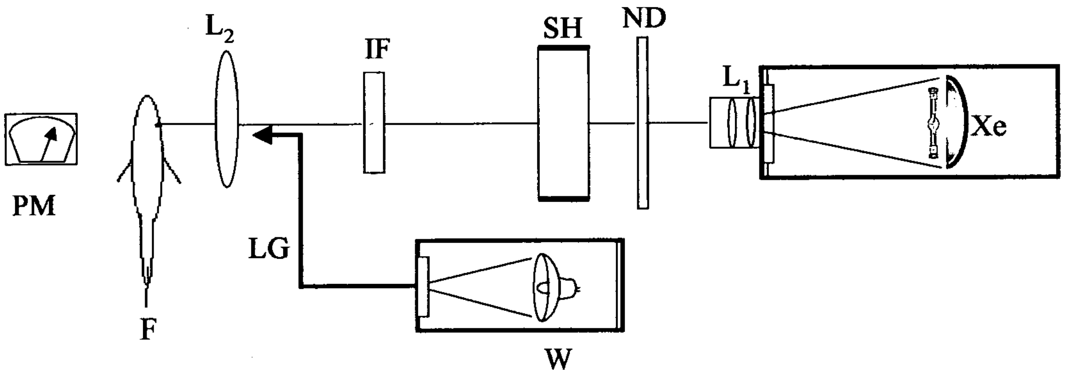


Figure 2.3 Schematic of two-channel optical system used to determine spectral sensitivity. Xe, xenon arc stimulus lamp, LG, light guide, W, tungsten halogen adapting lamp, ND, neutral density filter, IF, narrow band interference filter, SH, shutter, L_1, quartz collimating lens, L_2 quartz lens, PM, digital photometer, F, fish.

The optical system employed in this study was a two-channel system designed to deliver both stimulus and adapting background illumination to the retina of the fish (fig. 2.3). Light for the stimulus channel was provided by a xenon-arc lamp (XBO/75W, Osram) which was powered by a regulated voltage supply (Müller, Electronik Optik). The light from this lamp was focused onto the optic axis of the subject's eye using a quartz lens, and its wavelength composition controlled using a series of 10 narrow band interference filters (Edmund Optics, Inc; Appendix 1.3). The transmission maxima of these interference filters were positioned evenly across the human visible spectrum, 400-700nm, separated by intervals of approximately 25nm. The duration of the stimulus was 250ms and was controlled by a mechanical shutter placed in front of the quartz lens.

The light source for the neutral adapting background light was provided by a 250W tungsten-halogen lamp (L1500 Schott, Germany), which had a colour temperature of 4500°K. Using a quartz light guide (Fibreoptic Components Inc., California), the background light was directed onto the optical axis of the eye, where its image was superimposed onto the same area of the retina illuminated by the stimulus light. In order to achieve a desired background irradiance of 50mW /m^2 at the corneal surface, an appropriate neutral density filter was placed before the fibre optic cable. Previous studies involving both the goldfish and the zebrafish have indicated that this level of background irradiance is sufficient to suppress rod contribution, thereby ensuring the production of a photopic sensitivity curve (Chen and Stark, 1994; Hughes *et al.*, 1998). Preliminary trials also suggested that this intensity was sufficiently bright to maintain photopic conditions in the stickleback.

Light intensities were measured in all instances using a Tektronix J16 digital photometer in conjunction with a J6502 probe calibrated in mW^{-2}. The Tektronix photometer has a flat response across the human visible spectrum, and is ideally suited to measuring photon flux across the wavelengths tested in this experiment. All intensity measurements were converted into quanta (s^{-1}cm^{-2}) following the completion of the experiment.

2.7.3 Electroretinograms

Light-adapted ERG waveforms were collected electrophysiologically from the corneal surface. This was achieved by placing chlorided, silver electrodes on both the corneal surface and at the back of the eye, with the insertion of an accompanying earth electrode into the tail. Amplification of the resultant electrical signal was afforded by way of an AC microelectrode pre-amplifier (Grass, P15D) which fed into a dual channel oscilloscope (Tektronix, 2220 digital storage oscilloscope), operated in D.C. mode, and a data-acquisition board (National Instruments, AT-M10E series) of a microcomputer. Permanent records of the flash evoked ERGs were made using a proprietary software package (Virtual Bench; Wavelab Industries, Inc), which allowed wave images to be printed out for reference.

Of the several waves present in the ERG, spectral sensitivity in this study was determined from the response of the *b*-wave curve - Granit's PII (Granit, 1933; Granit and Riddell, 1934). It is believed that *b*-wave response reflects the activity of the ON-bipolar cells (Dick and Miller, 1978; Newman, 1980), representing the first layer of retinal neurons capable of colour opponency.

2.7.4 Procedure

Prior to testing, the subject fish was allowed to light adapt to moderate intensity broad band background light for a period of 1hr. The fish was then anaesthetised with MS222 at a dosage of 100mg/l. Once the fish exhibited a state of complete anaesthesia, it was removed, and a small incision made in the brain case above the right eye to allow access for the recording electrodes. Next, the fish was placed in a moulded wax holder where cable ties were used to hold the animal securely in position during the period of electrophysiological recording. Throughout this period, the fish was continually respired over the gills with a 50mg/l solution of MS222 maintained at a temperature of 20-24°C. To prevent desiccation, the fish was periodically moistened using a dilute saline solution.

Once preliminary ERGs had been obtained, a spectral sensitivity curve for each individual was obtained using an increment-threshold technique (Coughlin, and Hawryshyn, 1994). Using neutral density filters, each of the 10 tested wavelengths were presented to the eye in order of ascending intensity, increasing by 0.2 log units each time. In most instances, this series ran across one log unit of intensity. So that no photoreceptor became chromatically adapted to any particular stimulus, the next presented stimulus wavelength was chosen from a different part of the spectrum (Schellart *et al.*, 1987). To the same end, an 80sec interval between stimulus flashes was also observed (Chen & Stark, 1990).

The stimulus response functions obtained by using the ERG were found to be initially linear before exhibiting saturation. Hence, in choosing a suitable response criterion for the spectral sensitivity of the stickleback it is necessary to select a position on the stimulus response function that is linear, and in so doing, a response threshold of 50μV was chosen. This level was found in preliminary studies to consistently coincide with the linear section of the response vs. log intensity function. A threshold criterion of 50μV has been successfully employed by previous spectral sensitivity studies involving other fish species (Burkhardt, 1966; Hughes *et al.*, 1998).

2.8 Results

Figure 2.4 shows a representation of the typical electroretinogram responses of the three-spined stickleback to three different stimulus wavelengths. It can be seen from figure 2.4 that the amplitude of the *b*-wave response clearly changes as a function of stimulus irradiance. However, under the adopted experimental protocol only the *b*-wave of the ERG is evident, and it is likely that in this instance the threshold intensity for the relatively insensitive *a*-wave (Penn and Hagins, 1972) was not reached. Nevertheless, it is expected that under suitable conditions the eye of the stickleback would be able to elicit all ERG wave forms.

A total of nine fish were examined in this manner and the stimulus response functions plotted from the peak amplitude of the *b*-wave component of the ERG (Appendix 1.4). Figure 2.5 illustrates one such relationship for an individual showing clearly that the amplitude of the ERG response changes as a function of radiant flux. The ERG collected during this experiment could be isolated by if a suitable adapting background was used to bleach the functioning photopigments. The linear portion of the section of this amplitude intensity curve relationship was apparent for all of the tested stimulus wavelengths, and in each case coincided with the chosen 50μV criterion level. In all instances the criterion level was found to be above noise levels, a level typically around 15μV, and below response saturation.

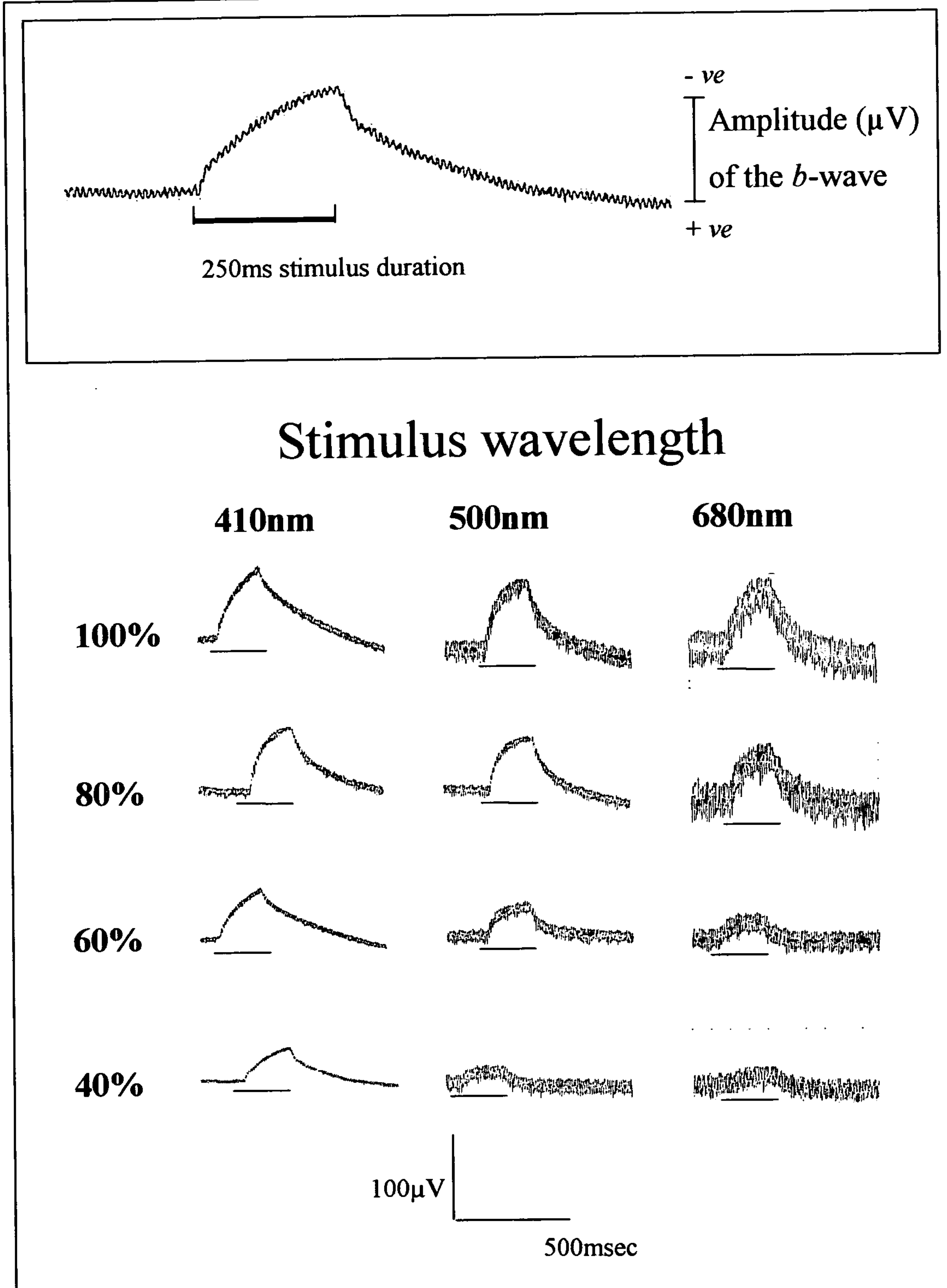


Figure 2.4 Representative electroretinogram responses of the three-spined stickleback to differing intensities of three selected wavelengths of light. Stimulus exposures were 250ms in duration (horizontal lines) and were superimposed over a 50mWm^{-2} adapting light. The degree of attenuation each stimulus was subjected to is denoted by the percentage to the left of each series. For the purposes of illustration, the light stimulus in each instance is equivalent in terms of photon number.

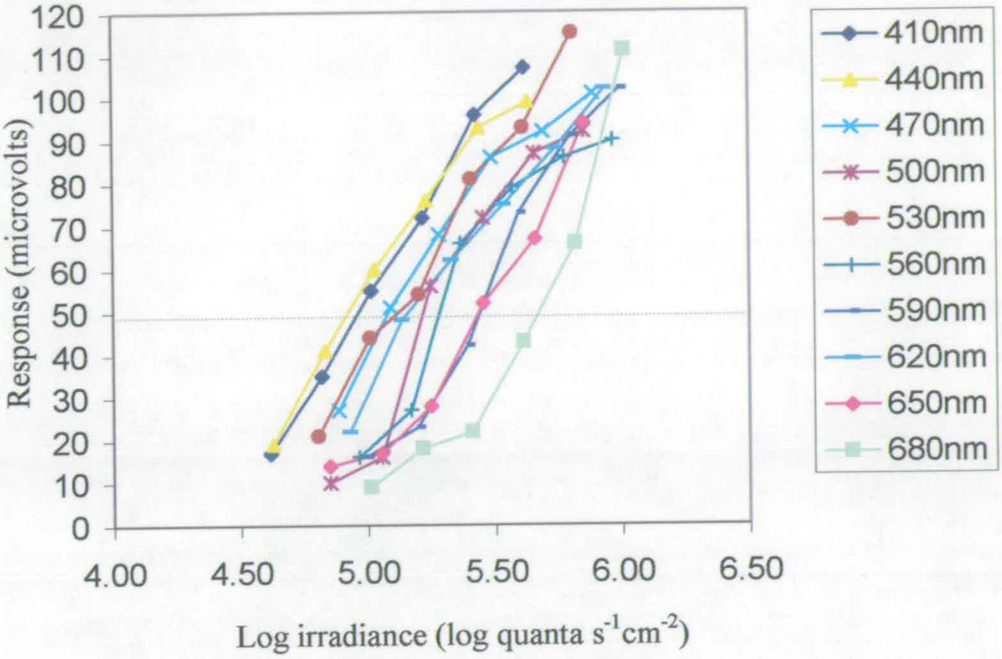


Figure 2.5 Response vs stimulus intensity plots for an individual subject collected across ten wavelengths. Measurements were recorded under a 50 mWm^{-2} light adapted regime. The dotted line denotes the chosen 50μV criterion level.

2.8.1 Spectral Sensitivity Curves

The stimulus irradiances required to produce the criterion amplitude response of 50μV were determined by interpolation from the constructed stimulus response functions for each individual (Appendix 1.4). Spectral sensitivity functions could then be produced by plotting the reciprocal of the log stimulus irradiance (quanta s^{-1} cm^{-2}) that yielded the criterion response at each wavelength, and once this was completed for each individual, an averaged spectral sensitivity curve could be produced. Figure 2.6 shows the averaged spectral sensitivity functions (squares) for the nine three-spined sticklebacks under the broadband background condition where curves have been given a relative log value with respect to the highest value obtained during experiment 2b. The electroretinal spectral sensitivity curve for the three-spined stickleback shows three peaks at 440nm, 530nm and 620nm. Of these peaks, both the short and medium wavelength peaks are found to coincide with preliminary

Microspectrophotometric (MSP) data for the three-spined stickleback (pers. comm. E. Loew). In contrast, however, the peak in the long-wavelength region of the spectrum at 620nm is somewhat displaced further into the red than the 608nm cone revealed by MSP. There are also two troughs in electroretinal sensitivity curve of the three-spined stickleback - at 500nm and again at 590nm. When redrawn for different levels of criterion amplitude, the shape of the average spectral sensitivity curve was not found to differ greatly (Appendix 1.5).

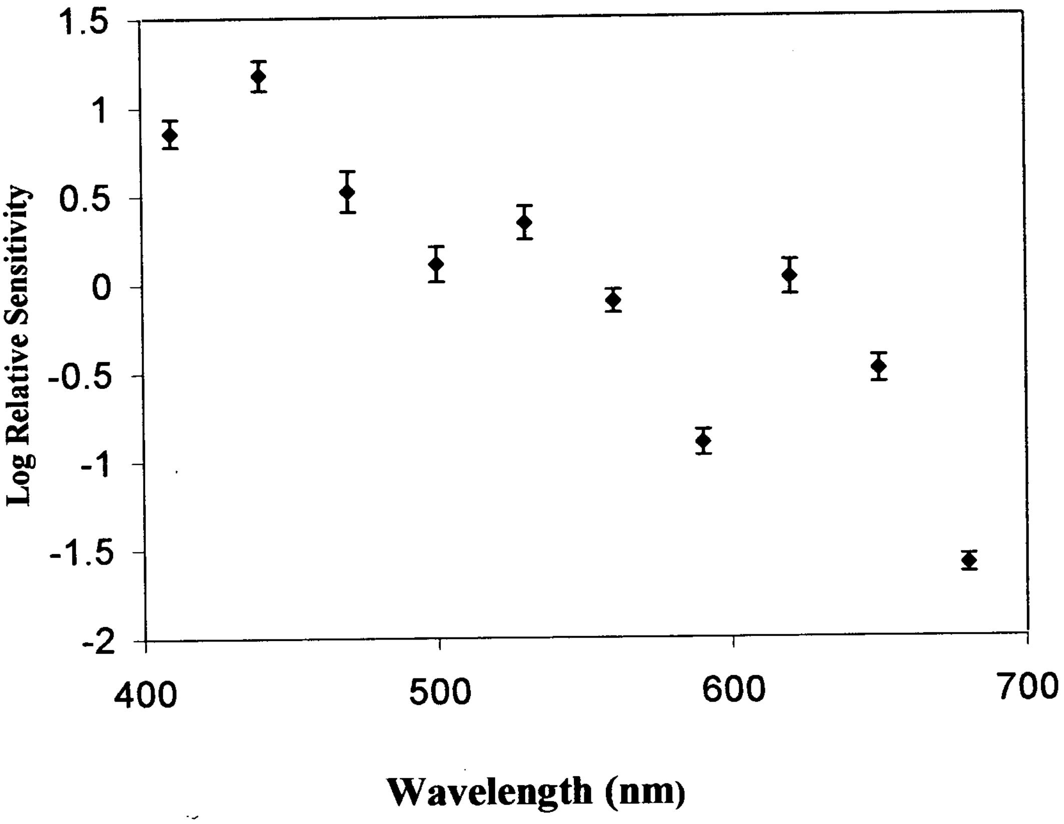


Figure 2.6 Averaged spectral sensitivity (n=9) of adult stickleback obtained under broadband background adapting conditions. Error bars represent ±1 standard error of the mean.

2.9 Discussion

Experiment 2b confirms the expectation from microspectrophotometric data that, across the human visible region of the spectrum, the visual system of the three-spined stickleback receives contribution from three spectrally distinct cone types (Lythgoe, 1979). Furthermore, the position of the absorption maxima of the short and medium-wavelength cones do appear to be largely in agreement with recent microspectrophotometric data collected for an estuarine population of three-spined sticklebacks (pers. comm. E. Loew). The long-wavelength cones found in this study appear, however, to be red shifted. These results also support the expectation that for a shallow dwelling fish such as the three-spined stickleback, spectral sensitivity curves are typically broad in their bandwidth, mirroring that of the photic environment (see Chapter 3).

2.9.1 Ultraviolet photoreception

Taken in combination with the results of experiment 2a, the electroretinal sensitivity curve shown in figure 2.6 is highly suggestive of ultraviolet photoreception in this species. The high level of sensitivity at short wavelengths of light suggests that the absorption function of the short-wavelength photopigment will continue into the near ultraviolet region of the electromagnetic spectrum. If this is so, the animal will be able to detect ultraviolet wavelengths using the photoreceptor maximally absorbent in the blue region of the spectrum. Furthermore, since the sensitivity of the β-band absorption peak of both the medium-wavelength and long-wavelength photoreceptors have been found in this species to be sensitive to ultraviolet wavelengths (McDonald and Hawryshyn, 1995), ultraviolet photoreception could also be conferred in the three-spined stickleback by aberrant sensitivity of these two photoreceptor classes. Indeed, such a mechanism for the photoreception of ultraviolet wavelengths has been widely documented in other species (Jacobs, 1992; Palacios *et al*, 1998).

That ultraviolet photoreception is implied in a freshwater pond population, rather than an oligotrophic estuarine population, is significant and suggests that ultraviolet sensitivity is more prevalent in this species than previously suspected. That this is so is supported by the fact that the waters of Balmaha Pond are very turbid and appear to be red shifted in the summer months (see chapter 3).

2.9.2 Red-shifted long-wavelength peak

The red shift of the long-wavelength peak in the spectral sensitivity of the Balamha Pond population could be due to one of two reasons. Since the microspectro-photometric data collected previously for this species was from an estuarine population of three-spined stickleback, the apparent red shift in this population may reflect spectral tuning of the photopigments in this species to differing photic environments (Lythgoe, 1979). Indeed, such population differences in the visual system of the fish originating from disparate photic environments have been found in three populations of North American three-spined stickleback (McDonald and Hawryshyn, 1995). In those fish found to inhabit red-shifted waters, the long-wavelength peak was found to be shifted further into the red region of the spectrum. One mechanism which might cause the difference in the peak sensitivity of the long-wavelength cone could be the vitamin A_1/A_2 ratio of the photoreceptor (Lythgoe, 1979). Another possibility is that the difference in cone absorption may be due to differences in the structure of the opsin moiety of the visual pigment (Bridges, 1956).

In examining the red shift, it should, however, be noted that the electroretinal spectral sensitivity curve is not the sole product of the input of the individual photoreceptors, but is the product of several spectrally distinct subsystems (Burkhardt, 1966). Therefore, a second mechanism underlying the shift in the long-wavelength peak could be due to strong antagonistic interactions between the medium-wavelength and long-wavelength cones. In such instances the simple "envelope model" predicted by the independent action of the photoreceptors would not apply (Yager, 1967). Previous studies have found the green minus red opponent mechanism to dominate the electrophysiological recording in this species (McDonald and Hawryshyn, 1995), a finding which has been borne out by behavioural evidence (Baube *et al.*, 1995). If such a mechanism is causing the shift to the electroretinal sensitivity curve produced in experiment 2b, it would provide further support to the notion that the three-spined stickleback may possess the necessary mechanisms for colour visual processing. However, in order to determine which mechanism underlies the red-shift in the electroretinal sensitivity curve, it would be necessary to carry out microspectrophotometric analysis of the photoreceptors in the Balamha Population.

2.9.3 Cone contributions to the electroretinal spectral sensitivity curve

When considering the relative contributions of the three cone types to the ERG in experiment 2a, it is important to consider the influence of the 4500°K tungsten-halogen background light used in this study. Although this source emits radiant energy across the visible and ultraviolet region of the spectrum, it does not do so in a uniform manner. Spectrometric data of the light used in this study revealed that medium and long wavelengths dominated the output of this light source. Thus, the relative depression of both the medium and long-wavelength mechanisms in the spectral sensitivity curve may be due in part to selective adaptation by the background light. Other studies have found that it is the medium and long-wavelength mechanisms that dominate the visual system of this species (McDonald and Hawryshyn, 1995; Baube *et al.*, 1995).

Experiment 2c

2.10 Independent ultraviolet photoreception

Taken together, the results in experiment 2a and 2b indicate that the retina of the three-spined stickleback is capable of ultraviolet photoreception. However, it remains possible that the retina of the three-spined stickleback may also have a fourth ultraviolet photoreceptor that is independent of the three cone photoreceptors maximally sensitive in the human-visible region of the spectrum. Such a mechanism was discovered in an estuarine population of the three-spined stickleback during a preliminary microspectrophotometric study by Crescitelli (pers. comm. E. Loew), but never published. If such a system does exist it opens the possibility of tetrachromatic vision in this species (Bowmaker, 1990). With this in mind, it is the aim of experiment 2c to determine if the retina of the population tested in experiment 2b contains a photoreceptor maximally sensitive to ultraviolet wavelengths, or if ultraviolet sensitivity is achieved by the short-wavelength tail of the blue-cone and the β-band absorption peak of both the medium and long-wavelength photoreceptors.

2.11 Methods

Ten fish, again from Balmaha Pond, were tested for sensitivity to ultraviolet wavelengths using the same apparatus used in experiment 2b. The method adopted in this experiment to produce an ERG remained the same with the exception that a 420-700nm long-pass (Appendix 1.2) adapting background light was used to isolate any potential ultraviolet mechanism. In so doing, contribution from photoreceptors operating in the visible region of the spectrum, which underpinned the spectral sensitivity curve obtained in experiment 2b, were suppressed (Chen and Stark, 1994). Isolation by appropriate background wavelengths is an accepted methodology to demonstrate cone multiplicity in vertebrates (Beauchamp *et al.*, 1979; Chen *et al.*, 1984). Once suppressed, if the retina was able to produce an ERG response to ultraviolet radiation, such a response could be attributed to an additional fourth photoreceptor, maximally sensitive in the ultraviolet region of the spectrum.

In addition to the ten stimulus wavelengths tested in experiment 2b, an additional filter treatment, transmitting maximally at 360nm was added to the procedure (Appendix 1.3). This wavelength was chosen as a stimulus as it was revealed in preliminary microspectrophotometric studies to be the position of an independent ultraviolet photoreceptor in this species (pers. comm. E. Loew). Also, as it was the intention of the experiment to suppress cone activity in the visible region of the spectrum, the subject was in this instance allowed to adapt to the background illumination for 20mins (Patterson *et al.*, 2002). After this period the incremental threshold procedure was applied across the eleven stimulus wavelengths.

As the digital photometer used in this study did not maintain its flat response curve over the ultraviolet region of the spectrum, it was impossible to assess accurately the true quantal flux of the 360nm stimulus. In an attempt to remedy this, the stimulus intensities tested across the human-visible wavelengths remained at the same levels as in experiment 2b, and in the case of the ultraviolet stimulus, only the narrow band interference filter was used in an attempt to maximise the quanta from the apparatus.

2.12 Results

Of the ten fish tested only three produced reliable ERG responses (n=3) in the ultraviolet region of the spectrum (Fig. 2.8). In all instances, no ERG response was detected for the tested stimuli positioned in the human-visible spectrum.

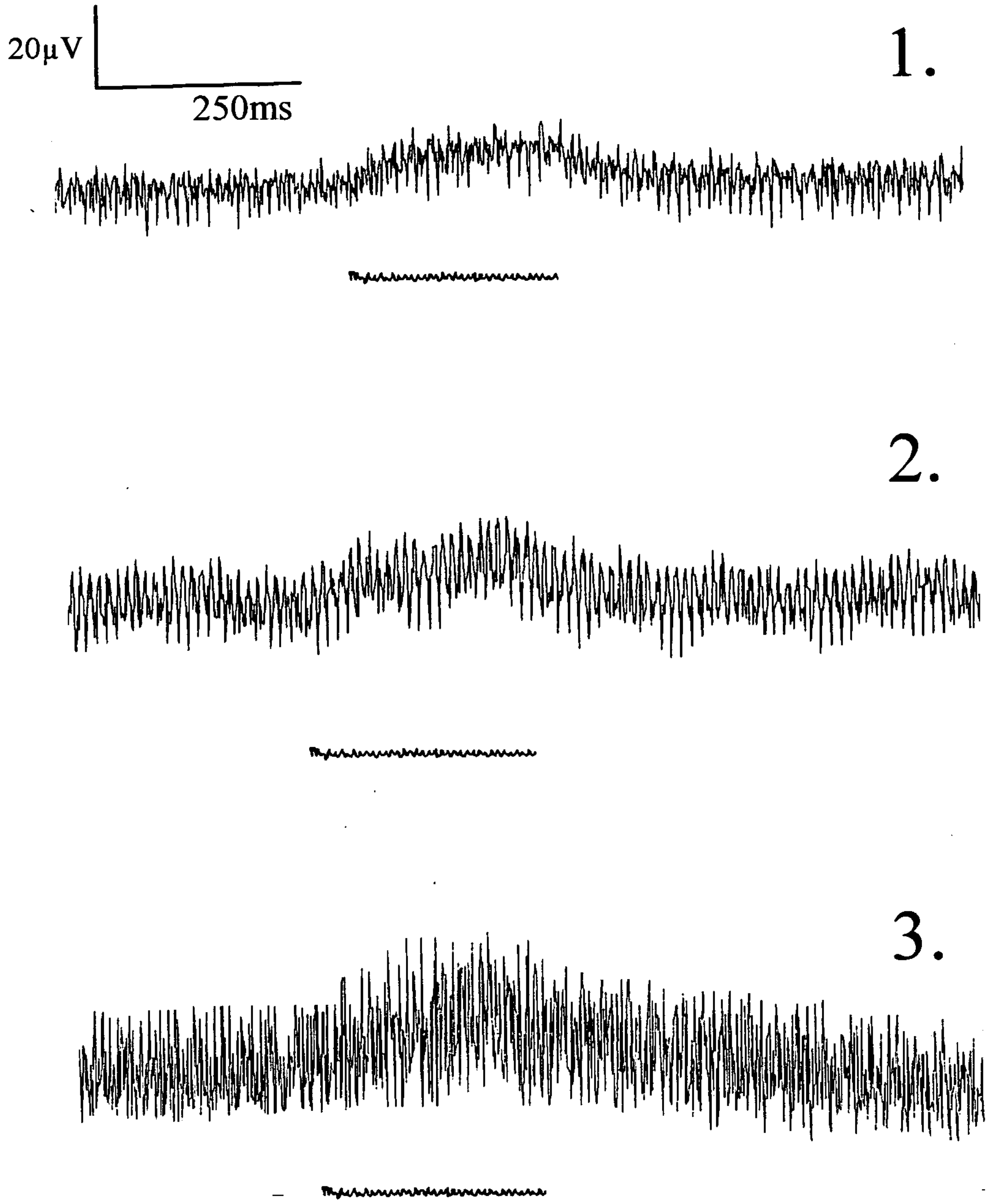


Figure 2.8 Light evoked ERG responses of the three-spined stickleback at 360nm taken from three subject fish. ERG's were obtained under 420-700nm long-pass light adaptation which suppressed ERG activity in the human-visible region of the spectrum.

2.13 Discussion

Of the ten fish tested, only three produced a reliable ERG response in the ultraviolet region of the spectrum. In the three instances recorded, ERG's obtained from a chromatically isolated retina produced an ERG response lower than 20μV; a figure below the 50μV criterion level used in experiment 2b. The difficulty associated with obtaining an ERG signal, however, means that it should not be assumed that, in those individuals where no response was found, ultraviolet photoreception did not take place. Several reasons could account for this low response level. First, it is possible that the ultraviolet cones may not contribute strongly to the ERG, and that this may be compounded by the fact that the ERG is not a very sensitivity technique (Pak, 1976). This explanation may, however, be somewhat overstated as a similar study investigating ultraviolet photoreception in the zebrafish, *Danio rerio,* was able to obtain strong ERG responses from its ultraviolet cone receptor (Hughes *et al.*, 1998). Second, it is also possible that the ultraviolet cone population may be relatively small when compared to other cone classes. For example, in cyprinids the relative number of ultraviolet receptors in the retina appears to be rather low and this would result in a lowering of the summed electrophysiological response (Dowling, 1987; Hawryshyn, 1991). Nevertheless, this may represent an exception rather than the rule as yearling trout and goldfish present ERGs which are consistent with high population numbers of ultraviolet cone classes (Bowmaker, 1990; Chen and Stark, 1994). Third, it is possible that those individuals where no response was found represent members of the population in which an ontogenetic loss of ultraviolet cones from the retina has occurred, and that those individuals where an ERG response was found are at an early stage of development. Whilst not characteristic of all fish with ultraviolet vision, such a loss of ultraviolet cones at maturity has been documented in both the trout and goldfish (Hawryshyn *et al.*, 1989; Chen and Stark, 1994); however, recent evidence suggests that the ontogentic loss of the ultraviolet cone receptor from the retina may not be entirely complete in some species, with ultraviolet sensitivity remaining intact in the dorsal region of the retina (Deutschlander *et al.*, 2001). To avoid this, care was taken at the time of collection to size match all individuals, however, size, in many instances it is not a reliable indicator of age in fish (Paul, 1992), and so it is possible that such an effect may be present. It is thought that the loss of the ultraviolet photoreceptor class may reflect a change in diet or a migration to deeper waters (Bowmaker and Kunz, 1989); however, such a change is not evident in the life history of the three-spined stickleback (Wootton, 1984; Ibrahim, 1988). Coupled with the fact that the stickleback is a short lived fish, living only one year or so (Wootton, 1984), a loss in ultraviolet photoreception with age might not be expected in this species. Finally, since the number of photons stimulating the retina is unknown in experiment 2c, the low response levels found could be due to the stimulus light operating at an intensity level close to the threshold level for this species.

Yet, since the 20μV responses recorded in experiment 2c were found to exhibit little latency, it is also possible that the results obtained in this experiment may be due to optoelectronic effect. Such an effect would have minimal latency and would be expected to mirror the single peak of the ERG's obtained in experiment 2b and 2c. However, that the ERG's obtained in experiment 2c is of a biological origin is suggested by the fact that the ERG's obtained from each subject in the visible range of the spectrum could be suppressed by chromatic adaptation - signal noise caused by an optoelectronic effect would not be expected to be affected such a procedure. Nevertheless, although the evidence obtained from experiment 2c is suggestive of an independent photoreceptor, due to the lack of several of the characteristic components of the ERG wave in the samples obtained, it would be preferable to re-test the ERG response of the three-spined stickleback with apparatus permitting the measurement of quantal flux in the ultraviolet region of the electromagnetic spectrum before it can be concluded that definitive evidence of an independent ultraviolet photoreceptor in the population at Balmaha exists.

If true, these results confirm unpublished microspectrophotometric data showing that there is an additional fourth ultraviolet cone receptor at 362nm in the retina of this species (pers. comm. E. Loew). Taken in conjunction with experiment 2b, these results also support the notion that tetrachromatic vision is possible for the three-spined stickleback. It should be noted, however, that to prove true colour vision in the visual system of the three-spined stickleback would require a behavioural colour mixing experiment such as that carried out in the goldfish (Neumeyer, 1992).

2.15 General Conclusions

In view of the evidence provided in this chapter which suggest that the sticklebacks at Balmaha Pond are capable of ultraviolet photoreception, and that such photoreception is potentially mediated via a fourth independent photoreceptor, it is appropriate to examine ultraviolet sensitivity in this species at a higher level in the visual system. Chapter 4 uses a behavioural technique to examine ultraviolet sensitivity. In so doing, it abstracts from the recording issues associated with the electrophysiological study, and also benefits from the fact that it examines the true visual capabilities of the animal. However, before commencing this study, it would be beneficial to understand the photic environment from which the study population have originated. Chapter 3 outlines an examination of the Balamha Pond environment.

Chapter 3

A survey of the photic environment of the three-pined stickleback

EXPERIMENT 3

3. A survey of the photic environment of the three-pined stickleback

3.1 Light in the aquatic environment

Light passing through the upper atmosphere into the depths of Balmaha Pond is modified in terms of both its spectral composition (quality) and intensity (quantity). The attenuation of solar radiation is not uniform, and it is the ultraviolet and visible short wavelengths of light that are most strongly attenuated in the upper atmosphere by an optical phenomenon known as Rayleigh scatter (Sastri and Das, 1968). Upon reaching the aquatic medium the same optical processes apparent in air now attenuate light more strongly, creating a peak in the maximum transmission of clear water at 470nm (blue)(Tyler, 1959). However, it would be erroneous to confuse all aquatic environments with the clear waters found in oligotrophic systems, as contaminants within the water can alter the optical properties markedly, and this will be reflected in the visual ecology of the animals that inhabit them. Critical to the photic environment of freshwater systems is the high degree of suspended particles and optical contaminants that are often apparent, and the precise nature of these contaminants can mean that the water transmits maximally in the green, yellow-green, or yellow region of the spectrum (Yentsch, 1962). For example, dissolved organic matter and decayed products of photosynthesis (Gelbstoff) so often found in freshwater systems, absorb strongly both short and long wavelengths of light and, as a direct consequence, the water will transmit maximally in the green region of the spectrum (Kalle, 1966). In fact, in some freshwaters systems Gelbstoff is found in such high concentrations that red wavelengths of light transmit furthest (Levine and MacNichol, 1979; Baker and Smith, 1982). Moreover, since the presence of optical contaminants varies, the photic environment cannot be regarded as static, with considerable changes in light transmittance often apparent over the course of a year. Typically, due to the breakdown of organic material in summer, freshwater systems are often observed to undergo a red-ward shift in their transmittance (Muntz and Wainright, 1978).

3.1.1 Ultraviolet light and the aquatic medium

Ultraviolet wavelengths are attenuated so strongly in the upper atmosphere that little natural light is available at wavelengths much shorter than 300nm (Sastri and Das, 1968). A similar story is found in aquatic environments where even ultraviolet wavelengths above 350nm are attenuated more rapidly than those wavelengths in the human visible spectrum (Lythgoe, 1979). As a consequence of this fact, it was not established until the 1950's that sufficient ultraviolet wavelengths for visual behaviour could penetrate to considerable depths in clear waters (Tyler 1959). In fact, in clear atmospheric conditions, it now seems likely that that ultraviolet photoreception should be possible down to 600m in oligotrophic oceanic waters (Frank and Widder, 1996). Also, in waters abundant in particulate matter, recent evidence suggests that the top metre has sufficient ultraviolet light for detection, and, rather than being poorly represented, the number of UV-A photons present near the surface can make up to 40% of the total photon flux for horizontal and downward directed lines of sight (Dowling, 1987).

3.1.2 The photic environment of Balmaha Pond

The visual system of an organism is constrained by the visual environment in which it operates. Hence, we will only be able to make sense of the visual system and visual behaviour of the three-spined sticklebacks in Balamha Pond if we have some understanding of the photic environment in which they live. Indeed, an understanding of the environment from which we are sampling fish is advantageous for two reasons. First, to establish the typical light intensities for each wavelength that are present in the environment, and to compare these to known stimulus threshold levels. In so doing, it is possible to ascertain whether there are sufficient photons to drive visually-mediated behaviour. Second, to note seasonal changes in the light environment that might drive changes in the visual sensitivity.

Since excellent treatments on the photic environments found within freshwater habitats have already been published (Levine and McNichol, 1976; Bowling *et al.*, 1986; Novales-Flamarique *et al.*, 1992), it was not the intention of this survey to document small-scale changes in the light environment and the physical processes therein. Rather, a simple optical survey of the Balmaha Pond was conducted during late winter and early summer of 2002 with the intention of examining the wavelength distribution of light present in the water column during periods which preliminary investigation revealed to be most contrasting. In conducting such a survey, it was hoped to determine to what extent ultraviolet light penetrates the water column and if seasonal shifts in transmittance exist.

Experiment 3

The light survey of Balamha Pond was performed during February and June of 2002. Balmaha Pond (Fig. 3.1) is a small pond on the east side of Loch Lomond, NS425905 (242775, 691050) GB Grid, and is located on forestry land surrounded by a mixture of deciduous and coniferous trees. In the centre of the pond is a deep trench of a depth of 1.6m, and while the northern side is shallow (around 0.5m in depth), the majority of the pond is 1m deep (Fig. 3.2). The northern side of the pond is dominated by a stand of rushes and freshwater grasses, whilst the southern bank is deeper and free of dense vegetation. This vegetation was composed of the water horsetail *Equisetum fluviatile,* the broad leaf pondweed *Potamogeton natans,* Canadian pondweed *Elodea Canadensis,* and yellow flag *Iris pseudacorus* (Girvan, 1999).

Figure 3.1 Balmaha Pond, Loch Lomondside, taken in June 2002.

3.2 Methods

3.2.1 Measurement of ambient light spectra at Balmaha Pond

To characterise the photic conditions prevalent in Balmaha Pond spectral measurements were made across eight days during late winter (February) and early summer (June) of 2002 when sticklebacks were found to be in non-reproductive and reproductive condition respectively. In addition to the fact that these two months coincided with the two different periods in the reproductive cycle of the stickleback, preliminary investigation during 2001 revealed that these two periods best illustrated the annual extremes in photic condition apparent in Balmaha Pond.

Spectral irradiance

Irradiance is the quantal flux per square meter incident over an angle of 180°, and has proved to be a useful measure of photic conditions in aquatic bodies (Tyler, 1965; Bowling *et al.*, 1986; Novales-Flamrique *et al.*, 1992). In all instances down-welling irradiance was recorded. Down-welling irradiance is relevant to the study of the visual ecology as light available for photoreception is likely to be dominated by light coming from both the down-welling and side-welling axes (Endler, 1990). Spectral irradiance was measured using a portable spectroradiometer (Ocean Optics, USB2000) which was fitted with an underwater cosine collector possessing a Lambert cosine response (Hobi Labs, Planar irradiance collector) with a functional range of 350nm to 700nm.

Since it was the intention to assess the lower most limits of ultraviolet light found in the habitat, down-welling irradiance scans were taken from the same site (site A) on the bottom of Balmaha Pond, 1.2m from the surface (Fig. 3.2). This site was in open water, clear of vegetation throughout the year, and was a site where nesting, male sticklebacks could clearly be seen to occupy. Measurements from a second site (site B) surrounded in dense vegetation were also made. All spectral measurements were taken at noon on cloudless days, with the caveat that days which followed strong periods of rain were avoided as this might produce short-lived changes in the colour and transmission characteristics of the water (although such an effect was not borne out by preliminary investigation). The total number of incident photons measured across the sampling range was calculated by measuring the area under the spectral curve. Median photon wavelength, λP_{50}, was calculated for each irradiance curve and can indicate the spectral position of prevailing ambient spectra (McFarland and Munz, 1975a, McDonald and Hawryshyn, 1995).

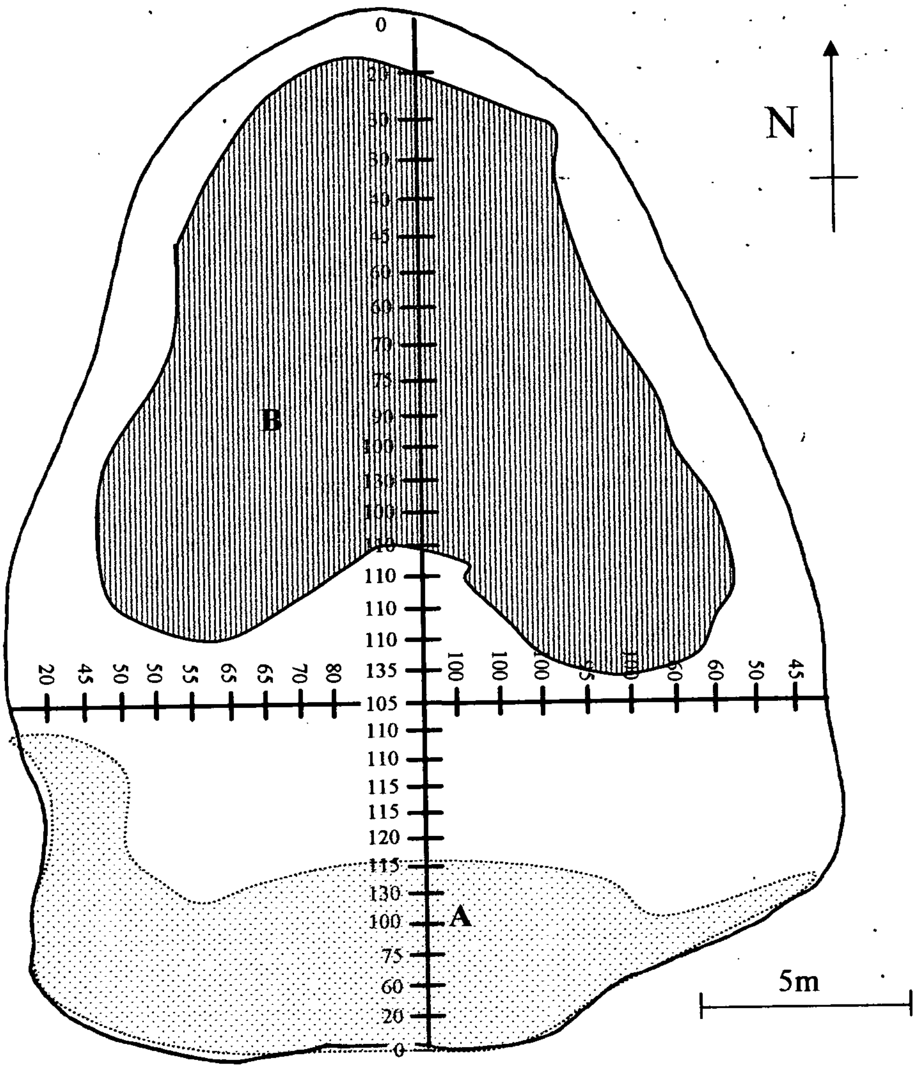


Figure 3.2 A relief map of Balmaha Pond, Loch Lomond, Stirlingshire. Cross-sectional scale indicates depth in centimetres taken every meter across the two orthogonal transects. Shaded areas represent: an area of firm clay substrate (dotted area), silt substrate (white area), and an area dominated by tall rushes. Letters indicate the position of sampling sites for which down-welling irradiance scans were taken.

Light transmittance

To determine if Balamha Pond underwent a shift in transmittance, measurements were made using the tube method (Endler, 1990), which uses a light-tight tube housing a collimated light source at one end and a radiance sensor at the other (Fig. 3.3). The apparatus is sealed underwater where excess water is forced out of the tube by exhaust holes at each end. These exhaust holes are then plugged, rendering the tube entirely light tight. To prevent sediment settling out of the water column all light scans were taken within 15 seconds of the tube being sealed underwater, preventing possible underestimation. This technique is suited to measuring changes in transmittance across the period of a year as it is not affected by associated changes in atmospheric illumination, and was found to be reliable across a spectral range from 350nm to 700nm. Since preliminary tests revealed the water colour in Balmaha Pond to be uniform, all samples were taken at the same site as the down-welling irradiance measurements (site A, Fig. 3.2). After taking appropriate reference scans it was possible to calculate the percentage transmittance of the water column using OOIbase.

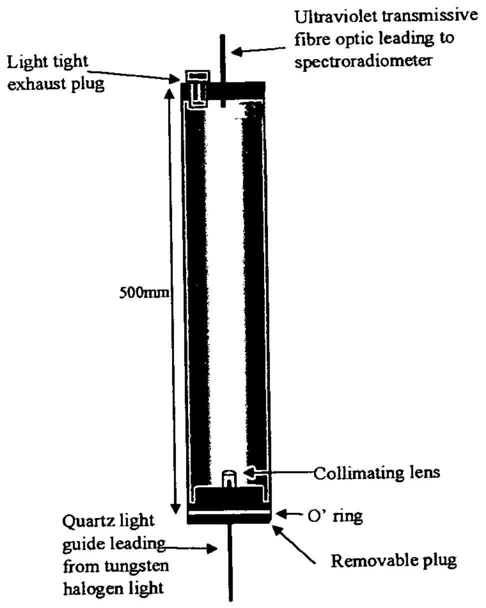


Figure 3.3

Schematic view of the light tube used to assess light transmittance in Balmaha Pond. Light was generated by a tungsten/halogen lamp directed onto the optical axis via a quartz light guide in conjunction with a quartz lens. Opposite was a radiance sensor with a visual angle of 6°. The amount of light transmitted was analysed using a spectrophotometer.

3.3 Results

3.3.1 Irradiance

Irradiance measurements indicate that ultraviolet wavelengths are able penetrate to the bottom of Balmaha Pond during both summer and winter (Fig. 3.4). The amount of ultraviolet light present during summer months was found to be higher in February, where the average amount of wavelengths under 400nm was 2.4%, than in June, where the average UV composition was 0.7 %; (two-tailed, t-test: T=13.922, $p<0.001$). The average wavelength position of the median photon during February was 514nm and 556nm in June 2002, indicating that Balmaha Pond undergoes a seasonal shift in its spectral characteristics, with longer wavelengths of light becoming more prevalent during summer (two-tailed student, t-test: T=-12.832, $p<0.001$).

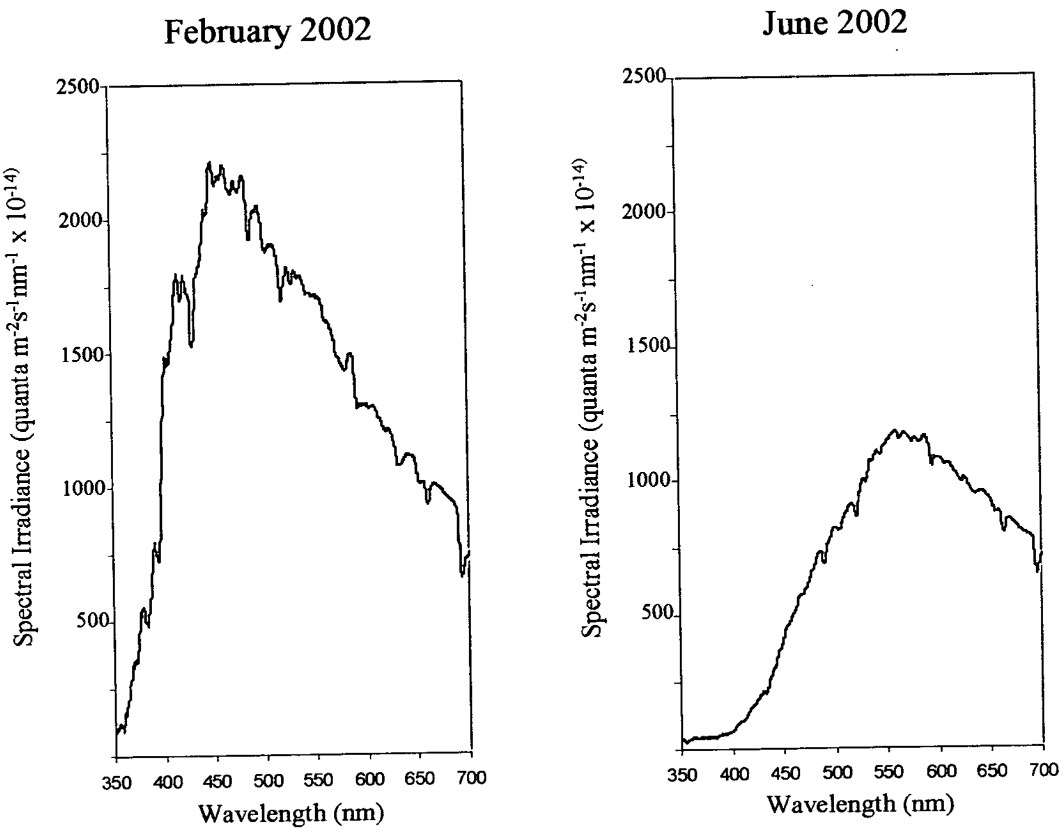


Figure 3.4 Two typical spectral irradiance scans taken during February and June 2002 respectively. Scans were taken at noon under clear skies at a depth of 1.2meter in open water at site A, Balmaha Pond.

Irradiance scans taken at site B, an area surrounded by dense vegetation, reveals considerable attenuation of shorter wavelengths in comparison to the area free of vegetation (site A), Fig. 3.5. This attenuation is reflected in the shift of the average median photon wavelength from 556nm in open waters to 583nm in dense vegetation (two-tailed, t-test: T=-9.023, p<0.001).

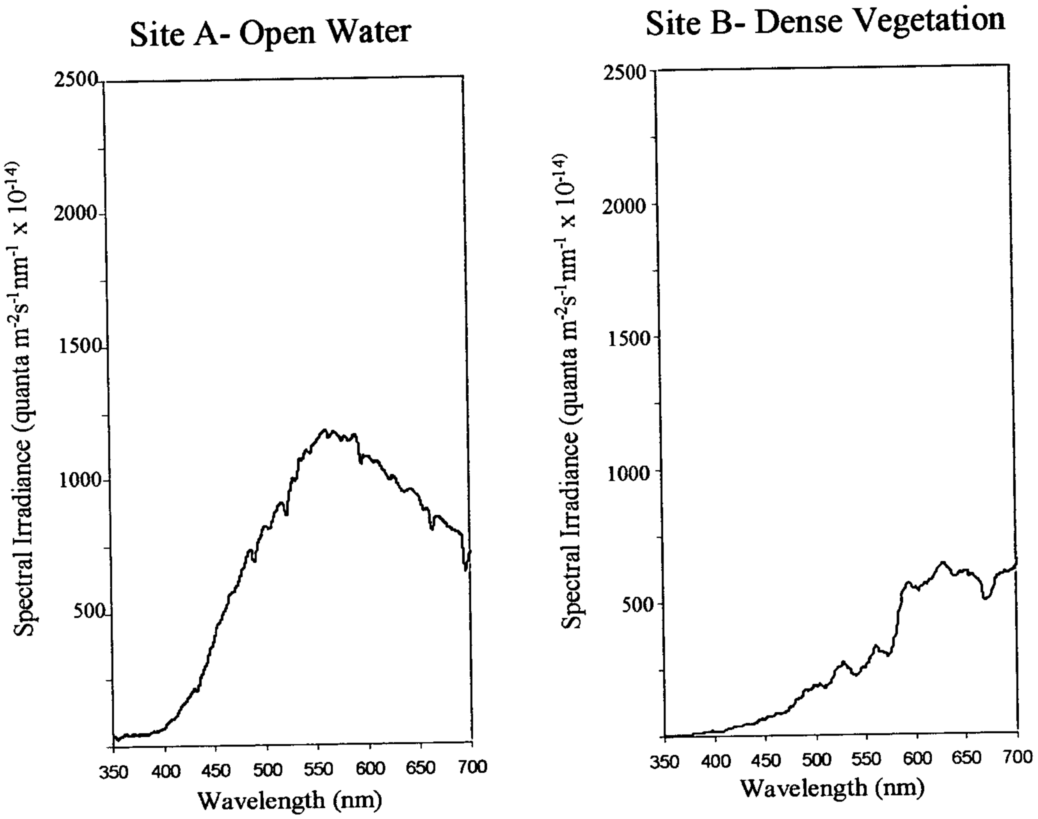


Figure 3.5 Two typical down-welling irradiance scans taken in June 2002 in open water (site A) and amongst dense vegetation (site B). Measurements were taken close noon on the same day. Both sites were approximately 1.2m deep.

3.3.2 Transmittance

Confirming the results of the irradiance scans, the tube method revealed a shift in spectral transmittance towards longer wavelengths in June 2002 (Fig. 3.6). This shift is represented by the shift in the average wavelength position of the median photon from 554nm in February to 613nm in June, (two-tailed, t-test: $T = -58.81$, $p < 0.001$).

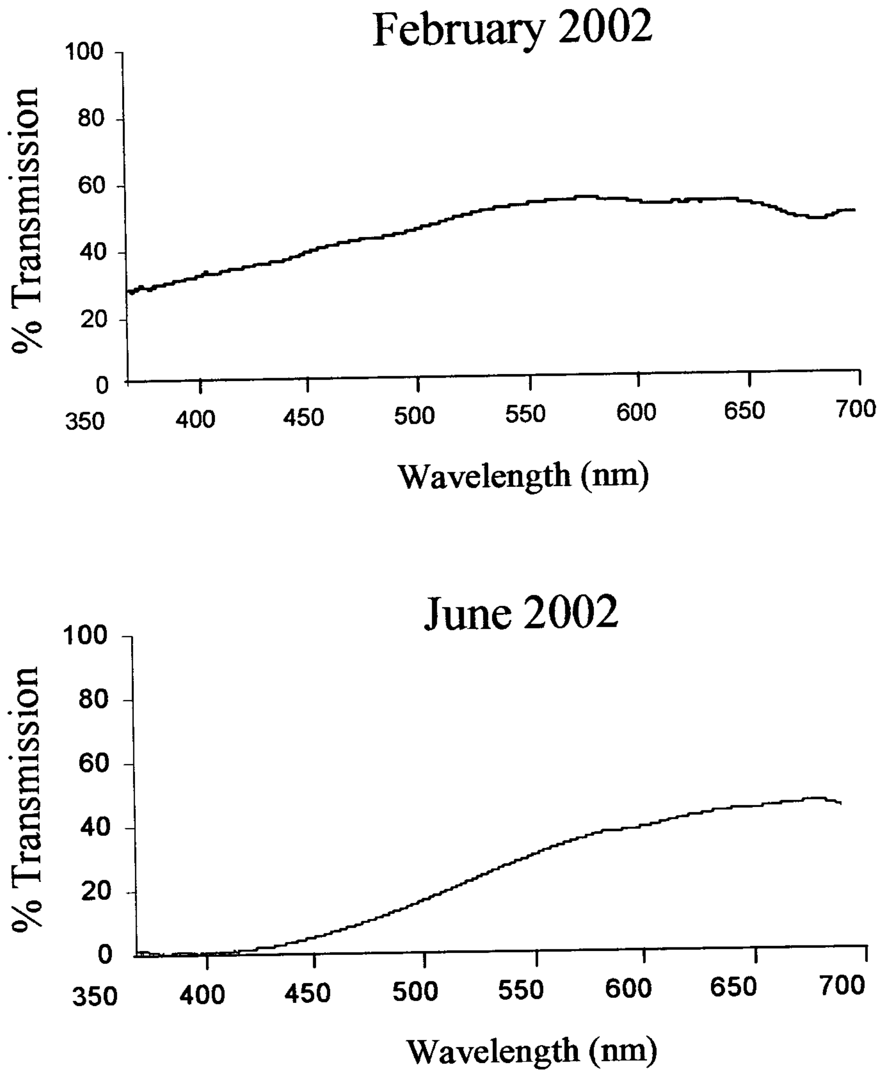


Figure 3.6 Two typical spectral transmission curves collected from Balamha Pond during February and June 2002. Transmittance scans were taken using the tube method (Endler, 2002) at site A.

3.4 Discussion

3.4.1 Ultraviolet wavelengths and the light environment at Balmaha Pond

Considerable extinction of short wavelength light was evident in Balmaha Pond during February and June 2002. This extinction is reflected in the fact that the wavelength positions of the maximum transmission peaks for all irradiance scans were shifted from the 470nm peak which is characteristic of clear water (Tyler and Preisendorfer 1962; Tyler and Smith, 1970). It is likely that the observed extinction was caused by the presence of dissolved organic material, such as tannins, in the water column, as this is responsible for the greater part of attenuation of ultraviolet wavelength in most inland waters (Kirk, 1983). In support, large amounts of leaf litter were in evidence in Balmaha Pond during the year, and these are known to be one of the main sources of tannins in aquatic systems (Bowling *et al.*, 1986).

Despite the high attenuation of ultraviolet light in Balmaha Pond, it is likely that the number of photons present in the water column is well above the visual threshold of the stickleback. Blaxter (1988) estimated the visual threshold of many fishes to be in the range of 10^{14}-10^{15} photons $m^{-2}s^{-1}$, and this figure is in line with the open water irradiance data collected throughout the year from Balmaha. In contrast, the irradiance data collected in areas of dense vegetation suggest that in some areas of Balmaha Pond ultraviolet intensities are close to this threshold level, and it is possible that in these areas there are insufficient ultraviolet wavelengths for the purposes of vision.

Rather than a problem of detection, perhaps a greater problem associated with the use ultraviolet wavelengths in the aquatic medium is due to the degradation of the visual image. The physical properties of water means that ultraviolet light is strongly scattered and, because of this, the optical path over which ultraviolet light is transmitted, in comparison with longer wavelengths, is shortened (Loew and McFarland, 1990). For this reason effective vision in the ultraviolet range of the spectrum is probably limited to distances of less than 5 metres from the target (Losey *et al.*, 1999). Such a figure is consistent with behavioural evidence obtained from the brown trout, *Salmo trutta* (Bowmaker and Kunz, 1987).

3.4.2 Seasonal variation in the light environment

Data collected in experiment 3 using the tube method suggests that Balmaha Pond undergoes a shift towards longer wavelengths during June. The presence of a red shift was confirmed by irradiance scans and is reflected in the shift in wavelength position of the median photon wavelength towards longer wavelengths. Moreover, the scans collected in February and June were found to be consistent with other scans collected during these periods and did not, therefore, represent a temporary instability in the photic regime of the pond during this period. Rather, the observed shift was found to be stable, with the red shift in the water's colour remaining until heavy rainfall during October and November. The tendency for higher extinctions and lowest transparencies to be evident during summer months is in line with previous studies of similar water bodies (Muntz and Wainright, 1978).

3.4.3 The optical environment and the visual ecology of the three-spined stickleback

Chapter 3 provides evidence that functional amounts of ultraviolet light pervade the Balmaha Pond. Coupled with the finding that the visual system of the fish sampled from this environment is capable of ultraviolet photoreception, it is possible that ultraviolet light may be important to the visually mediated behaviour of the three-spined stickleback. In order to establish whether this is true, it is necessary to examine their visual behaviour, and this is done in Chapters 4 & 5.

Chapter 4

Ultraviolet light and visual behaviour in the three-spined stickleback

EXPERIMENT 4

4. Ultraviolet light and visual behaviour in the three-spined stickleback

4.1 Introduction

The results presented in chapters two and three suggest that the three-spined sticklebacks in Balmaha Pond are capable of ultraviolet photoreception. Yet, while the presence of a fourth, independent photoreceptor is indicative of ultraviolet sensitivity, its existence is not sufficient to demonstrate that the stickleback is capable of perceiving ultraviolet radiation. This is because perception, the act of actually seeing an object, is more than the simple process of photoreception, and is a phenomenon of eye and brain rather than a property of the eye alone (Hubbel, 1995; Gregory, 1997). Indeed, whilst electrophysiological studies indicate that the retina can respond to single quanta (Douglas and Hawryshyn, 1990), the actual perception of an object requires significantly more than the photoreception of a single quantum (Pirenne, 1962). It is also theoretically possible that the independent ultraviolet photoreceptor discovered within the stickleback retina subsumes a lower function in the visual system than perception, being involved only, for example, in movement detection or phototaxis, and is not involved in higher vision (Jacobs, 1992). Hence, in order to demonstrate that the three-spined stickleback is capable of perceiving ultraviolet light, it is necessary to test the visual system in its entirety using a psychophysical technique, for only at the behavioural level will the true capabilities of the visual system be revealed (Neumeyer, 1992).

4.1.1 Techniques for the analysis of ultraviolet sensitivity

The visual capability of an organism, its power of detection and discrimination, will always require behavioural substantiation, and in meeting this end there a number of different psychophysical techniques that can be applied. Broadly speaking, the psychophysical techniques that have been successfully employed by investigators when examining an animal's visual system fall into two distinct categories: those that use the animal's innate stereotyped response to visual stimuli, and those that employ a visual task which has to be learned by the subject prior to testing (see Douglas and Hawryshyn, 1990, for a review). It is important to note, however, that the stimulus response function derived from these studies may differ according to the specific visual behaviour under examination, with these behaviours reflecting the part of the visual system underlying each task. Yet, when interpreted correctly, such variation can be advantageous, as it allows the neural circuitry of the visual system to be examined in more detail. What is somewhat more problematic, however, is that these behavioural methods are susceptible to experimental variation. For example, the behaviour of individuals under observation is often influenced by non-visual cues, can be difficult to construe, or requires care in its interpretation. Despite this failing, behavioural studies have been successfully used to study aspects of the visual ecology of fish in areas such as: absolute sensitivity (Powers and Easter, 1978), spectral sensitivity (Blaxter, 1964; Silver, 1974; Muntz *et al.*, 1996), shape recognition (MacKintosh and Sunderland, 1963), motion detection (Meester, 1940; Herter, 1953), flicker fusion, (Protastov, 1968) acuity (Nakamura, 1968), visual range (Vinyard and O'Brien; Aksnes and Utne, 1997) and colour vision (Von Frisch, 1913; Neumeyer, 1992).

4.1.2 A behavioural test of ultraviolet sensitivity in the three-spined stickleback

Using a simple foraging experiment, Merker (1939) demonstrated that, in one population at least, the three-spined stickleback was able to respond behaviourally to ultraviolet wavelengths. Subjects in this experiment were housed in a light tight tank illuminated only by ultraviolet wavelengths, but were nevertheless able to accurately locate and forage upon red larvae. In contrast to previous behavioural studies investigating ultraviolet sensitivity, the experiment carried out by Merker controlled for the effect of fluorescence – the re-emission of ultraviolet wavelengths as human-visible wavelengths – by minimising the fluorescent properties of the experimental apparatus and by examining visual angle. In so doing, Merker was able to determine that a foraging fish was able to perceive the food item rather than detecting a fluorescent bounded shadow cast upon the retina.

Whilst this experiment does establish ultraviolet sensitivity in the stickleback, it is important to note that it falls short of establishing ultraviolet sensitivity in this species under full spectrum conditions. Such a distinction may be crucial as different photoreceptors come into play at different light intensities, and so the ultraviolet photoreceptors may not be utilised under the lower intensity range experienced during the normal life history of the stickleback (Neumeyer and Arnold, 1989). Furthermore, criticism has been levelled at some visual tasks performed in the lab, for although they may be within the animal's capabilities, it is unsafe to conclude that these abilities can be extrapolated to encompass visual tasks that are thought to be more typical of the wild. In this instance, if experimental conditions abstract too much from natural conditions, it is possible that observed visually mediated behaviours may merely be an artefact of the test conditions themselves. Only ultraviolet light was used in Merker's experiment, and this was presented at an intensity level higher than would be expected in the typical aquatic environments in which the three-spined stickleback lives. To rely on this evidence alone is unsound as recent behavioural studies have found disparities between results obtained under full spectrum lighting and those collected under ultraviolet light sources where the human-visible component of the spectrum is poorly represented (Rocco *et al.*, 2002). In order to establish the true visual repertoire of an animal, it is, therefore, necessary to examine the visual behaviour of the organism under conditions that mirror those of the environment (Lythgoe, 1979).

Experiment 4

4.2 Methods

To assess if the three-spined stickleback can respond behaviourally to ultraviolet wavelengths under full spectrum conditions a two-choice operant conditioning technique was employed. At the start of each experimental trial an individual was presented with a two-door maze in which a food item was hidden in one of two compartments (Fig, 4.1). During the trial the fish would be required to choose between the two compartments in order to find the food reward. Once the fish entered the compartment a trapdoor closed behind it removing the opportunity to forage in the other compartment. The location of the reward was randomised at each trial and food could only be successfully located by the relative position of two sets of landmarks mounted at each entrance. Crucially, these landmarks differ only in their ability to reflect ultraviolet light, and hence, the fish were only able to discriminate between the two landmarks if they were able to perceive ultraviolet radiation. The trained behaviour used in this technique has been demonstrated to be a task which is well within the cognitive and perceptual capabilities of the three-spined stickleback (Girvan and Braithwaite, 1998; Girvan, 1999; Odling-Smee and Braithwaite, in press). Hence, if the fish can perceive ultraviolet wavelengths it is expected that the subjects would be able to find the food reward more frequently than indicated by chance alone.

4.2.1 Subjects

During late summer of 2002, 16 adult sticklebacks were caught from Balmaha Pond, Loch Lomond (see chapter 3). The fish were initially held in groups of eight in two 45x30x30cm holding tanks. Lighting was provided primarily by a 5,500°K full spectrum fluorescent bulb (Arcadia Ltd, UK), placed 40cm above each tank, and was supplemented by two standard 75W fluorescent tubes in the room. The colour output of the full spectrum light is close to that of natural sunlight and provides a spectral illumination that consists of approximately 12% of ultraviolet wavelengths (see Appendix 1.1). The holding tanks were maintained at a temperature of 19°C and subject to a light/dark regime of 13L:11D. This light regime was chosen as it has been found to be sufficient to suppress the onset of sexual reproduction in the three-spined stickleback (Hoar, 1962). Before testing commenced, the tips of the spines of the 16 fish were trimmed, each in a unique pattern, so as to facilitate identification. In order to maintain a high degree of motivation for the experimental task, the fish were only fed during experimental trials by way of the food reward, *Tubifex*, and were not fed whilst in the holding tanks.

4.2.2 Experimental apparatus

The experimental apparatus consisted of a long tank (90x40x30cm) lined with gravel which was divided by green plastic partitions into three sections - a start box, a central choice chamber, and a reward section (Fig. 4.1). The reward section was further divided into two isolated, compartments that could only be accessed from a small entrance (3cm x 5cm) cut into the lower section of the partition. In order to reduce the influence that external cues or the presence of the observer may have on the behaviour of the fish, black plastic sheeting surrounded the entire apparatus. Observations were made via a small slit cut into the upper section of this sheeting. A full spectrum fluorescent tube was extended across the length of the tank, approximately 40cm from the waters surface (Appendix 1.1). Water temperature was maintained at a temperature of 19°C, mirroring that of the holding tanks.

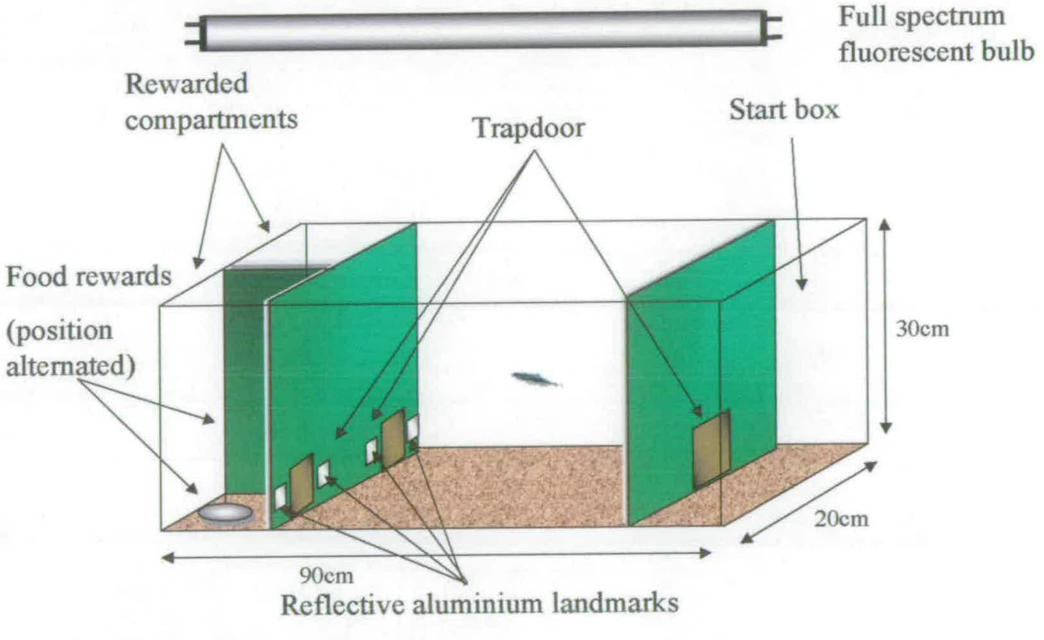


Figure 4.1 The two–choice operant conditioning apparatus employed to examine the perception of ultraviolet light by the three-spined stickleback. Once acclimatised, individual fish were released from the start box and allowed to seek hidden food situated within one of the two rewarded compartments. The location of the food reward was randomised at each trial and food could only be successfully located by the relative position of two sets of landmarks mounted at each entrance. Crucially, landmarks differed visually only in their ultraviolet content, appearing identical to the human observer. Once the subject entered either compartment, a trapdoor was lowered and the subjects exit barred.

4.2.3 Aluminium Landmarks

The experimental apparatus presented test fish with a simple two choice paradigm where fish were rewarded with food if they correctly located the designated compartment. Since the food was hidden, and reward position designated at random, the subject could only determine the position of the rewarded compartment by paying attention to two sets of contrasting aluminium landmarks placed at either side of each entrance. The landmarks were squares of polished aluminium, sandwiched together with a Perspex filter, and differed only in terms of the filter's transmission (Fig. 4.2). Theses two filters were designed to differ only in their transmission of ultraviolet wavelengths: one permitting the transmission of ultraviolet wavelengths in addition to visible light (hereafter referred to as UV+), and the other blocking ultraviolet light (UV-) whilst allowing other wavelengths to pass. Aluminium was chosen for the reflective background as it possesses a flat reflectance spectrum in both the visible and ultraviolet range of the spectrum, and has proved in the past to be useful reflectance standard in field studies (Wyszecki and Stiles, 1982). The intention of this set-up was to establish a situation where only those fish who were able to perceive ultraviolet wavelengths could differentiate between the landmarks and thereby successfully locate the food reward.

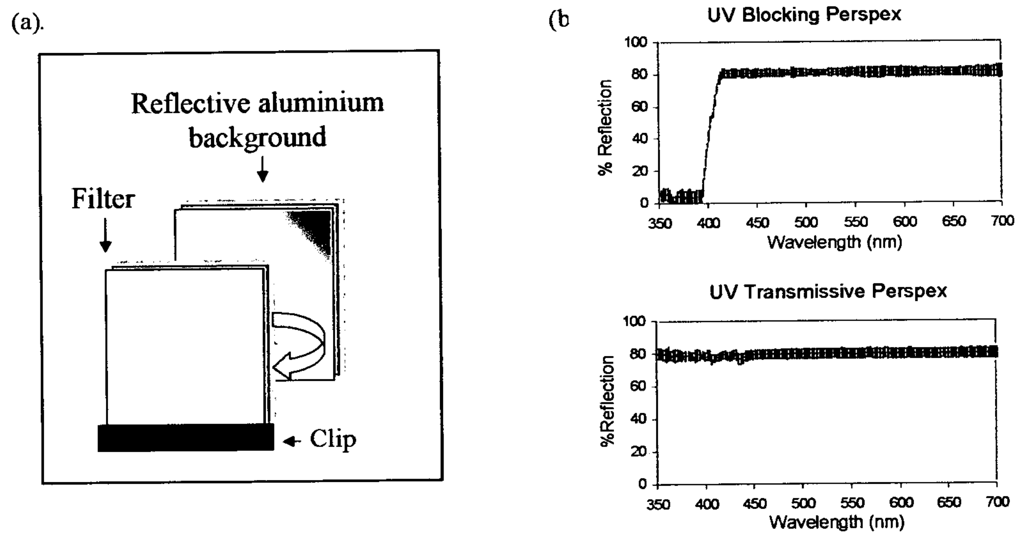


Figure 4 .2 (a) Schematic diagram of reflective aluminium targets used in experiment 4 (n=5). Targets are fitted with interchangeable Perspex filters (b) that when measured appeared to differ only in their reflection of ultraviolet wavelengths. Vertical bars denote standard error.

Although both filter and aluminium backgrounds appeared to the naked human eye to be without flaw in their manufacture, to ensure that subjects were not using some property of the landmarks other than the difference in ultraviolet reflection, five filters of each type, and nine different backgrounds, were used during the experiment. In so doing, each trial could be randomly allocated different combinations of filters and aluminium backgrounds. This precaution meant that fish were never consistently presented with the same filters and backgrounds, and so could not distinguish between the landmarks using cues other than ultraviolet light; such as scratches and adhesions on the filter.

The position of the food reward, and hence the aluminium landmarks, was pseudorandomly assigned at the onset of each trial, with a balanced design of five rewarded trials taking place on each side. This precaution was deemed to be prudent as previous work with this type of apparatus has found that individual fish often exhibit a side bias (Odling-Smee, 2003) and so could potentially visit one side more than chance without the aid of any cue.

4.2.4 Treatment groups and trial allocations

The experiment consisted of four phases: group training, individual training, testing, and a control phase. Due to spatial restrictions, the sixteen subjects involved were split into two groups of eight and housed in separate tanks (Fig. 4.3). These two pseudo-randomly assigned groups of eight were divided into two groups of four, with each individual being pseudo-randomly assigned to one of the two filter types, UV+ or UV-. In so doing, the food reward was consistently linked with either the UV+ or UV- filter in each trial. Such an assignment served as a control for any predisposition that the fish might have in associating the food reward with a particular filter type, and also controlled for any holding tank effects that might exist.

4.2.5 Training

i). Group training

To acclimatise the fish to the test conditions, fish were allowed an initial group training period in which they were housed in the experimental apparatus in the company of their entire holding group. During this period, both compartments were baited copiously with food clearly visible from the choice chamber so that the eight fish in each group might become accustomed to feeding under the test conditions. Six sessions of group training were performed for every fish, each session lasting a period of three days. No filters and backgrounds were present at this stage.

ii). Pre-training

During the second training period, fish were trained in conditions that were identical to the test trials. Subjects were placed individually in the start box and given a 10 minute acclimation period. The trapdoor of the start box was then raised, and the time taken for the fish to locate the food reward recorded. In an attempt to familiarise the subject with the task, the food reward, whose position left or right was assigned at random, was initially placed in front of one of the rewarded compartment entrances. In the trials that followed, as the individual became better at locating the food, the reward was gradually moved out of sight into the rewarded compartment. Reflective landmarks were present during this period. If any individual took longer than 10 minutes to locate the food in any given training session, then the reward was kept in a similar position relative to the designated entrance in the subsequent trial.

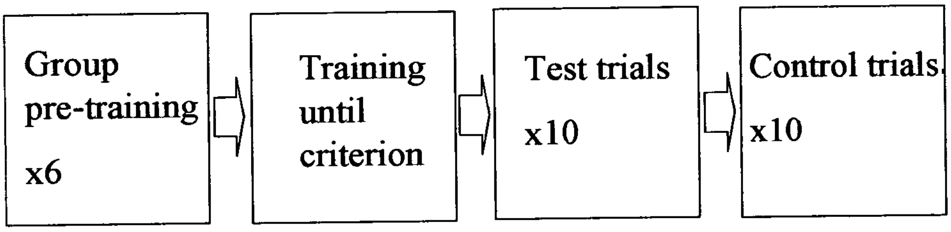
When a fish entered either of the rewarded compartments, a trapdoor was lowered to prevent the fish leaving the compartment and seeking food elsewhere (Fig. 4.1). Hence, any fish that made an incorrect decision would not get fed, and was returned to the holding tank five minutes later. The presence of a trap door was designed to introduce a cost to the choice experiment, limit the benefits of exploratory behaviour, and reinforce the choice decision. To ensure that no fish was deprived excessively of food, if an incorrect choice was made for three successive days, the subject was released from the non-rewarded compartment after five minutes and allowed to locate the food reward in the other compartment. In all instances, fish were not removed from the experimental tank immediately after the successful location of the food reward, but were allowed a five-minute grace period in order to prevent an association developing between the feeding task and the potentially unpleasant experience of being netted. An individual was considered to have completed training if it consistently located food, now hidden in one of the two rewarded compartments, within five minutes on three consecutive occasions. Typically this process took between ten and fourteen trials. Each individual carried out one trial per day between the 09:00h and 18:00h, the order of which having been randomly assigned for each trial session.

4.2.6 Test Trials

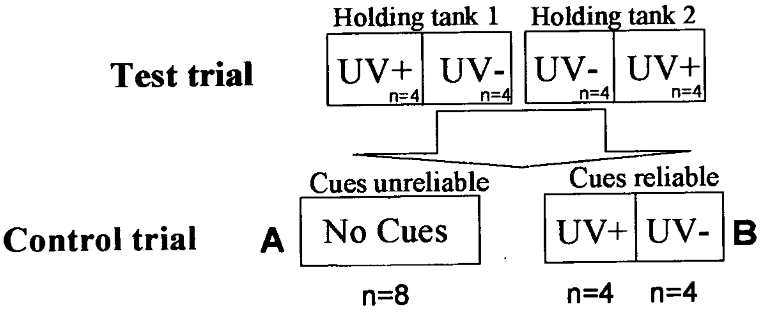
Test trials followed on immediately after each fish reached criterion performance and were identical to the training sessions. A series of 10 trials in total, two per day, were carried out for each of the 16 fish. In each trial, the status of the compartment entered – whether correct or incorrect – was recorded.

4.2.7 Control Trials

The intention of experiment 4 was to examine whether individuals were able to track ultraviolet cues, and in so doing, are able to perform during the test trial phase at a performance level better than the 50% chance level. However, since it was possible that subjects may be using cues other than the aluminium landmarks, two control trials were carried out over a final series of ten trials. To do so, the 16 individuals used in the test phase were pseudo-randomly assigned to two groups of eight, control group A and control group B, with the proviso that each group possessed four representatives from both the UV+ and UV – treatments and contained an equal number of representatives from the two holding tanks (Fig 4.3). Control group A continued a further 10 trials identical in design to that of the test trials with the exception that all aluminium cues used in the trial were now the same, either all UV+ or UV-, and did not, therefore, reliably indicated the presence of the food reward. Thus, if only the ultraviolet reflectance properties of the aluminium cues were utilised by individuals during the test trial phase, it is expected that the performance of control group A will be at level consistent the 50% chance level. Such a precaution controlled against the possibility that individuals were using olfactory cues to locate the food rewards during the test trial phase. Indeed, whilst the olfactory sense is much reduced in the three-spined stickleback (Wooton, 1986), it has been recently suggested that olfactory cues may be used when feeding (Mussen and Peeke, 2001). Control group B repeated 10 more trials which were identical to the test trials. These trials were carried out at the same time as control group A, and included subjects from the same two holding tanks as control group A. Hence, the addition of this control trial allowed us to determine whether the expected drop in performance in control group A was due to the removal of the ultraviolet cues or a coincidental drop in motivation to complete the foraging task.



Sequence of training schedule



Allocation of groups to trials

Figure 4.3 Summary of training and test procedure

4.2.8 Statistical analysis

The performance level of an individual fish in both the test and control trials were expressed as the proportion of trials in which the correct reward chamber was chosen. Then, prior to statistical analysis, all proportional data was arcsine square root transformed. The resultant data set was found to be normally distributed and suitable for statistical analysis. General linear models (GLM's) were initially fitted to the data and included all explanatory variables and their interactions (see Appendix 1.9). Terms were then removed in a stepwise deletion. Where possible, the minimal model for the main factor is reported for each experiment in the form of a two-tail student t-test. One tailed T-tests were used to compare trial performance against the expected level of performance predicted by chance alone as a lowering of performance below this level was not expected.

4.3 Results

i) Test trials

During the test trial period, fish achieved a mean performance score of 66.3% ±3.6 S.E. (Fig. 4.4). A one-sample, one-tailed t-test revealed this to be significantly better than the 50% performance level that would have been predicted by chance (T= 43.56, df = 15, $p < 0.001$). A GLM, in which performance during the test trial was examined, indicated that performance was not affected according to whether fish were rewarded by UV+ or UV- filter type ($F_{1,15}$= 0.29, p= 0.60) or holding tank ($F_{1,15}$=0.13, p= 0.729).

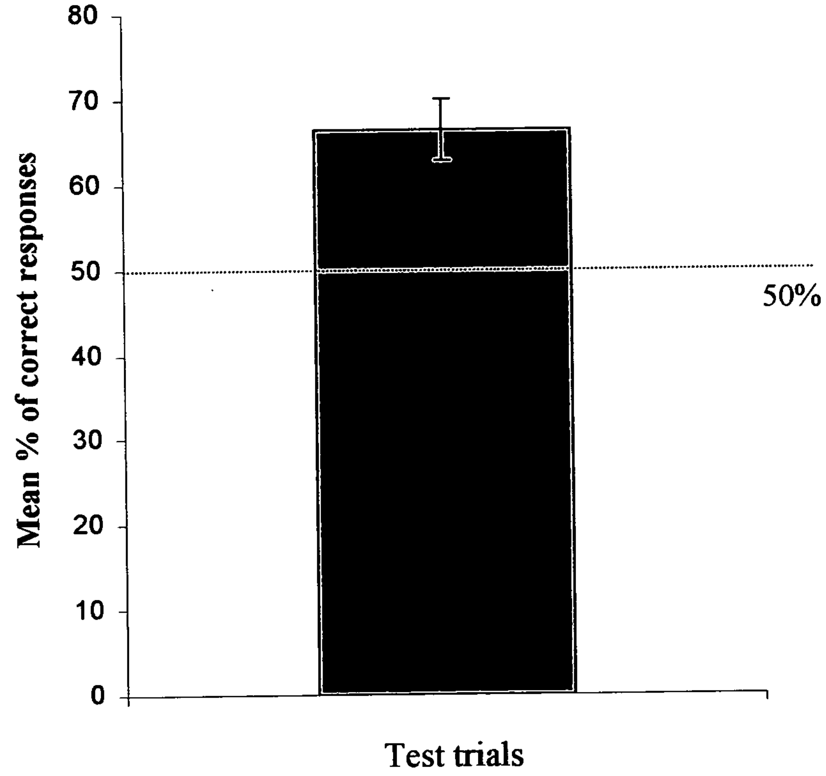


Figure 4.4 Mean performance score of fish during the test trials (n=16). The error bar indicates standard error. The dotted line indicates the level of performance that would have been predicted by chance alone.

ii) Control trials

A two-tailed t-test revealed a significant difference in performance between the two control groups (T= -3.37, p= 0.004). Performance during the test trails was not affected according to whether fish were rewarded by UV+ or UV- filter type (GLM: $F_{1,15}$= 0.54, p= 0.47) or holding tank (GLM: $F_{1,15}$=0.23p= 0.642). The mean performance score for control group A, where ultraviolet cues were absent, was 48.7% ±3.5 S.E. The performance level of control group A was found not to differ significantly from the 50% chance level (one-tailed, t-test: T = -0.36, df = 7, p = 0.72). Control group B, where ultraviolet cues were present, achieved a mean performance score of 63.3% ±1.6 S.E. (Fig. 4.5), a performance level which did differ significantly from the 50% chance level (one-tailed, t-test: T = 7.806, df = 7, p < 0.001).

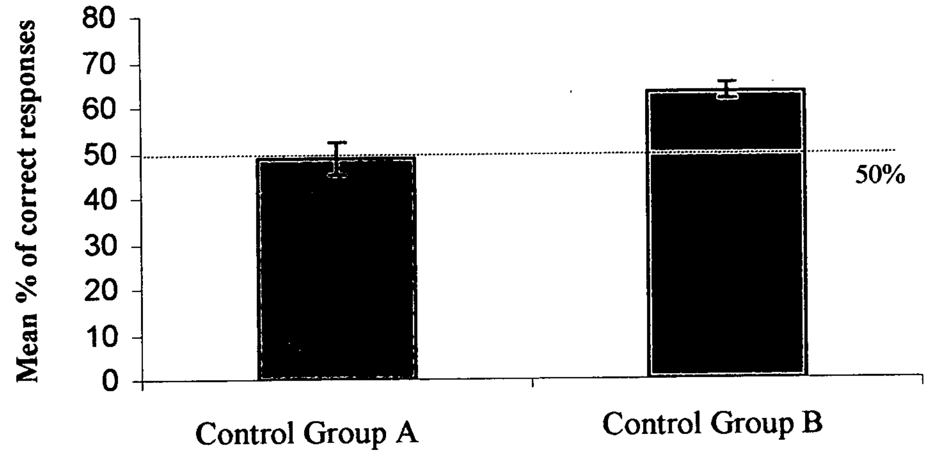


Figure 4.5 Mean performance score of the two control groups (n=8): control group A, where UV cues were unreliable, and control group B, where UV cues remained reliable. Error bars indicate standard error. The dotted line indicates the level of performance that would have been predicted by chance.

A comparison of control group A with their previous performance during the test trial phase revealed a significant difference in performance between trial and control phases (paired, t-test: T = 2.62, df = 14, p = 0.02; Fig. 4.6).

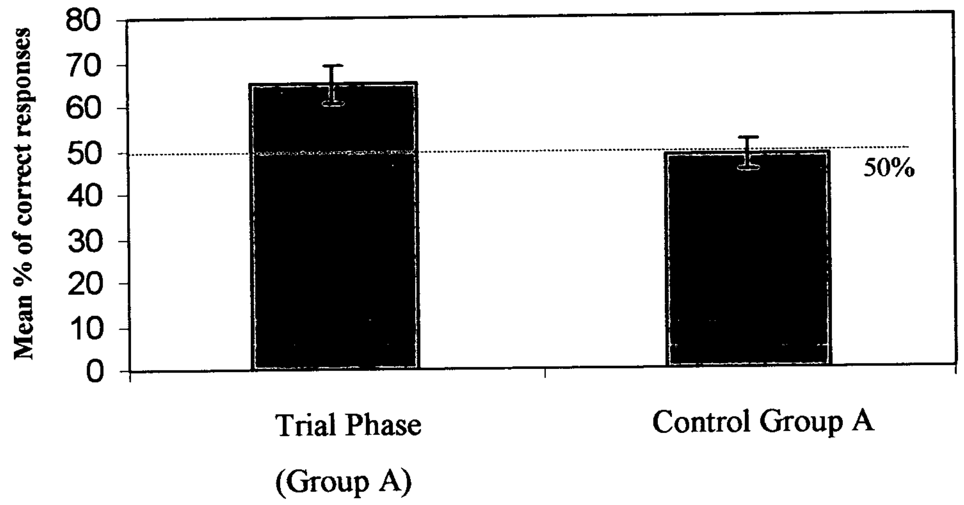


Figure 4.6 Performance during the two test phases of the eight individuals in control group B when tested in the presence of reliable landmarks (trial phase) and when landmarks did not differ in ultraviolet reflectance (Control Group B). Error bars indicate standard error.

In contrast, a comparison of the performance of control group B with their previous performance during the test phase of the experiment revealed no difference in performance (paired, t-test: T = 0.46, df= 14, p= 0.65; Fig. 4.7).

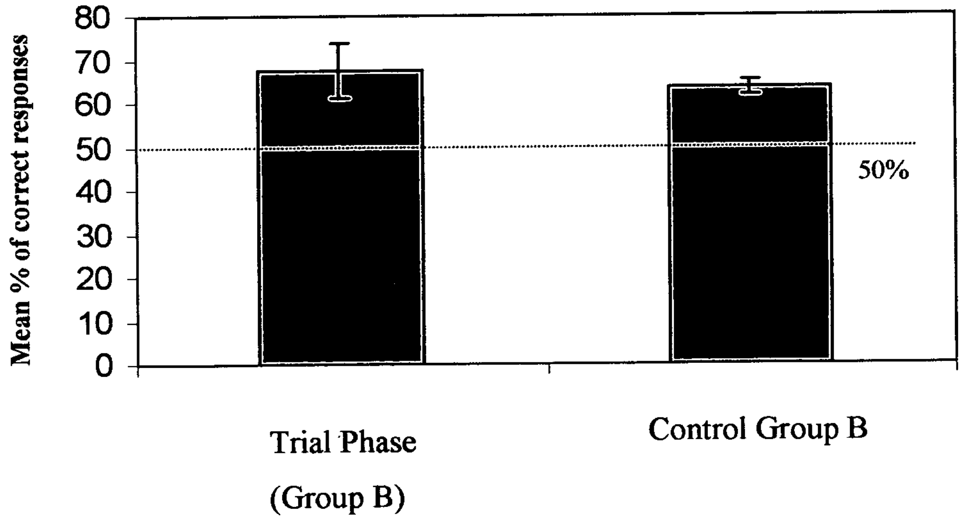


Figure 4.6 Performance during the two test phases of the eight individuals in control group B when tested throughout the experiment in the presence of reliable landmarks. Error bars indicate standard error.

4.4 Discussion

During the test trial phase, fish performed better in the two-choice learning task than predicted by chance alone. This suggests that fish were able to discriminate between the two aluminium landmarks that differed only in terms of ultraviolet reflectance. In contrast, control group A, where ultraviolet cues had been made unreliable during the control phase, fish were found to perform no better than the 50% chance level, and were found to differ significantly from control group B. This finding provides evidence that performance above the 50% chance level during the test trial phase must have been due to visible differences in the aluminium cues, and that other cues, such as olfactory cues, were not used by the fish to locate the food reward in the test trial phase. Furthermore, since control group B, which was provided with reliable cues throughout the experimental series, did not show change in performance from the test trial phase to the control phase, the drop in performance of control group A could not reasonably be attributed to a general drop in motivation to complete the behavioural task as the experiment proceeded.

The findings of experiment 4 are in accordance with Merker (1939), and provide strong support for the hypothesis that the three-spined stickleback can respond behaviourally to ultraviolet wavelengths. That these findings were collected under conditions similar to the ambient environment adds further weight to the argument that such visual capabilities form a natural part of this species behavioural repertoire. The results obtained in this experiment do, however, contradict the behavioural analysis of colour vision in the stickleback carried out by Baube *et al.* (1995). These authors found that the perceptual discrimination displayed by a population of sticklebacks was best modelled by a green-red dichromatic colour model, finding no behavioural evidence to indicate sensitivity to ultraviolet wavelengths. Nevertheless, it should be noted that the behaviours examined in their study were not carried out under full spectrum conditions and were not, therefore, suitable for testing visual behaviour in the ultraviolet range of the spectrum. It is possible that the authors permitted such an omission because of the widely held belief that the three-spined stickleback is unable to perceive ultraviolet wavelengths (Künzler and Bakker, 2001). Yet in view of the results reported here, where a freshwater population of sticklebacks, despite living in fairly turbid waters, are able to respond behaviourally to ultraviolet wavelengths, it is likely that ultraviolet sensitivity may be a widely occurring phenomenon in this species complex.

4.4.1 Performance Levels

During the test trial phase of experiment 4, fish were able to locate the food reward with an average of 66.3%, a level greater than chance. Yet, if we consider that fish were able to discriminate between the landmarks with ease, we might expect the revealed level of performance to be nearer 100%. An explanation for such a low figure could lie in the nature of the discrimination task given to the fish. Such a task makes two assumptions: first, that the fish could learn the task involved, and second, that once learnt, individuals performing the task would be only motivated to visit the rewarded chamber first. That the first assumption is true is borne out by several studies on the spatial ability of the stickleback (Millinski, 1994; Girvan and Braithwaite, 1998; Odling-Smee and Braithwaite, 2003). In addition, similar two-choice colour discrimination tasks have been successfully used to determine the spectral sensitivity of other species fish (Neumeyer, 1985). The validity of the second assumption is, however, questionable on several accounts. While present in the choice chamber, the participating fish were subject to contrasting motivational drives; the motivation to take flight from the potentially stressful experience of remaining in open area of the choice chamber competing with the need to undergo foraging behaviour. Fear, therefore, may have caused the fish to choose at random a compartment irrespective of whether they could distinguish between the two landmarks. Alternatively, having learnt the correct response, the fish may still be exhibiting exploratory behaviour during the trials (Odling-Smee, 2003). Such behaviour may be selectively beneficial where food sources are distributed in an unpredictable pattern, such as that presented by the natural environment of these fish (Milinksi, 1994). In confirmation of this, the performance level found in this experiment was not untypical of other such learning experiments (Neumeyer, 1992; Girvan and Braithwaite, 1998; Girvan, 1999).

4.4.2 Ultraviolet sensitivity and colour vision

Although the results presented in this chapter indicate that the three-spined stickleback is capable of discriminating between cues differing in their ultraviolet content, it be cannot determined if this discrimination is based on the detection of a difference in hue, or if their choice is based on differences in brightness. This is because it is theoretically possible that not all the cone classes contribute to colour vision, or that not all cone types are processed independently from each other during neural processing (Hubel, 1995). Hence, the fish tested in experiment 4 could have employed an achromatic mechanism in the performance of the task, reliant on either the single response of one cone type, or the additive responses from several cones types, without the need for colour vision. In such an instance, the ultraviolet photoreceptor is not used in the hue-discrimination mechanism, and could operate in a similar way as the rod system by merely providing an achromatic input (D'Eath, 1998). To establish colour vision in this species, it is necessary to undertake an additive colour mixture experiment. One such experiment was carried for the goldfish, *Carassius auratus*, by Neumeyer (1992). In so doing, she was able to demonstrate that a minimum of four colours were required to match a white light stimulus which included an ultraviolet component, signifying that the colour vision in this species is tetrachromatic.

4.4.3 The functional significance of ultraviolet vision

The ability to perceive wavelengths extending into the ultraviolet region of the electromagnetic spectrum will serve the animal in the simplest case by expanding the spectral window over which photoreception occurs (Jacobs, 1992). In so doing, many of the visual behaviours displayed by the three-spined stickleback would be advantaged by the added amount of information provided by this spectral range. However, in addition to the benefits connected with extra bandwidth, several properties of ultraviolet light may predispose its use during visual behaviour. The benefits associated with ultraviolet wavelengths are linked to the fact that ultraviolet radiation has the potential to be scattered more than light of longer wavelengths, and when this scattering is caused by small particles, the scattered light will also be plane polarised (see chapter 1). Furthermore, since a high proportion of light at dusk and dawn falls within the ultraviolet region of the electromagnetic spectrum (Lythgoe, 1979; Endler, 1993a), animals that are particularly active during these periods will benefit from an ability to perceive ultraviolet radiation. Given this, it has been postulated that ultraviolet light may be especially beneficial to many of the biologically significant tasks performed by the fish over its life history (Tovée, 1995).

i). Prey detection and foraging

It has been hypothesised that the presence of an independent, ultraviolet photoreceptor could contribute to the prey searching behaviour of fish foraging on small mid-water zooplankton (Loew and Wahl, 1991). Data collected from juvenile perch, *Perca flavescens* (Loew *et al,* 1993), pumpkinseed sunfish, *Lepomis gibbosus*, and the rainbow trout, *Onchorhynchus mykiss* (Browman *et al.*, 1994) suggest that these fish were able to detect small prey at shorter distances when ultraviolet wavelengths were present in down-welling light column. Such prey items pose a test for the visual system due to their size and low contrast against the background illumination (McFall-Ngai, 1990). As ultraviolet wavelengths are readily scattered, it is unlikely that ultraviolet photoreceptors confer improved prey detection in this instance by being a part of the visual system mediating higher spatial resolution. Rather, two alternative mechanisms are most likely to cause the efficiency of the ultraviolet photoreceptor. Due to the wavelength dependent nature of light when passing through medium, some small prey items will differentially absorb or scatter ultraviolet radiation more than human-visible wavelengths. In the case where an object absorbs more ultraviolet than other wavelengths and it is viewed from below against a background rich in ultraviolet, the object will appear as a dark spot. Conversely, where an object differentially scatters ultraviolet radiation, if viewed from above, the object would appear brighter than a background relatively poor in terms of its ultraviolet component (Novales-Flamarique *et al.*, 1992). In support of this, a chromatic action spectrum derived by Loew and co-workers from a simple foraging task suggests that visual sensitivity to prey is greater in the near ultraviolet region of the electromagnetic spectrum than at any other region (Loew *et al.*, 1993). However, a recent re-analysis of foraging behaviour under solar illumination rather than an artificial light source strong in ultraviolet radiation has cast some doubt about the significance of ultraviolet wavelengths in enhancing feeding performance in the natural environment, with reaction distance remaining similar for both natural daylight and ultraviolet omitted daylight regimes (Rocco *et al.*, 2002). Counter to this argument, ultraviolet sensitivity has been found in several adult and larval forms of planktivorous fish (McFarland and Loew, 1994, Britt *et al.*, 2001), suggesting that it may in fact play a role in prey detection. The loss of polarisation sensitivity in the brown trout, *Salmo-trutta*, as the fish becomes larger and changes its diet also adds weight to this hypothesis (Bowmaker and Kunz, 1987).

ii). Orientation

In their unpolarised state, the electric fields (*e*-vectors) of photons are permitted to oscillate in all directions. However, when incident light is scattered by particles smaller than their wavelength, a phenomenon known as Rayleigh scatter, a plane of polarisation will be established which is perpendicular to the angle of incidence (Novales-Flamarique and Hawryshyn, 1997). Accordingly, a large number of animals have been found to respond to *e*-vector direction (Waterman, 1975). In the case of fish it is thought that the ultraviolet photoreceptor working in conjunction with the long wavelength cone may form the sensory basis of a mechanism which facilitates the discrimination of plane-polarised light (Coughlin and Hawryshyn, 1995). With the capability of discerning e-vector orientation emerges the possibility that fish could potentially use polarised light as a navigational compass, permitting large scale migrations or allowing the fish to determine its position in the water column (Hawryshyn and Bolger, 1990; Parkyn and Hawryshyn, 1993). The use of the *e*-vector in large scale navigation by fish rather than spatially distinct tasks is borne out by evidence collected from the rainbow trout, *Oncorhynchus mykiss*, where the fish were found to be unable to discriminate between *e*-vector orientations that differed by less than 45° (Degner and Hawryshyn, 2001). Whilst strongly associated with the possession of an independent ultraviolet photoreceptor, it should, however, be noted that that not all fish are able to detect the angle of polarisation as sensitivity may also require the possession of an ordered double cone mosaic in the centro-temporal area of the retina (Novales-Flamarique and Hawryshyn, 1997; Novales-Flamarique and Hawryshyn, 1998a).

iii). Sexual selection and species recognition

The ability to perceive ultraviolet radiation may also be involved in intraspecies communication, particularly in sexual display. Where tested, the reflectance spectra of fish have revealed structural and reflectance colours that extend into the ultraviolet region of the spectrum (Endler, 1991; Barry and Hawryshyn, 1999a). Accordingly, such ultraviolet colour patches could be involved in sexual selection, informing the mate choice decisions of the female. The influence of ultraviolet wavelengths in mate choice decisions has been established over the past decade in birds (Bennett *et al.*, 1996), but has more recently been discovered to be of importance in the mate choice decisions of the guppy, *Poecilia reticulata* (Smith *et al.*, 2002b; Kodric-Brown and Johnson, 2002). As colour patches in the human-visible region of the spectrum have been found to be important in the mate choice decisions of the three-spined stickleback (Milinski and Bakker, 1990; Boughman, 2001), it would be of interest to determine what role ultraviolet perception plays in the mate choice decisions of this species.

Chapter 5

The role of ultraviolet wavelengths in mate choice

5. The role of ultraviolet wavelengths in mate choice

5.1.1 Secondary sexual characteristics and mate choice

During the reproductive season a male three-spined stickleback develops pronounced secondary sexual characters, acquiring a red throat and a blue iris, which reach a maximum in intensity during courtship (Rowland, 1994). The development of this mosaic colour pattern, which is driven by androgen production in the interstitial cells of the kidneys, runs counter to the usually cryptic colour pattern of the three-spined stickleback and, as such, might be regarded deleterious to the bearer. Indeed, evidence suggests that the mosaic pattern is costly to a male: both in terms of an increase in the risk of predation (McPhail 1969; Moodie, 1972; Wootton, 1984); and the initial development of the colour patch itself, as the sequestration of the necessary carotenioid pigments deny their use by the immune system (Frischknecht, 1993; Barber *et al.*, 2000). Against such selective pressure, the mosaic pattern of a male will, therefore, only be maintained if it confers some benefit to its owner. Since variation in the mosaic signal pattern is evident within individual three-spined stickleback populations, if this variation in signal expression is detectable, the benefits to be gained in the display of a mosaic pattern might be apparent during antagonistic interaction among males (intra-sexual selection) and the courtship of females (inter-sexual selection), (Hamilton and Zuk, 1982; Keyser and Hill, 2000).

More than sixty years ago Niko Tinbergen (ter Pelkwijk and Tinbergen, 1937; Tinbergen, 1951) first drew attention to the use of sign stimuli in the courtship rituals of the three-spined stickleback, finding females responsive to a male's conspicuous red nuptial coloration. In the years that have followed, it has become clear that colour plays a central role in the sexual selection of this species (Selmer, 1971; Rowland, 1994; Kirkpatrick, 1987). Under competition for potential mates - whilst controlling for factors such as courtship vigour and size - female three-spined sticklebacks have been found to prefer males displaying blue irises rather than silver irises, and in particular, more intensely coloured red throats (McLennan and McPhail, 1990; Milinski and Bakker, 1990; Bakker and Mundwiler, 1994). Since the expression of these signals comes at a cost to the male, presenting in some sense a handicap to the individual, it has been hypothesised that the mosaic pattern serves as a signal, honestly communicating information to other conspecifics about the general quality of the individual without the possibility of deception (Zahavi, 1975; Zahavi, 1977; Grafen, 1990). This may be especially true for the red carotenoid pigment of the mosaic as carotenoids may be a dietary limited resource for many species and, hence, the intensity of this colour patch may reflect an individual's ability to accumulate protein and other limited nutrients (Endler, 1980). In view of this, it has been proposed that the strength of the red signal in a male three-spined stickleback may reveal information relating to: resistance to parasites and physical condition (Milinski and Bakker, 1991; Barber *et al.*, 2000; Barber *et al.*, 2001), the provision of paternal care (Candolin, 2000), dominance status (Bakker and Svenster, 1983; Rowland, 1983; Bakker, 1986), or aggression and performance in fights (Rowland, 1982, 1984; Baube, 1997). However, despite strong evidence for natural selection of male traits in the three-spined stickleback, it should be noted that the precise nature of the mechanism which has generated female preference is still a topic of much debate (Ryan, 1990; Pomiankowski and Møller, 1995; Bakker *et al.*, 1999). Indeed, a competing hypothesis of relevance to the visual ecology of the three-spined stickleback suggests that male traits may have evolved to exploit some pre-existing sensory bias of the viewing female (Ryan and Rand, 1990; Ryan *et al.* 1990) - see Chapter 6.

Whilst the benefit to a male in being chosen is obvious, the benefits to a female in being choosy are, in some instances, less so. In being choosy a female will expend unnecessary energy assessing potential mates, and may forgo viable mating opportunities while attempting to find a more acceptable partner in the future. Indeed, females will only benefit directly from being choosey if their choice of a mate improves paternal care, and in the three-spined stickleback there is some evidence that it does (Candolin, 2000). Maternal females might also benefit indirectly from being selective if male quality is heritable, where females may benefit from the genetic advantages conferred to their offspring. By preferentially mating with colourful males, female are choosing, on average, potentially more viable individuals. To the extent these "good genes" are heritable (Zahavi, 1975), in so doing the female will enhance the lifetime reproductive success of its offspring (Reynolds and Gross, 1992, Barber *et al.* 2001).

Nevertheless, the role of breeding colour in sexual selection in the three-spined stickleback is not entirely clear cut. Evidence collected over the past 15 years suggests that the function of the red signal is not a universal feature of the *Gasterosteus aculeatus* species complex, with black-throated and, in some instances, white-throated populations being found (McLennan and McPhail, 1990; Fitzgerald, 1993). Even in populations where males are red-throated, female preference for one male over another may also depend on the degree of difference in the signals displayed by two competing males (Braithwaite and Barber, 2000), her motivational state (Rowland *et al.*, 1995), or prior experience of the female (Bakker and Milinski, 1991; Milinski and Bakker, 1992), and these may mask any preference the female will have for high intensity red males.

5.1.2 The role of ultraviolet colour in mate choice

To date, experiments investigating the role that colour plays in mate choice decisions of the three-spined stickleback have used techniques more appropriate to the human visual system (Rowland 1984; Milinski and Bakker, 1990; Bakker and Mundwiler, 1994; Firschknecht, 1993; Baube *et al.*, 1995; Barber *et al.*, 2000). In adopting such an approach, it is likely that colour choice has been misclassified in this species (Bennett *et al.*, 1994). For example, it is possible that the red belly of the male stickleback, commonly associated with reproductive behaviour and fitness (Tinbergen, 1951; Milinski and Bakker, 1990), might be more correctly described as an "ultraviolet purple" – essentially a mixture of ultraviolet and red wavelengths, in the same way that red and blue wavelengths make purple in the human visible region of the spectrum. Also, in omitting ultraviolet wavelengths, researchers have arbitrarily omitted a region of the electromagnetic spectrum to which the three-spined stickleback is sensitive, and which may be involved in visual signalling. By considering ultraviolet wavelengths, it is also possible that the function of other elements of the male's colour mosaic may be better understood. Indeed, the blue iridescent iris of a male, which is only apparent when in breeding condition, might also be highly reflective of ultraviolet light as well as blue light. If this were so, the conspicuousness of such a feature would be enhanced when viewed by a visual system capable of perceiving ultraviolet light.

Reflectance of colour patches

The extent to which ultraviolet photons are reflected from the colour patches of an animal cannot be predicted simply by its human-visible appearance (Siebeck and Marshall, 2001). Thus, a re-appraisal of the coloration of the stickleback could have implications for the study of sexually selected traits in this species and, as such, it would be of interest to examine their reflectance characteristics in more detail. Using reflectance spectrometry, experiment 5a assesses the components of a male three-spined stickleback's colour mosaic across the ultraviolet and human-visible wavelengths.

Female choice

In addition to the fact that we may have misclassified colour, it has become apparent over the past ten years that vertebrates may use ultraviolet wavelengths during mate choice. Ultraviolet reflectance has been found to influence the mate choice decisions of birds (Bennett *et al.*, 1996, 1997; Andersson and Amundsen , 1997; Andersson *et al.*, 1998; Hunt *et al.,* 1998; Keyser and Hill, 2000; Maddocks *et al.*, 2002; Smiseth *et al.*, 2002; Smith *et al.*, 2002a), lizards (Fleishman *et al.*, 1993; LeBas and Marshall, 2000), butterflies (Arikawa *et al.*, 1987; Knüttel and Fiedler, 2001), and two species of fish - the guppy, *Poecilia reticulata* (Smith *et al.* 2002b; Kodric-Brown and Johnson; 2002) and the Amarillo, *Girardinichthys multiradiatus* (Garcia and de Perera, 2002). Given that the three-spined stickleback is capable of responding behaviourally to ultraviolet wavelengths (see chapter 4), and that colour plays an important part in the mate choice decision of this species, it would be useful to assess if ultraviolet wavelengths are important. Experiment 5b, parts (i) to (iv), examines the role of ultraviolet wavelengths in the mate choice decisions of three-spined stickleback females.

Experiment 5a

5.2 Methods

The colour patches of the three-spined stickleback were assessed using reflectance spectrometry sampling across a spectral range of 350nm to 700nm. All reflectance readings were taken using the beam method (Endler, 1990) with a USB2000 spectrophotometer (Ocean Optics, California). Coloured areas were illuminated by an XBO75W/2 xenon arc lamp (Orsam, Munich), powered by a regulated voltage supply (Müller, Electronik Optik, TYP XH 100), and focused by way of a quartz light guide (Edmund Scientific). Compressed, medical grade, barium sulphate was used as a reflectance standard throughout the procedure. Barium sulphate has been found to have a flat reflectance spectra across the region of the electromagnetic spectrum tested and, in addition, has a near perfect diffusing surface (Erb and Budde, 1979; Wyszecki & Stiles, 1982). All measurements taken from the surface of the fish were then expressed relative to the reflectance curve produced by the reflectance standard. Since, in structural colours at least, reflectance has been found to alter with angle of illumination (Endler, 1990; Osorio and Ham, 2002), all surfaces were illuminated at a constant angle of 45° to the dorsal axis of the fish. All reflectance measurements were taken from a sample diameter of 1mm collected at an angle of 90° from the measured surface. Fish were dabbed with an absorbent material to remove excess water and then temporarily mounted on a light bench lined with black velvet to minimise stray light. Although the presence of an ultraviolet absorbing mucus layer has been found to be present on some fish, leading to the possibility that this might be damaged during preparation, preliminary experiments did not find evidence to support the existence of such UV absorbing mucus in the stickleback. Four main regions of a sexually reproductive male stickleback could then be sampled: the blue iris (1), red ventral belly (2), silver iridescent flank (3), and melanated dorsal surface (4), (Fig.5.1).

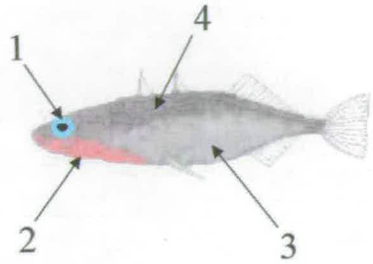


Figure 5.1

Numbers indicate the four anatomical areas measured by reflectance spectrophotometry on sexually reproductive males: (1) iris, (2) belly, (3) flank and (4) dorsal surface.

5.2.1 Subjects

Eight male three-spined sticklebacks in full nuptial coloration were caught from Balmaha Pond, Loch Lomond during June 2002. Since many animals exhibit colour changes that reflect different physiological or motivational states, and this is especially true for fishes where colour patterns form an important function in communication (Baerends, 1976; Rowland 1979), it was important to minimise any changes in colour exhibited by the caught fish. To this end, once caught, males displaying nuptial coloration were stored in a light-tight, black bucket for a maximum of five minutes whilst they were moved a short distance to the reflectance spectrometry equipment which was set up in a light tight area. All measurements were made out of water, and fish were returned to the light tight container prior to re-introduction. A preliminary experiment revealed that this procedure caused minimal disruption to the nuptial coloration of a male stickleback. This method was preferred to the use of anaesthesia - used by other reflectance studies investigating fish colour (Barry and Hawryshyn, 1999a) - as it was found to alter the intensity of the red patch and green melanated flanks in this species.

5.3 Results

Figure 5.2 shows the average spectral reflectance curves measured from the three predominant colour patches of reproductively active male three-spined sticklebacks. Due to its small size, however, no reliable curves were obtained for the blue colour patch evident in the iris of the males tested. Of the three colour patches where measurements were obtained, only the silver flank of the fish was found to reflect ultraviolet wavelengths strongly, possessing a broad spectrum with a peak in the ultraviolet region of the spectrum. The dark melanated dorsal surface of the three-spined stickleback reflected the least amount of light, but was found to possess a broad spectral reflectance with a maximum in the green region (approx. 500nm) of the spectrum. Due to the visible diversity of the red throat colour displayed by the eight tested males, a feature which is typical of this species, the spectral curves for the red throat colour are displayed as two groups: those having an intensely coloured throat patch, and those displaying a less intense red. The red patch of the more intensely coloured males were found to reflect about 20 % of incident light in the red region of the spectrum, possessing a fairly sharp cut-off from 600nm to 550nm. In those males that were found to possess a less intense throat colour, this cut-off was less pronounced and shifted further towards the short-wavelength end of the spectrum (600nm to 500nm).

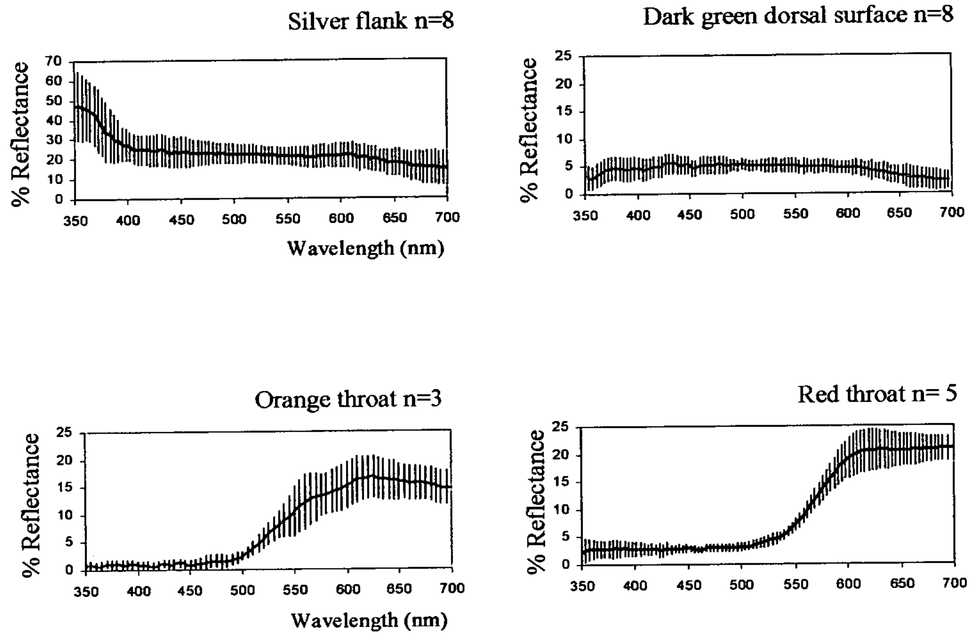


Figure 5.2 Average absolute reflectance curves of the four tested colour patches of eight, wild caught, male three-spined sticklebacks in reproductive condition. Curves represent mean reflectance percentages and are expressed relative to a barium sulphate reflection standard. Since a high degree of variation in the colour of the throat patch was evident, reflectance curves obtained from scans of the throat are split into two groups according to the degree of red displayed. No reliable data could be obtained for the iris. Vertical bars enclosing the curves indicate standard error from the mean.

5.4 Discussion

The results of experiment 5a suggest that the silver flank of a male three-spined stickleback, in contrast to the other components of the colour mosaic, strongly reflect in the ultraviolet region of the spectrum. The melanated dorsal surfaces of the flank also possessed a broad flat reflectance spectrum, from 375nm to 700nm, but did not exhibit a peak in the ultraviolet region of the spectrum. Similarly, the saturated red colouring of the male throat was not found to reflect strongly in the ultraviolet, thus, it can be concluded that the red throat of the three-spined stickleback, in contrast to the "red" bar found on species such as the saddle wrasse, *Thalassoma duperrey* (Barry and Hawryshyn, 1999a), is not a "UV-purple". Rather, the red patch on a male's throat and belly acts essentially as a short wavelength cut-off, maximising contrast against reflectance spectra rich in short-wavelength light. This finding is consistent with other studies which have shown that carotenoid based red pigments rarely have a strong ultraviolet component (Johnstone, 1995). Indeed, such a red patch, when viewed against the melanated flank, aquatic vegetation, and ambient space light will produce a high spectral contrast, and it is for this reason that red is often used by freshwater species as an advertisement (Lythgoe, 1979). The importance of the red-green region of the spectrum in producing spectral contrast in this species is also borne out by the fact that it is the medium and long wavelength cone receptors that dominate opponency mechanism in the three-spined stickleback, and it is this region of the spectrum that the stickleback is most readily able to discriminate between on a chromatic basis (McDonald and Hawryshyn, 1995; Baube *et al.*, 1995).

When a colour pattern such as that displayed by the three-spined stickleback has high and low luminance patches adjacent to each other it will be conspicuous in almost all light conditions (Barry and Hawryshyn, 1999b). This will be true even for those animals which do not possess colour vision as differences in contrast can be detected by brightness perception alone (Endler, 1992, 1993a). In viewing the mosaic pattern of a male three-spined stickleback across both the human visible and the ultraviolet regions of the spectrum, the addition of the ultraviolet region will serve to enhance both luminance contrast and spectral contrast (Endler, 1978). Hence, environmental conditions permitting, the perception of ultraviolet wavelengths would seem to be adaptive in a territorial species such as the stickleback in which a male's colour may serve to advertise its status as a territory holder in reproductive condition (Tinbergen, 1951; Lorenz, 1963). Indeed, there is increasing evidence to suggest that preference for signals in the three-spined stickleback is more likely to be a function of efficiency in generating a visual contrast than a feature for its intrinsic spectral characteristics (McDonald *et al.*, 1995; LeBas and Marshall, 2000), with the importance of red in this species being overstated for the species complex as a whole. Furthermore, as the silvery flank of a fish produces constructive interference when illuminated by ultraviolet light, the ultraviolet region of the spectrum may in some instances produce a higher target contrast than other areas of the spectrum (Tovée, 1995). Since the three-spined stickleback is capable of ultraviolet photoreception, such a mechanism may assist further in the detection of conspecifics.

5.4.1 Colour and the light environment

The spectral composition of light reflected from the mosaic pattern of a male three-spined stickleback is contingent upon the spectral composition of illuminating light and the transmission of the aquatic medium (Wright, 1991). Hence, in examining the colour patch of an organism it is necessary to consider the light conditions of the environment in which it resides (Endler, 1980; Levine *et al.*, 1980). For this reason both luminance and spectral contrast produced by the colour pattern of a fish will vary under changing light conditions. Nevertheless, changes in environmental illumination will affect spectral contrast more than luminance, as changes in illumination tend to change the radiance from all patches proportionally, whilst this cannot be said for their shape (Land, 1977; Barry and Hawryshyn, 1999b). However, due to the principle of colour constancy - the perceived stability of the colour of objects under changing spectral illumination - under broad illumination the appearance of individual colours will largely depend on surface reflectance properties of the colour patch (King-Smith, 1991). Hence, in the shallow water of Balmaha Pond, where the spectral distribution of down-welling illumination is fairly broad, the colours measured in experiment 5a will be perceived largely according to their properties of reflectance. In contrast, light coming from side-welling elevations may differ markedly, and may be restricted to a narrow part of the spectrum (Lythgoe, 1979; Novales-Flamarique *et al.*, 1992). In such conditions, colour constancy will not be achieved (Mollon, 1985). Here, colours perceived by the viewer will alter according to angle and distance from the target, where at short distances colours are least affected by the effects of selective absorption and scatter. Vorobyev and colleagues (2001) modelled this phenomenon for the reef fish *Scarus spinus,* and found that colour constancy only broke down at longer distances. Given this, although illumination spectra in aquatic environments such as Balmaha Pond do change considerably in comparison with terrestrial habitats, at short distances it is possible that this may not be a major consideration. In fact, much of the visually mediated behaviour of the three-spined stickleback takes place over short distances.

Experiment 5b

5.5 Introduction

The results from experiment 5a suggest that the colour mosaic pattern of a male three-spined stickleback has a strong ultraviolet component, and since colour has been found to play an important role in the mate choice decision of this species it is possible that this region of the spectrum may also be involved. Indeed, in the guppy, *Poecilia reticulata*, the attractiveness of males to sexually mature females was found to be enhanced when viewed under full spectrum conditions in comparison with light conditions lacking an ultraviolet component (Krodic-Brown and Johnson, 2002). Subsequent analysis revealed that 4-24% of the male's colour pattern is reflective across the ultraviolet waveband. In view of this, it might be useful to examine female choice in the three-spined stickleback in a situation where the ultraviolet content of potential mates can be manipulated.

Colour in the three-spined stickleback is not, however, limited to inter-sexual communication, but may also be implicated in their shoaling behaviour. Experimental evidence suggests that individual shoal choice in the stickleback is strongly assortative according to body length, colour, and general phenotypic similarity with respect to other group members (Krause *et al.*, 1996, 1998; Peuhkuri *et al.*, 1997; McRobert and Bradner, 1998). In behaving in this manner it is generally supposed that the resultant increase in shoal homogeneity may enhance the predator confusion effect, thereby decreasing the risk of predation to the individual (Ohguchi, 1981; Landeau and Teborgh, 1986; Krakauer, 1995). Since the iridescent silver flanks of the three-spined stickleback have high reflectance, it is possible that ultraviolet light may also play an important role in shoaling behaviour.

Given this, if ultraviolet light is implicated in the mate choice decisions of the three-spined stickleback it will be necessary to demonstrate that a preference for ultraviolet colour patterns relates solely to sexually motivated mate preferences (Bennett *et al.*, 1996), rather than ultraviolet visual signals *per se*. For example, it is possible that conspecifics without an ultraviolet component may appear abnormal to the female, and that preference during mate choice is a general preference for any conspecific, male or female, displaying across the full spectral range to which the stickleback is sensitive. Similarly, when presented with two males displaying under full spectrum and ultraviolet deficient conditions, female choice may represent a preference for the environmental light conditions rather than a sexual preference for the male itself (Pearn *et al.*, 2001). The following series of experiments, experiments 5b(i) through to 5b(iv), aims to provide one such test by manipulating the visual environment.

5.6 Methods

5.6.1 Subjects

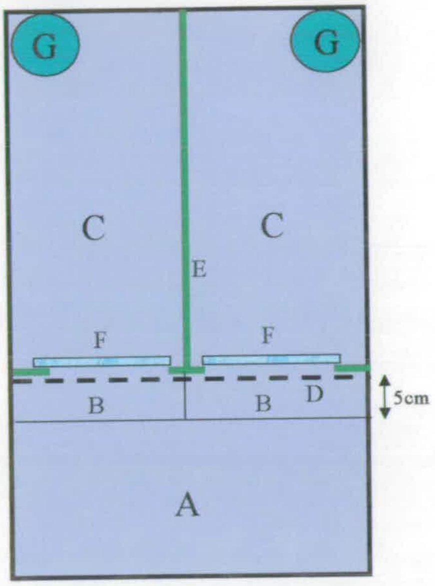
Adult three-spined sticklebacks were collected from the Balmaha Pond, Loch Lomond during March 2002 using several wire minnow traps. All fish were caught in early March, and, since they were not in reproductive condition, could not be sexed visually. Whilst in the lab, fish were held in two main stock tanks (92cm x

39cm x 30cm), each illuminated by a 5,500°K full spectrum fluorescent bulb (Arcadia Ltd, UK; Appendix 1.1) in addition to eight ceiling 80W fluorescent tubes, and were fed twice daily on a diet of live and frozen *Tubifex* worms. In order to encourage the onset of reproductive condition for subjects in experiments 5b(iii) and 5b(iv), the ambient temperature of these two stock tanks was maintained at approximately 20°C. For similar reasons the light regime was set to a 16/8 hour light/dark cycle (Hoar, 1962; Baggerman, 1968). Once the fish had reached a sexually reproductive condition, males were temporarily removed to smaller holding tanks (30cm x 22cm x 20cm) prior to testing. Temperature and light regimes in these tanks replicated those of the stock aquaria.

5.6.2 Experimental Apparatus

The four experiments carried out for this mate choice series involve a simple two choice comparison as used by Smith *et al.* (2002b) in their ultraviolet mate choice experiments with the guppy, *Poecilia reticulata.* Such a set-up has the added benefit that it has been used successfully during the development many of the stickleback mate choice experiments, and fish mate choice work in general (Houde, 1997).

The experimental apparatus consisted of five rectangular tanks (50x40x30cm), each divided by an opaque plastic partition into three sections – a central viewing chamber and two adjacent choice-chambers (Fig. 5.3). The two choice chambers were sealed-off using aquatic sealant from the central viewing-chamber at the front of the tank by a single sheet of UV transmissive Perspex. In so doing, it was possible to prevent the use of olfactory cues during the experimental trials (Steck *et al.*, 1999). In addition, prior to assembly, tanks, filters, and gravel were disinfected in order to remove all odour cues; a precaution that was repeated at the outset of each new trial. Two interchangeable light filters were placed immediately behind the Perspex barrier, and were used to manipulate the appearance of the viewing chambers. To minimise visual disturbance from the laboratory the outer walls of each tank were covered with black plastic. Test tanks were maintained at the same temperature as holding conditions and were illuminated by a single full-spectrum fluorescent tube suspended 40cm above the tank.



A	No preference zone (viewing-chamber)
B	Preference Zone (viewing- chamber)
C	Choice-chamber
D	UV transparent screen
E	Opaque dividing wall
F	Interchangeable light filters (UV+/UV-)
G	Water filters

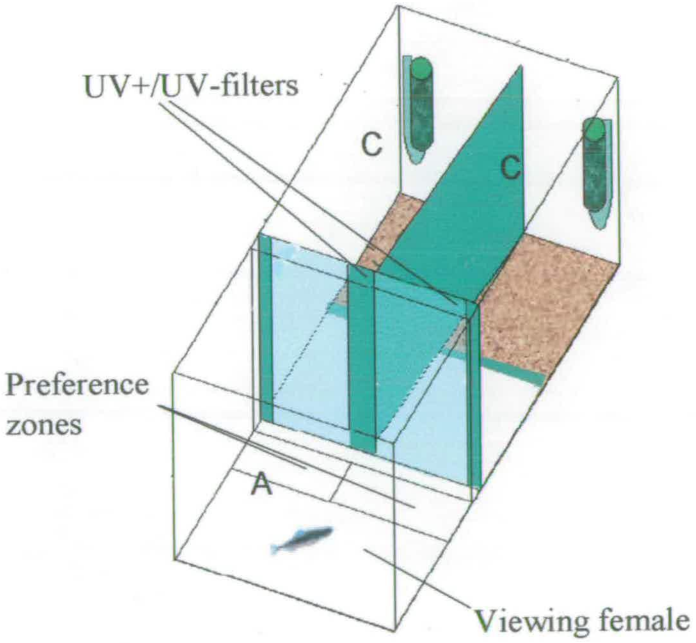


Figure 5.3 Overhead and elevation view of two-way mate choice apparatus used in experiments 5b(i)- 5b(iii).

5.6.3 General experimental protocol

Four experiments were carried out to examine the role that ultraviolet wavelengths might play in the shoaling and mate choice decisions of the three-spined stickleback, with experiments (i) and (iii) in this series acting as controls. In each experiment, the presentation of the two choice-chambers to the test fish differed in terms of two interchangeable filter treatments placed in front of each viewing-chamber (Appendix 1.7). These filters were either ultraviolet transmitting or blocking, and were allocated psuedo-randomly to each chamber (Fig. 5.3). For an animal with the appropriate visual system, identical objects viewed through these filters will differ in terms of their hue and brightness.

Individual preference of the test fish was quantified in terms of the proportion of time the fish spent orientated towards the choice chambers within a 5cm zone of either of the two the filters. Each test fish was only adjudged to have entered a new zone in the experiment once its head and pectoral fins had crossed the zone boundary. For the data to be included in any subsequent analysis, the test fish must have entered both test zones during the first 2 minutes of the trial period. Such a measure of preference has proved to provide a useful estimate of preference in previous mate choice experiments (Rowland, 1995; Smith *et al.*, 2002b).

Once transferred from the holding tank, at the outset of each trial all test individuals were allowed a 10 minute settling period. During this period the two choice-chambers were screened-off using an opaque screen. This screen could be raised remotely, signifying the beginning of a trial, and was only lifted when the test fish was situated centrally in the non-preference zone. Each trial lasted 20 minutes during which period observations were recorded remotely via a video camera (JVC, GX-NFE) suspended one meter above the tank in order to minimise disruption. All trials were carried out between 10:00h and 16:00h.

Experiment (i) - filter preference of non-reproductive individuals

Experiment (i) consisted of a series of ten trials and was designed to determine if subject fish exhibited a preference for either filter type *per se*. For each trial, one test fish was selected at random from either of the two stock tanks and placed centrally in the non-preference zone for a 10 minute period of acclimatisation, after this period, fish preference was recorded. All ten test fish used in the experiment were not yet in reproductive condition.

Experiment (ii) – ultraviolet perception and shoaling behaviour

Experiment (ii) examined whether ultraviolet wavelengths are used in the shoaling decisions of the three-spined stickleback. This experiment was identical to experiment (i) with the exception that two groups of stimulus shoals, each containing three non-reproductive individuals, now occupied the two choice-chambers. Both shoal fish and test fish used in each of the ten trials were naïve to the set-up. Shoal members were size matched with a corresponding partner in the other stimulus shoal in order to minimise size selection bias during the shoaling process (Peuhkuri *et al.*, 1997). All fish in any given trial had been pooled in the same stock tank for a minimum of 14 days and had, therefore, a similar degree of familiarity with each other. Such a period has been found to be sufficient long to promote the establishment of shoaling preferences (Barber and Wright, 2001). This precaution was thought to be necessary as a number of studies have demonstrated a tendency in shoaling fish to shoal with familiar conspecifics rather than those unfamiliar to them (Griffiths and Magurran, 1997; Barber and Ruxton, 2000).

Experiment (iii) – filter preference of gravid females

The experimental procedure in experiment (iii) replicated experiment (i) with the exception that ten gravid females were selected as test individuals. As it was important to ensure that these gravid females were sexually responsive, females were chosen only if they exhibited the characteristic “head-up” display in response to a sexually reproductive male. To avoid the problems associated with prior experience, the male used in this procedure was not used in the later mate choice experiment.

Experiment (iv) – ultraviolet perception and mate choice

Experiment (iv) examined the role that ultraviolet wavelengths might play during mate choice decisions of the female three-spined stickleback. The experimental apparatus used in the prior three experiments was adapted and is illustrated in figure 5.4. Each choice-chamber now contains a gravel substrate, 40g of sand held in a petri-dish, and 50 strands of black cotton thread, providing suitable nesting material for the each male. In order to encourage the males to build nests, gravid females were introduced as stimuli into the viewing compartment for 20mins each day. Again, these gravid females were not used in any test trials to avoid the effect that prior experience may have on their choice (Jenkins and Rowland, 1997). Males were encouraged to build the nests in the rear of the nest chamber by removing all nest-building material, including gravel, from the front of the nesting chamber. Furthermore, during each trial the water filter in each viewing chamber was moved in front of the nest to minimise the use of visual cues from the nest, as these may be used in the female's assessment of the two stimulus males (Braithwaite and Barber, 2000). Once both males had constructed a nest from the materials provided and were observed to court the stimulus female vigorously, they were judged ready for test trials. In total, 10 trials were carried out for 10 gravid females all of whom were naïve to the test apparatus.

5.6.4 Pairing stimulus males

Males used in the trial did not develop the same intensity of nuptial coloration despite being reared in identical conditions. However, evidence suggests that females may select males based on their degree of red nuptial coloration and that selection may also depend on the extent of the difference between the two males (Braithwaite and Barber, 2000). In view of this, and since several other morphological cues have found to influence female choice (Wootton, 1984), each pair of males was matched for size, general morphology and, as far as practicably possible, colour. To obtain an estimate of the degree of intensity of the red belly of each fish, males were scored visually by two observers from 1-5, and the average score for each fish taken (Rowland, 1984; Ward and Fitzgerald, 1987). No trials were undertaken where the males differed in redness by more than one point. Whilst this technique is necessarily subjective, it does reduce the influence of factors other than ultraviolet content which are known to affect female choice. Once paired, males were then randomly assigned to one of the two filter treatments: UV transmissive or UV blocking.

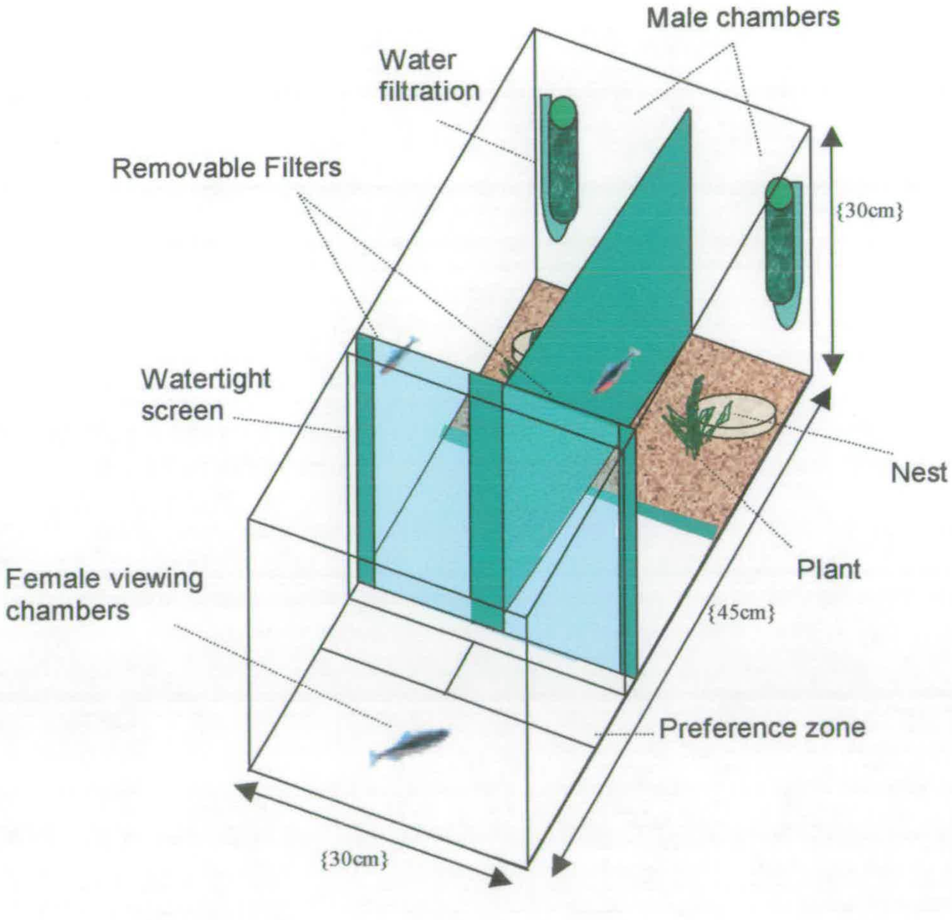


Figure 5.4 Experimental apparatus used in the female mate choice experiment, 5b(iv).

5.6.5 Sexual receptivity

Females used in the mate choice experiment were required to be sexually receptive and ready to spawn. One obvious signal to determine this is the distended abdomen of ripe females. In addition, females were only regarded to be sexually responsive if they assumed a characteristic head-up posture when viewing a separate, test male in reproductive condition. The head-up posture in female sticklebacks has been found to correlate strongly with her probability of spawning (McLennan and McPhail, 1989; Bakker and Rowland; 1995, Rowland *et al.*, 2002). Trials in which viewing females failed to exhibit a clear head-up response were not analysed. In a similar manner, trials were only analysed if both stimulus males were sexually receptive. This was judged to be the case if males performed a characteristic zig-zag dance, which has been found to be a useful measure of sexual tendency in this species (Tinbergen, 1951; Rowland, 1984; Bakker and Milinski, 1991).

5.6.6 Statistical analyses

Female preference, the proportion of time spent within either of the two preference zones, was analysed using a General Linear Model, the maximal models for which are given in the appendix. General linear models were initially fitted to the data and include all explanatory variables and their interactions. Terms were then removed by stepwise deletion. Where possible, the minimal model for the main factor is reported for each of the four experiments in the form of a student t-test. Prior to analysis, all proportional data was arcsine square root transformed. An Anderson-Darling test was performed on the data to assess normality. Male display rates in experiment 5b(iv) were analysed using a Wilcoxon matched signed-pairs ranked test.

5.7 Results

Experiment (i)

Ten non-gravid females were tested in experiment (i). During the twenty minute test period the proportion of time each individual spent time orientated forwards in either of the two preference zones was recorded. No effect of tank was found (GLM: $F_{4,5} = 1.36$, $p = 0.365$). Similarly, there was no difference in amount of time each fish apportioned between each filter was found (two tailed t-test: T= -0.26, df= 17, p = 0.80; Fig. 5.5). Hence, fish were not found to exhibit a preference for either choice chamber despite the different filter types.

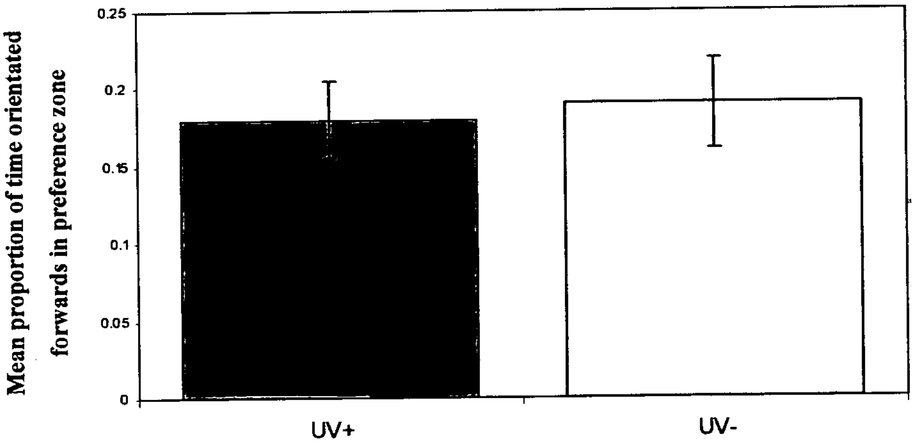


Figure 5.5: Comparison of female preference according to filter type in experiment (i) (n =10). Preference in this instance is denoted by the proportion of time each individual spent in either of the two preference zones (UV+/UV-) facing towards the choice compartments. Error bars denote standard error from the mean.

Experiment (ii)

Data from experiment (ii) revealed no effect of tank (GLM: $F_{4,5} = 0.96$, $p = 0.503$). The females tested did not display a preference to shoal with conspecifics viewed through either the UV+ or UV- filter (two tailed t-test: T= -1.27, df= 15, p = 0.22; see Fig. 5.6). The evidence, therefore, does not support the prediction that ultraviolet wavelengths are utilised in the shoaling decisions of this species. When comparing the time each female spent in the preference zones across experiments (i) and (ii), no significant difference was found (two tailed t-test: T= -1.88, df= 12, p = 0.08; Fig. 5.7).

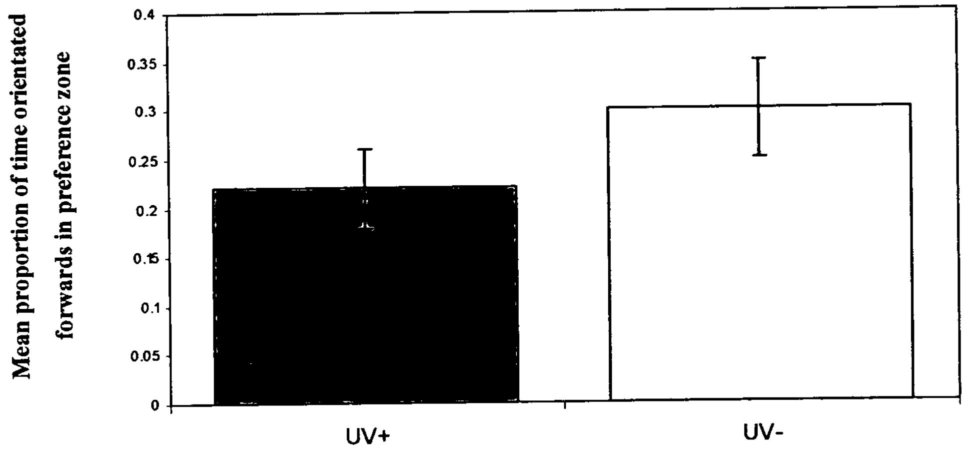


Figure 5.6: Comparison of female preference according to filter type in experiment (ii), where n =10. Preference is denoted by the proportion of time during the 20 minute test period each individual spent in either of the two preference zones (UV+/UV-) orientated towards the two stimulus shoals. Error bars denote standard error from the mean.

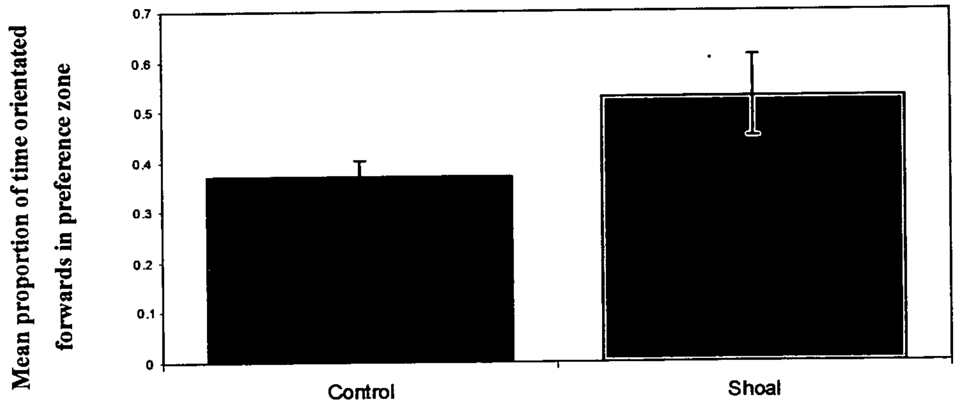


Figure 5.7: Comparison time spent in preference zone by subject females in experiment (i) and experiment (ii), where n =20. Each trial lasted 20 minutes. Preference is denoted by the proportion of time each individual spent in either of the two preference zones orientated towards the two filters. Error bars denote standard error from the mean.

Experiment (iii)

No tank effect was revealed for the ten gravid females tested during experiment (iii) (GLM: $F_{4,5} = 2.09$, p = 0.219). Similarly, no significant difference was found for the time gravid females spent orientated forward towards the UV+ or UV- filter (two tailed t-test: T= -1.52, df= 17, p = 0.15). Hence, gravid females, in common with their non-gravid counterparts in experiment (i), did not express a preference for either filter type (Fig. 5.8).

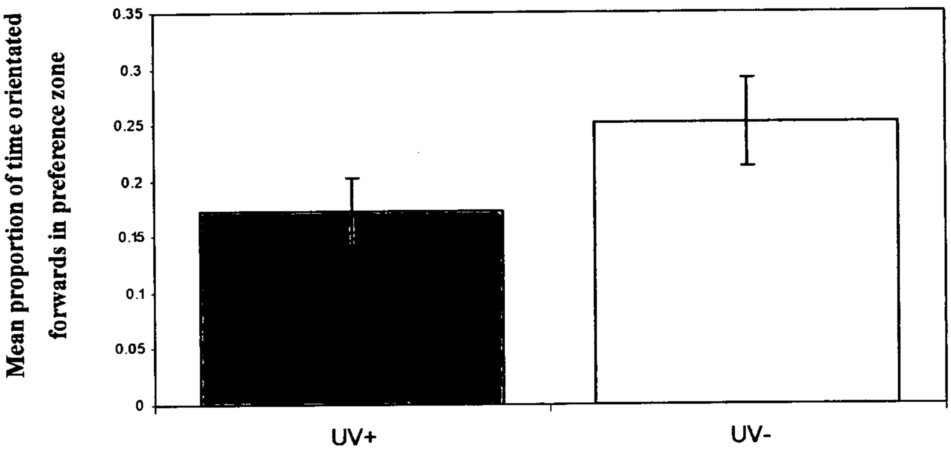


Figure 5.8: Comparison of female preference according to filter type in experiment (iii), where n =10. Females were gravid and were found to respond sexually to a stimulus male prior to experimentation. Preference is denoted by the proportion of time during the 20 minute period each individual spent in either of the two preference zones (UV+/UV-) orientated towards the two stimulus shoals. Error bars denote standard error from the mean.

Experiment (iv)

Throughout all mate choice trials, males were seen to exhibit zig-zag display. The rate of this display was not found to differ significantly according to the filter type during the mate choice experiment (Wilcoxon matched signed-pairs ranked test: Z =

-0.255, P = 0.79). Similarly, all females, whilst in the preference zone, exhibited the classic head up display. No effect of tank was found (GLM: $F_{4,5}$ = 0.41, p = 0.79). Gravid females did, however, exhibit a statistically significant preference for those males viewed through the UV+ filter over those viewed through the UV- filter (two tailed t-test: T= 3.88, df = 12, p = 0.002), Fig 5.9. Gravid females also spent more time orientated forwards in the preference zone when males were present than in experiment (iii) where no conspecifics were present (two tailed t-test: T= -4.46, df = 17, p < 0.001), indicating that the gravid female is attracted towards the choice chambers in the presence of the sexually reproductive males, Fig 5.10.

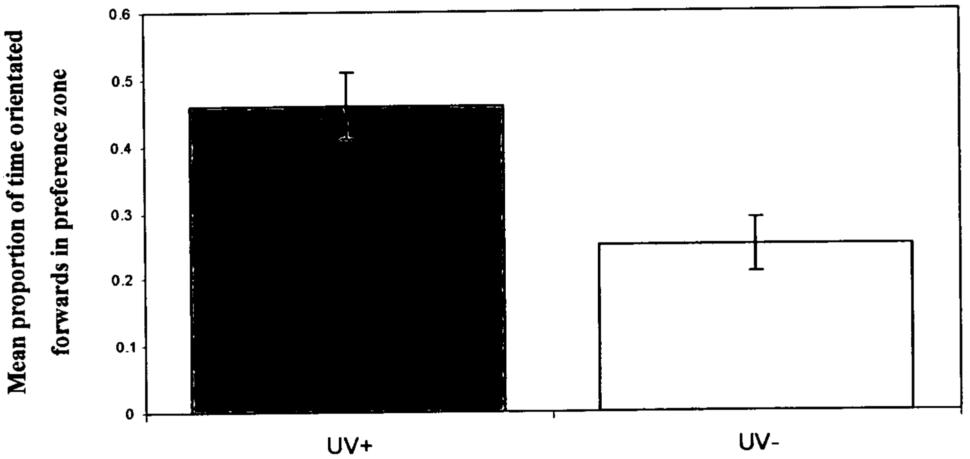


Figure 5.9: Comparison of female preference according to filter type in experiment (iv), where n = 10. Females were gravid and were found to respond sexually to a stimulus male prior to experimentation. Preference is denoted by the proportion of time during the 20 minute period each individual spent in either of the two preference zones (UV+/UV-) orientated towards the two stimulus shoals. Error bars denote standard error from the mean.

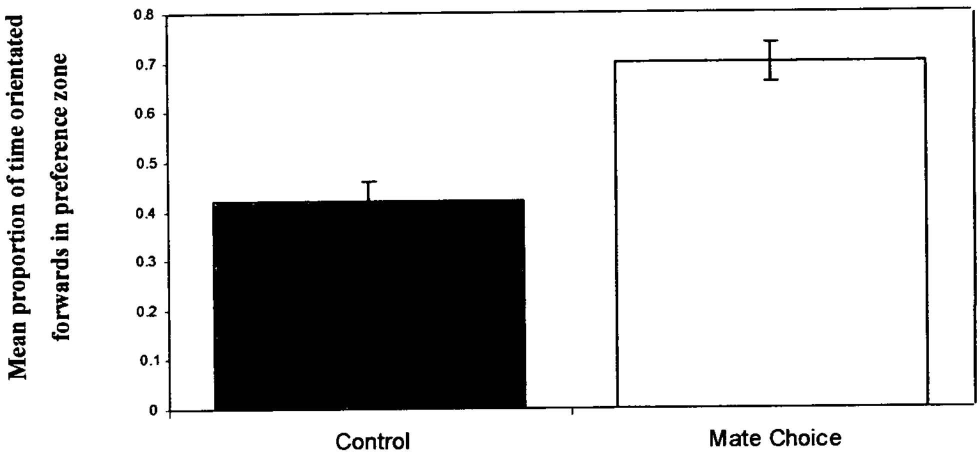


Figure 5.10: Comparison time spent in preference zone by subject females in experiment (iii) and experiment (iv), where n =20. Each trial lasted 20minutes. Preference is denoted by the proportion of time each individual spent in either of the two preference zones orientated towards the two filters. Error bars denote standard error from the mean.

5.8 Summary of findings

- Both control trials, where no conspecifics were present in the viewing chambers, did not reveal a preference for either filter.
- The presence or absence of ultraviolet wavelengths does not influence shoaling choice.
- Female mate choice is influenced by the presence or absence of ultraviolet wavelengths during mate assessment.

5.9 Discussion

5.9.1 Ultraviolet wavelengths and mate choice

The results in experiment 5b(iv) indicate that female three-spined sticklebacks use ultraviolet cues during mate choice, strongly preferring males with an ultraviolet content when other mate choice factors such as red coloration and size were minimised. That this preference is not found in experiments 5b(i) and 5b(iii), where no conspecifics were present in the two choice chambers, suggests that preference is not based on a preference for filter type *per se*. Females performed clear head-up responses to both males throughout the 20 minute period of the mate choice experiment, confirming that females were sexually responsive to both males and did not view either individual as unsuitable. In addition, gravid females were found to spend more time in the two preference zones adjacent to the displaying males in comparison to the time they spent in the same preference zones during experiment 5b(iii) when no males were present.

It should be noted, however, that these results are unable to indicate whether the mate choice decisions of the female are based on chromatic or achromatic brightness cues; whether based on a trichromatic or tetrachromatic system. Although it is likely that the stickleback possesses an independent ultraviolet photoreceptor (see Chapter 2), it is possible that such a photoreceptor may contribute by simply enhancing achromatic brightness, rather than adding a specific perception of a hue by the viewing female. Nevertheless, where tested, in the goldfish (Neumeyer,1992) and guppy (Smith *et al.*, 2002b), the ultraviolet cone in teleosts has been found to contribute to chromatic vision. It is likely, therefore, that the discrimination of stimuli apparent in experiment 5b(iv) is based on specific spectral composition regardless of relative intensity. However, it should be noted that the "blue" photoreceptor, acting as part of a trichromatic system, would be able to discriminate between the stimuli used in experiment 5b as the UV+ stimulus would appear brighter, and it is possible that such a visual mechanism could account for our results in the absence of a fourth independent photoreceptor sensitive in the UV range.

Male behaviour

Female sticklebacks, following the onset of sexual receptivity, develop a dark bar-like melanated pattern down their silver flanks (Wootton, 1976). Previous study has revealed that this characteristic pattern might be used by males when assessing female receptivity (Rowland *et al.*, 1991). Thus, as the ultraviolet waveband will enhance contrast of this signal, and female mate choice in the stickleback has been found to be affected by the vigour of the displaying male's courtship (Rowland, 1995), it might be argued that the female's choice in experiment 5b(iv) could be driven by the two males responding to the female differentially since they viewed her under different spectral conditions. Such a mechanism would operate despite the random assignment of filter type to each pair of male. The results, however, suggest that male display rate does not differ significantly with filter, and that each male courts the female vigorously throughout the period. It is likely, therefore, that the observed differences in female behaviour in experiment 5b(iv) were driven by the visual appearance of the males alone and not by a causal difference in male display activity associated with how the female was viewed through the filter type. This finding differs from similar mate choice studies carried out in the guppy, *Poecilia reticulata*, where the addition of the ultraviolet waveband was found to enhance the attractiveness of potential mates in both males and females (Smith *et al.*, 2002b; Kodric-Brown and Johnson, 2002).

5.9.2 The role of ultraviolet wavelengths during shoaling behaviour

In contrast with the mate choice experiment, the results from experiment 5b(ii) indicate that ultraviolet cues do not influence the shoaling behaviour of the three-spined stickleback. Previous studies have revealed that the three-spined sticklebacks have been found to be strongly assortative (Wootton, 1976), preferentially associating with conspecifics with a familiar appearance more than would be expected by a model of random assortment (Barber and Ruxton, 2000). In displaying such a preference for familiar over unfamiliar individuals, shoaling fish are thought to gain advantages from improved anti-predator defences and more stable dominance hierarchies (Barber and Wright, 2001). That the two ultraviolet treatments used in this experiment did not influence shoaling choice implies that females did not regard the "tinted" individuals viewed through the ultraviolet blocking filter as abnormal, and that the decisions taken by the viewing female in experiment 5b(iv) were based on a sexually motivated mate preference for one mate over another, rather than a natural tendency to preferentially associate with individuals, male or female, displaying across the ultraviolet and human-visible region of the spectrum. Such a finding is particularly persuasive as the anti-predator benefits to be gained from shoaling behaviour rely on a high degree of phenotypic homogeneity between the individuals in the shoal (McRobert and Bradner, 1998).

When comparing the time spent by the test individuals within the preference zones in experiments 5b(i) and 5b(ii), no difference was found. This observation runs contrary to what might be expected, as it might be argued that the viewing individual should spend more time closely associated with the conspecifics viewed across the barrier and, indeed, a trend in the predicted direction did in fact exist ($p = 0.06$). Despite every effort to minimise disruptions, such a result could have been masked by the position of the observer causing the test individuals in experiment 5b(i) to spend more time in the preference zone than might be predicted as this site was furthest away from the observer. Test individuals may also have been attracted to the novel environment revealed to them when the opaque screen was raised. This desire to explore novel environments is a common feature of the three-spined sticklebacks' behaviour, and may mask to some extent the effect of the shoal being present in the subsequent trial.

Improvements to experimental design

Regrettably, the design of the study in experiment 5b is incomplete as the shoaling preferences of gravid females, such as those used in the mate choice experiment, were not tested, and in so doing, the results lack a useful control. This omission from the experimental design was largely dictated by the availability of reproductive individuals and the duration of their breeding activity. Nevertheless, behavioural studies with the three-spined stickleback suggest that changes in shoaling behaviour with reproductive status are generally associated with the newly territorial males rather than females (Wootton, 1976). Indeed, it is the female who remains in small shoaling groups, where individuals often differ in terms of sex and reproductive status (Black and Wootton, 1970; Fitzgerald, 1983).

5.9.3 Possible mechanisms for female choice

Does ultraviolet colour play a special role in mate choice?

The results of experiment 5b(iv) confirm the findings of chapter four, indicating that the three-spined stickleback can respond behaviourally to visual targets differing in their ultraviolet content. It remains unclear, however, whether ultraviolet wavelengths *per se* are particularly important to the mate choice decisions in this species in comparison with the other regions of the electromagnetic spectrum (Banks, 2001). If this is so, ultraviolet wavelengths might contribute a special communication channel during the mate choice decisions (Hunt *et al.*, 2001). Indeed, Endler (1991) in looking at the reflectance spectra of the guppy, *Poecilia reticualta,* suggested that if predators are not capable of ultraviolet photoreception, then ultraviolet wavelengths might form a secret communication channel, enabling the individual to signal to other conspecifics without alerting predators (Burkhardt, 1989; Hunt *et al.*, 2001). Also, since ultraviolet wavelengths are scattered more readily than any other wavelength visible to the stickleback, this might prove advantageous when it is desirable to advertise to females within the territory boundaries, which are typically 250mm – 1000mm in diameter (Wootton, 1972; Kynard, 1978; Bakker 1994), but not to predators further a field. One further mechanism in which ultraviolet light may be regarded to be beneficial over other wavelengths during courtship is due to the reflection properties of the small platelets that make up the flank of each fish. These platelets have flat surfaces and are orientated at different angles in the body surface, hence, during display, the movements of the fish will change their angle relative to the ambient light field and this will produce dramatic changes in the brightness of the fish (Denton and Nichol, 1964; Denton and Rowe, 1994). Taken in combination, these effects may establish the importance of ultraviolet wavelengths during the courtship displays of the male three-spined stickleback.

Care, however, should exercised with regard to such hypothesises as evidence from the zebra finch, *Taeniopygia guttata*, suggests that whilst ultraviolet light plays a role in mate choice, female preferences were affected more by the removal of longer wavelengths in the spectrum than the removal of ultraviolet wavelengths (Hunt *et al.*, 2001). The importance of longer wavelengths in this species has also been found during foraging behaviour (Church *et al.*, 2001; Maddocks *et al.*, 2001). Given the development of the red throat by males during the breeding season, a similar conclusion might also be drawn for the three-spined stickleback. Indeed, using incident light of different colours across the human-visible region of the spectrum, Milinski and Bakker (1991) found that female mate choice preferences in this species relied almost exclusively on the intensity of the red throat. However, as this experiment did not test ultraviolet wavelengths, it would be of interest to repeat this experiment without such an omission. Furthermore, since the existence of a private communication channel will necessarily depend on the visual performance of the predators, it would be prudent to investigate further the capabilities of potential predators to perceive ultraviolet light (Guilford and Harvey, 1998).

Visual contrast and mate choice

The individual sensory channels of the visual system of the three-spined stickleback do not function in isolation, rather they are integrated, and, hence, are more appropriately regarded as components of a more complex system (McDonald and Hawryshyn, 19995; Novales-Flamarique and Browman, 2001). As the mosaic pattern of the male's nuptial coloration - red belly, dark melanation and silver flank - is a high contrast signal (Milinski and Bakker, 1990), in addition to allowing the three-spined stickleback to display in the ultraviolet region of the spectrum, the further effect of permitting the female to view the male under full spectral conditions would be to enhance both hue and luminance contrast – thereby increasing total contrast. If the output of the cone identified in chapter 2 is used achromatically in visual tasks, male sticklebacks viewed under the ultraviolet transmissive treatment would appear brighter, with the increased brightness of the silver flank enhancing contrast against melanated patches. Furthermore, if the fish is viewed against a background less reflective of ultraviolet light, a situation which is likely if the fish is viewed against the pond weed and muddy substrate of Balmaha Pond, visual contrast of the complete fish against its background would also be increased. It is, however, possible that the ultraviolet photoreceptor class contributes to the colour opponent pathways of the three-spined stickleback's visual system. If this is so, then the addition of ultraviolet wavelengths will also serve to increase the colour contrast of the red throat signal displayed by the male. This maximisation of contrast will depend on the peak absorbance of the cones relative to the cut-off wavelengths of the colour patches (Endler, 1992).

It is thought that both colour and brightness contrast are important in the mate choice decisions of the three-spined stickleback (Milinski and Bakker, 1990 Baube *et al.*, 1995). Baube and co-workers found that 100% red dummies used to elicit mate choice behaviour in females brought no greater reaction that 100% beige coloured dummies (Baube *et al.*, 1995). Those dummies which possessed some degree of visual contrast produced the greatest reaction, and this was independent of the area of shading. Such findings suggest that not only are abnormal signals disregarded, and that key stimuli must have context (Tinbergen, 1959), but that the stickleback's visual system is tuned to detect signals, both in terms of form and colour, that produce a high contrast target – both within the mosaic pattern and against the space-light of the aquatic environment. Here the typical vertebrate "centre surround" ganglion receptor field organisation predisposes visual systems to respond to achromatic and chromatic contrast between patches (Hubbel, 1995; Endler 1991). The function of the mosaic signal as an epigamic optical signal is supported by studies investigating mate choice in populations of black throated three-spined sticklebacks which suggest that contrast rather than the red throat colour is important during courtship (McDonald *et al.*, 1995).

5.9.4 Does female choice reliably predict the outcome of mating?

Due to the position of the filters in the test tanks, the result in experiment 5b(iv) was obtained using a situation where the female was afforded an unhindered choice of the two males. While the adoption of such a protocol is valid in this instance as our interest lies primarily in the visual signalling of this species, it is important to remember the outcome of reproductive behaviour in the three-spined stickleback may also be dependent on several other factors such as: olfactory cues; male aggression (Bakker 1994); the presence of eggs in the nest from a previous mating (Rowher, 1978; Ridley and Rechten, 1981); tactile cues (Sevenster 1968; McPhail and Hay, 1983); territory size (van den Assem, 1967); and the expectations of the female with regard to available male quality (Bakker, and Milinski, 1991). Furthermore, in trials where three-spined sticklebacks were permitted to interact freely during mate choice, male-male interactions will override female choice (Nilsson and Nilsson, 2000). Nilsson and Nilsson (2000), in studying the mate choice of the stickleback found that the dominant male of a pair deprived the female of the opportunity to interact with the other male, a bias which was not apparent when females were allowed an uninterrupted choice. These tests took place in aquaria and it is likely, in the less confined areas of the wild, that this effect may not be so pronounced. Nevertheless, in assessing the role that colour might play in the ultimate fitness of an individual, such factors must be taken into consideration.

5.9.5 Signals and display

The effectiveness of any signal is dependent on the ambient light during sexual display, the reflectance spectra of the mosaic nuptial pattern, and the visual background against which the display is viewed (Endler and Théry, 1996). Hence, since visual signals are important in the mate choice decisions of the three-spined stickleback, then the situations which affect these three factors, such as time of day, weather, and the specific characteristics of the microhabitat where the display takes place, might be expected to affect the evolution of colour patterns and display behaviour in this species (Endler, 1993a).

The male three-spined sticklebacks studied in experiment 5b(iv) were seen to display in the areas of Balamha Pond clear of vegetation, as reed beds in this area were too dense to allow the building of nests. In avoiding the area of the pond covered by macrophytes the males are able to display in an area relatively richer in ultraviolet light than those under shaded reeds (Endler, 1991, Chapter 3). In so doing, it is possible that the male is exploiting these patches in an attempt to maximise their attractiveness to potential mates; maximising visual contrast between their own colour patches and with the environmental background. The exploitation of light patches to advertise to conspecifics has been documented for the guppy, *Poecilia reticulata*, and in three species of tropical lekking birds (Endler, 1991; Endler and Théry, 1996). Similarly, as the periods of dusk and dawn are associated with light rich in both ultraviolet and red wavelengths (Endler, 1987, 1991; Loew and McFarland 1990) it is also possible that males may prefer to display during these periods of the day.

The importance of the background space-light during signalling is reflected by the fact that the *Gasterosteus aculeatus* species complex exhibits considerable variation in male nuptial display. A recent mate choice study by McDonald and co-workers (1995) on red and black-throated populations suggests that females belonging to each population preferred males that contrasted strongly with their background, displaying a preference for an epigamic signal rather than a preference for the intrinsic spectral characteristics of the red or black colour patch. Such a finding may also be true for colour patches which reflect ultraviolet wavelengths strongly, and in those environments in which ultraviolet light is strongly attenuated, nuptial coloration in the three-spined stickleback may exhibit a reduction in its relative ultraviolet component. This may be an oversimplification, however, as the greater amount of ultraviolet reflection from the silver flanks of the three-spined stickleback found in the population at Balmaha Pond may simply be an unavoidable structural consequence of the platelets themselves, where selection only acts strongly on the red throat in order to produce mosaic signal of high contrast.

Chapter 6

Spectral tuning in the three-spined stickleback

A RE-ANALYSIS OF CRONLY- DILLON AND SHARMA (1968)

EXPERIMENT 6

6. Spectral tuning in the three-spined stickleback

6.1 Introduction

Natural selection will favour those males in a population that are able to successfully communicate their readiness to reproduce to potential mates. In a cluttered visual environment, however, the display of this information risks being overlooked as it must compete with other visual stimuli. In such environments natural selection will favour signalling behaviour that maximises the strength of the received signal relative to background noise whilst at the same time minimising the attenuating affect of the environment (Dusenbury, 1992; Endler, 1992). Yet the evolution of signals and signalling behaviour cannot be regarded in isolation, as, in a similar manner to the evolution of signals, receivers will be favoured by natural selection if they too increase the chance of signal detection during mate choice. Crucial to the success of this communication process is the close alignment of the waveband over which both signal transmission and reception occurs (Ryan, 1990). Hence, it is appropriate to regard signals, receivers, and indeed display behaviour, as functionally related; influencing the evolution of one another rather than operating as independent evolutionary traits (Sargent *et al.*, 1998). Put another way, as sensory systems determine how the male traits are perceived, mate choice criteria will evolve in a direction favoured by the sensory characteristics of the organism, implying a co-evolution between male sexual ornamentation and female preference (Endler, 1992, Verrell, 1999).

6.1.1 Vision and the co-evolution of signal and receiver

Conditions which affect the generation, transmission and detection of a signal alter the effectiveness of the received signal, and will, therefore, exert selective pressure on the sensory system (Guilford and Dawkins, 1991; Ryan and Wilczynski, 1991; Endler *et al.*, 2001). With regard to vision, the direction in which the evolution of the visual system and visual ornamentation proceeds is influenced by the photic environment (Fuller, 2002). In fact, since visual communication in the aquatic habitat is often severely limited by photic conditions (Lythgoe, 1979), it is likely that the evolution of both visual signal and visual system will be constrained by the optical limits imposed by the environment (Endler, 1992). This position is further complicated by the fact that optical conditions in the aquatic environment are liable to change both temporally and spatially, and as such, the evolution of signals and receivers will also be governed by the timing and location of the biologically significant tasks performed by the animal during its lifetime (Endler, 1992; Partridge and Cummings, 1999). Furthermore, as the nature of these different biologically significant tasks may themselves impose different optical requirements, the evolution of both signal and receiver may represent a balance of these requirements. For example, if predation presents a relatively strong selective force, then colour patterns displayed by males will evolve to be less conspicuous to the visual system of the predator (Endler, 1978, 1980, 1983). Differences in the visual tasks performed during feeding behaviour and courtship may also necessitate a compromise in the evolution of both the receiver and signal, with the evolutionary outcome representing a functional balance. Indeed, if the factors which influence the expression and detection of signals are found to vary between habitats, then this may cause the populations of a species to diverge in terms of their signal and receiver properties (Ryan *et al.*, 1990; Schluter and Price, 1993; Boughman, 2001; Scott, 2001). If signals diverge to such an extent the frequency of reproductive interactions decreases between two populations, then this divergence might drive speciation (Ryan, 1990).

6.2 Sensory Drive

Ryan (1990) defines the mechanisms which lead to biases in the direction of signal and receiver evolution as "sensory drive". During this process sensory systems and sensory conditions drive the evolution of mate choice criteria in a particular direction (Endler and McLellan, 1988; Endler 1992). Crucially, the evolution of these criteria should not be random as they are driven largely by the environmental conditions and the behaviour of the animal, with particular conditions favouring particular sensory and signalling characteristics. With regard to the evolution of sexually selected traits, if pre-existing sensory biases were to exist, then this might provide a mechanism with which to drive mating preferences, and this in turn, might initiate a Fisherian runaway process (Kirkpatrick, 1987). If true, such a mechanism might prove sufficient to account for the evolution of secondary sexual characteristics in males, and there is evidence in the swordtail, *Xiphophorus*, that the characteristic extension of the male's caudal fin may have evolved through a sensory or pre-existing bias in female preference (Ryan and Wagner, 1987; Basolo, 1990, 1995). However, such a hypothesis is not without criticism, and whether phylogenetic data support the concept of sensory exploitation in the evolution of secondary sexual characteristics is inconclusive (Meyer *et al.*, 1994; Wines and Morris, 1996; Sargent *et al.*, 1998). Nevertheless, even in natural selection models of sexual selection, such as the good genes or handicap systems (Zahavi, 1975; Grafen, 1990), sensory drive may be implicated, with the exact suite of traits used to determine these factors being themselves determined by the process of sensory drive (Endler, 1992).

6.2.1 Evidence for sensory drive in the three-spined stickleback

Work carried out with three-spined stickleback populations originating from red-shifted waters suggests that environmental conditions may affect both spectral sensitivity and mate choice in this species (McDonald and Hawryshyn, 1995; McDonald *et al.*, 1995). McDonald and Hawryshyn (1995), examining the spectral sensitivity of three stickleback populations originating from three different photic regimes, found that populations living in red, peat-stained waters were relatively more sensitive to longer wavelengths of light than those populations living in habitats rich in short wavelength light. It is in such red-shifted waters that black throated populations of male stickleback are often found (Reimchen 1994; Scott, 2001), and it is possible that the black nuptial coloration displayed by these populations represents an adaptation to environmental conditions, as the more usual red signal provides low chromatic contrast in such waters (Reimchen, 1989). Indeed, using a video imaging technique, McDonald *et al.* (1995) found that female sticklebacks selected males on the degree of colour contrast relative to the background rather than any spectral quantities of the mosaic signal *per se*. If the mosaic signal of the three-spined stickleback does function as an epigamic signal, it provides further evidence in support of the sensory drive hypothesis, where the environment affects both the spectral sensitivity of the female and the signal design of the courting male.

That the efficiency of such a signal will be enhanced if the receiver is tuned to detect it, finds relevance in the three-spined stickleback literature. Since redder males are preferred by female sticklebacks (Selmer, 1971), it might be advantageous to the female to tune into this signal during the breeding season. Work carried out by Cronly-Dillon and Sharma (1968) suggests that females do become increasingly sensitive to red wavelengths of light during the summer. Whilst the tuning of sensory systems to match mating signals has been documented in other animals (Capranica, 1976; Wilczynski *et al.*, 1992; Hornstein *et al.*, 2000; Cronin *et al.*, 2000), what is particularly convincing about this result is that it is only the female that undergoes such spectral shift, and that this shift in sensitivity is timed to coincide with the development of the red throat signal by the male. Indeed, if the detection of the males red throat presents the visual system with a visual task different in nature from that experienced during other months, it might be expect that such a shift in visual sensitivity will be restricted to summer months, as by so doing the female maximises her chance of mate detection without compromising the functional balance of her visual system at other times of the year. That the males do not change despite the fact that they perform similar visual tasks in an identical environment provides compelling evidence for the sensory drive hypothesis in this species.

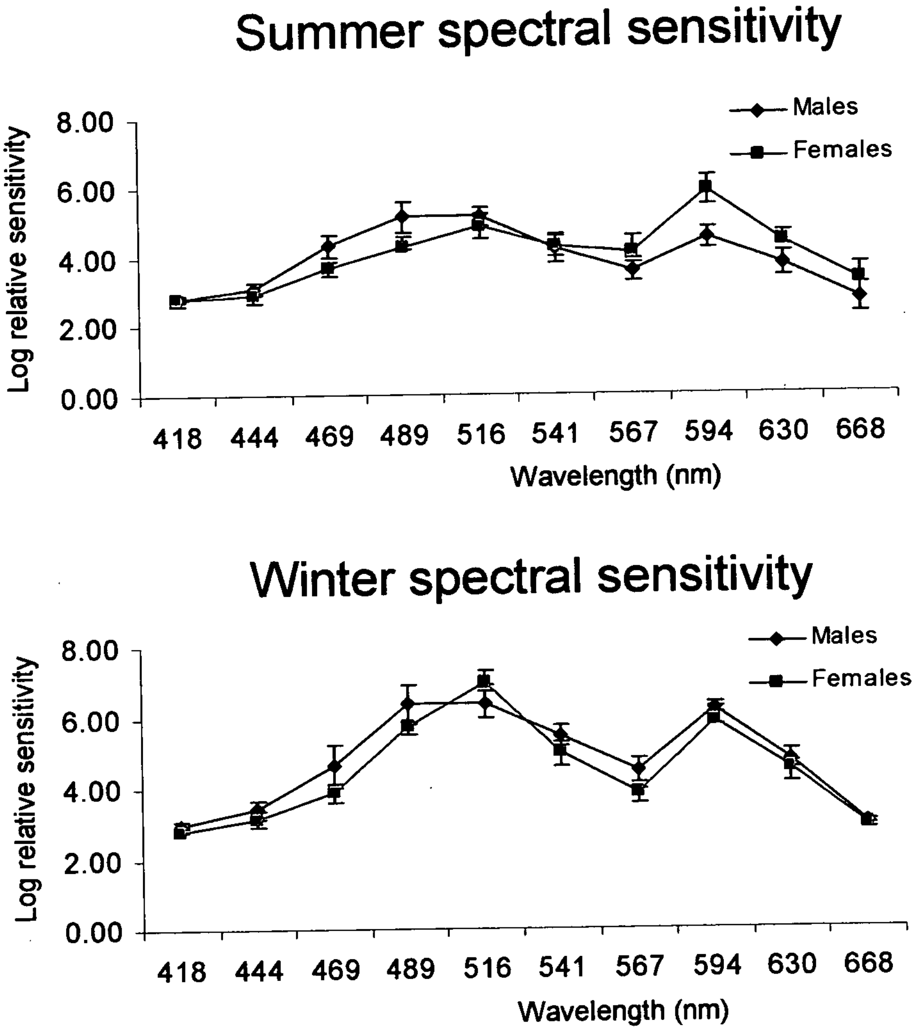
6.2.2 Plasticity and red sensitivity in the three-spined stickleback

The development of the sensory drive hypothesis has attracted much interest in the work of Cronly-Dillon and Sharma, 1968. Yet, in the intervening years since its publication, little work has been carried out to determine the cause of the shift in female sensitivity, or whether this shift is commonplace across the *Gasterosteus* *aculeatus* species complex. It is also unclear what drives the change in red sensitivity in the female. Two mechanisms would appear to be most likely. First, females could be changing in response to a red shift in the environment. Second, since the red sensitivity of the male does not alter in a reddening environment, it is possible that the changes in the female's visual system are hormonally driven. If this is so, the increase in red sensitivity exhibited by the female might anticipate changes in the light properties of the aquatic environment in a similar manner to many migratory species of fish (Carlisle and Denton, 1959; Beatty, 1969; Wood and Partridge, 1993). In attempting to address such questions, an obvious starting point would be to revisit the work carried out by Cronly-Dillon and Sharma in 1968.

6.3 Cronly-Dillon and Sharma, 1968: The effect of season and sex on the photopic spectral sensitivity in the three-spined stickleback

By exploiting the innate following, or optomotor, response of the three-spined stickleback to a moving light stimulus, Cronly-Dillon and Sharma were able to determine the threshold sensitivity of males and females in reproductive and non-reproductive condition (Fig. 6.1). Their results indicate that the long wavelength peak of the female's spectral sensitivity curve becomes more sensitive to red light during summer months when all subjects were in reproductive condition.

While the shape of the spectral sensitivity curves in figure 6.1 are easy to interpret visually, the statistical analysis of them is somewhat problematic. Cronly-Dillon and Sharma chose to solve this problem by comparing the vertical difference between the two pronounced peaks apparent in each sensitivity curve, expressing this difference as units of optical density. Using these units they were then able to analyse possible sex differences in threshold sensitivity using a Wilcoxon test. However, since Cronly-Dillon and Sharma also found a consistent difference between males and females in the wavelength position of the shorter wavelength peak – a difference somewhere in the order of 10nm - it may be that in choosing optical density units they were not comparing like with like. Given that Cronly-Dillon and Sharma published the data set for this experiment, it may, therefore, be appropriate to re-analyse their data using modern statistical methods.



Summer spectral sensitivity

Winter spectral sensitivity

Figure 6.1 Results of Cronly-Dillon and Sharma (1968) showing the spectral sensitivity curves of the three-spined stickleback during reproductive (summer) and non-reproductive (winter) condition. Errors bars indicate standard error.

A Re-analysis of Cronly- Dillon and Sharma (1968)

6.4 Methods

A repeated measures ANOVA was performed on the data published by Cronly-Dillon and Sharma (1968). Due to the apparent sex differences in the wavelength position of the green peak, data relating to the green and blue region of the spectrum were omitted from the subsequent analysis. The analysis is therefore restricted to the four tested wavelengths in the red region of the spectrum: 567nm, 594nm, 630nm, and 668nm. Also, that only the red peak is considered is desirable as it is possible that the inclusion of the further data points might mask potential main effects occurring at the red end of the sensitivity curve. To ensure normality, an Anderson-Darling test was performed on all data prior to analysis.

The spectral sensitivity curves produced by Cronly-Dillon and Sharma (Fig 6.1) were produced under photopic conditions. Crucial to this procedure was that background illumination, used to keep the fish light adapted, was kept at a constant intensity throughout each trial period. Unfortunately, an error in setting up the apparatus meant that this was not the case. The alteration of test conditions between the two trial periods has caused the spectral sensitivity curves in winter to be artificially lowered and, as a direct consequence of these changes in sensitivity, changes in red sensitivity across season could not be included in the following analysis.

6.5 Results

The analysis revealed a significant sex difference in red sensitivity during summer months when both males and females were in reproductive condition (Repeated measures ANOVA: $F_{1,7} = 12.22$, $p = 0.01$). These results were in agreement with the results of Cronly-Dillon and Sharma, suggesting that the visual threshold of females at the longer wavelength end of the spectrum was lower in females than males during summer.

However, in contrast to their results, during winter months, when subjects were outside reproductive condition, a statistically significant difference in red sensitivity between males and females was also found (Repeated measures ANOVA: $F_{1,6}$ = 21.37, p = 0.0036). During winter the visual threshold at the longer wavelength end of the spectrum was lower in males than females, representing a reversal of the trend found during summer.

6.6 Discussion

The results of the re-analysis are counterintuitive. While the data obtained during summer confirms the existence of a red shift in sensitivity predicted under the sensory drive hypothesis, it is difficult to imagine why females might become less sensitive to red wavelengths of light than females during winter months. During this period both females and males are outside breeding condition and will be expected to perform similar visual tasks. This is confirmed by the fact that sex differences in feeding behaviour and diet have not been found in this species (Ibrahim, 1988). The

Two explanations for the results of the re-analysis are possible. First, it might be case that the low sample numbers used by Cronly-Dillon and Sharma - five males and four females in summer, and four males and four females in winter - have resulted in a type one error. If so, variation inherent in the spectral sensitivity of the population may be driving the observed sex differences rather than any real difference. Second, if spectral sensitivity changes with age, it may be that the age structure of the sample group tested could be responsible for the observed differences in spectral sensitivity. Changes in spectral sensitivity with age have been documented in a number of species of fish and can be driven by a number of mechanisms such as: the ontogenic loss of a photoreceptor (Bowmaker and Kunz, 1987), a change in the pigment ratio (Bridges and Yoshikami, 1970), and the yellowing of the cornea with age (Walls and Judd, 1933; Douglas, 1989). The three-spined stickleback is a short lived fish, living on average only a year, where considerable changes to the age structure of the population occur with the cessation of the breeding season (Wootton and Smith, 2000). If males and females differ in their life span, it is possible that sex differences in age structure may drive the differences in sensitivity revealed in the re-analysis. In using such a small population sample size, Cronly-Dillon and Sharma may have inadvertently strengthened this effect.

Experiment 6

It is the aim of experiment 6 to re-examine the effect of season on the spectral sensitivity of the three-spined stickleback using a methodology designed to address some of the problems highlighted in the previous analysis.

6.7 Methods

In common with Cronly-Dillon and Sharma (1968), photopic spectral sensitivity curves for the three-spined stickleback population at Balmaha Pond during reproductive and non-reproductive periods were obtained using an optomotor technique. The optomotor method has proven to be a useful psychophysical technique in the study the visual system of fish (Northmore and Yager, 1975), and provides one of the simplest techniques in the measurement of spectral sensitivity (Grundfest, 1932; Cronly-Dillon and Muntz, 1965; Antis *et al.*, 1998). Nevertheless, care should be exercised with regard to the application of this technique as spectral sensitivity curves produced by the optomotor response often differ from those derived using other psychophysical methods (Neumeyer and Schaerer, 1992). This is due to the fact that different visual pathways are used for the detection of motion and, hence, it is often the case that only a subset of the photoreceptor types present in the retina contributes to the optomotor response. Indeed, in a number of species spectral sensitivity curves produced by the optomotor method are often limited to the long wavelength receptor in the retina, leaving motion detection in these species effectively "colour blind" (Goldsmith, 1994; Schaerer and Neumeyer, 1996). Hence, in estimating the true visual capabilities of an animal, the relevance of the spectral sensitivity curves derived from the optomotor response is somewhat questionable. However, since our interest lies in observing a change in sensitivity rather than the production of a precise estimation of spectral sensitivity in the stickleback, the application of the optomotor technique is in this instance appropriate.

6.7.1 Subjects

Subjects were caught from Balamha Pond during June and October of 2001, and February and April of 2002. These months were found to coincide with the reproductive and non-reproductive seasons of the three-spined stickleback (Wootton, 1984). Once caught, subjects were held for a maximum period of two weeks in full spectrum holding conditions provided by two full spectrum fluorescent bulbs (Arcadia Ltd), each with a colour temperature of 5600°K, suspended 40cm above the holding tanks (Appendix 1.1). All fish were held under ambient temperatures and light cycles and were kept for a maximum duration of two weeks prior to experimentation in order to minimise the effect of holding conditions on visual sensitivity (Kröger, 2003). Differences in environmental conditions were maintained in the holding tanks as they served to maintain reproductive condition in the summer cohort and suppress the onset of reproductive activity in the winter group (Baggerman, 1968). All fish were fed once daily on a diet of bloodworm, *Tubifex*.

6.7.2 Sampling protocol

Spectral sensitivity was measured across two reproductive cycles: from summer to winter during 2001, and from winter to summer during 2002. In each reproductive cycle, a minimum of twenty new individuals, ten males and ten females, were tested. This design allowed spectral sensitivity to be measured from reproductive condition to non-reproductive condition during 2001, in the same manner as Cronly-Dillon and Sharma, and from non-reproductive condition to reproductive condition during 2002.

In so doing, it is possible to guard against the effect of changing population demographics as samples taken from February and April represent fish from the same age class (Wootton and Smith, 2000). Since it was not possible to determine the sex of individuals outside breeding condition, after the completion of the test trials the sex of each individual was determined by dissection.

6.7.3 Apparatus

A schematic diagram illustrating the apparatus used in the optomotor experiment is shown in figure 6.2. The apparatus consisted of a white cylindrical aquarium which contained water to a depth of 15cm kept at temperature of approximately 22°C. This aquarium was positioned directly below a projector with a 250W tungsten halogen bulb (colour temperature approximately 3050°K), allowing a pattern of light to be projected onto the walls of the aquarium during trials. Stimulus light from the projector was passed through a collimating lens before its spectral content was adjusted using one of a series of narrow band interference filters (Edmund Optics, Inc; Appendix 1.3).

To examine photopic sensitivity in the three-spined stickleback, 4 stimulus wavelengths were chosen in the red end of the spectrum: 590nm, 620nm, 650nm and 680nm. Stimulus illumination was then passed through a rotating template which enabled a pattern of stripes to be projected onto the outer walls of aquarium. The image cast by this template was aligned orthogonally to the base of the aquarium and coincided with its central axis. As it was found during preliminary trials that the optomotor response exhibited by the fish was less reliable when fish were very close to the central axis, a white spindle was placed at this point in order to position the fish further into the revolving striped pattern. The direction of rotation could be reversed by an observer via a remote switch. The thickness of each stripe was 5cm, where the angle subtended between adjacent stripes was 45°. Such dimensions have been used successfully in previous studies to derive a photopic sensitivity in the three-spined stickleback (Cronly-Dillon and Sharma, 1968; Boughman, 2001) and were found during preliminary experiments to elicit a clear optomotor response in the direction of the moving stripes. The intensity of the stimulus stripes was controlled with a series of neutral density filters where subjects were exposed to stimulus wavelengths descending in intensity by 0.2 log units across each trial.

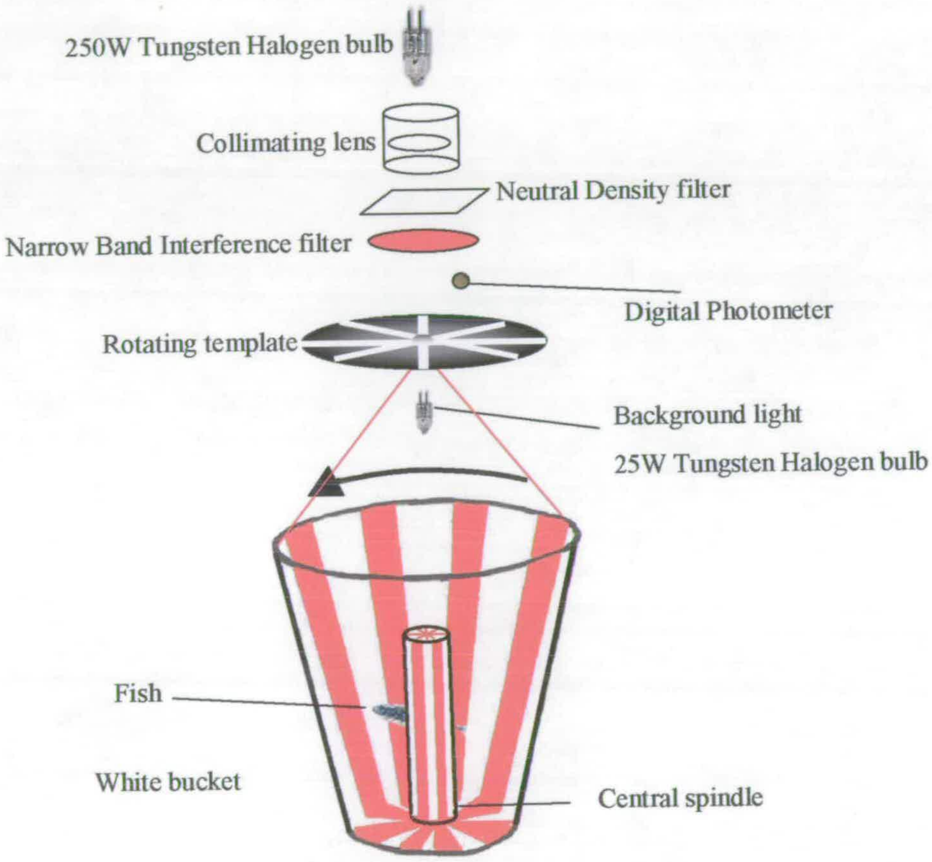


Figure 6.2 Schematic diagram of the optomotor apparatus used to estimate photopic sensitivity in the three-spined stickleback. Groups were tested during the summer and winter months of 2001 and 2002 when the fish were in full reproductive condition and non- reproductive condition respectively. Subjects were exposed to stimulus wavelengths descending in intensity by 0.2 log units across each trial. Wavelengths tested were: 590nm, 620nm, 650nm, 680nm.

The absolute energy of light transmitted by the stimulus light was measured in mWm^{-2} using a Tektronix J16 digital photometer operated in conjunction with a Tektronix J6502 probe. Using the reading taken from the first experimental trial as a reference, it was possible to calibrate the stimulus lamp prior to each test, thereby ensuring that the intensity of the stimulus light remained constant across the experiment. Diffuse background illumination was provided by a 20W tungsten halogen bulb (approximate colour temperature 2900°K) positioned above the bucket. Light from this bulb was attenuated by way of a neutral density filter. Preliminary trials enabled the establishment of a situation where there was sufficient background light to maintain a photopic vision in the test individuals, signified by the production of two characteristic peaks in both the green and red region of the spectrum (Cronly-Dillon and Sharma, 1968). No other illumination was present in the laboratory during the experiment, allowing the observer to remain in relative darkness whilst viewing the behaviour of the fish in the illuminated apparatus.

6.7.4 *Experimental protocol*

Spectral sensitivity curves were derived for each fish, the results of which were grouped according to reproductive status and year. Prior to experimentation, fish were removed from the stock tank, transferred to the apparatus, and allowed one hour in which to settle. During this period fish were kept under background light conditions in order to maintain the subject's light adapted state. Once this settling period was over, for a given wavelength, the striped stimulus pattern was rotated at its highest intensity in either a clockwise or counter-clockwise direction. The initial direction of rotation was varied at random over the entire test sequence. If the fish was found to follow the moving striped pattern, the direction of rotation was reversed, and the behaviour of the fish to the change in direction noted. A fish was judged to have been able to see a particular stripe pattern if it responded to at least eight of the ten rotational changes initiated by the observer.. Once it was established that the fish was able to respond to the pattern of rotating stripes, the intensity of the striped pattern was lowered 0.2 log units, and the experiment re-run at the new intensity level. If the fish failed to respond to a stimulus, the fish was tested for a second time at the level of intensity 0.2 log units higher. In so doing, it was possible to discern if the lack of response was due to the fact that the visual threshold for the particular stimulus wavelength had been reached, rather than a general lack in motivation to keep station with the stripes. This process was repeated for a second time if doubt existed over the subject's response to the stimulus. Data that were more consistent with a drop in motivation were disregarded from the final data set, and the fish replaced with another that was naïve to the experimental set-up. The level of stimulus intensity above that at which the fish failed to respond was deemed to be the threshold level of the optomotor response. In total, four stimulus wavelengths were presented to the fish in pseudo-random order, with the caveat that adjacent wavelengths were not tested one after the other. In combination with a five minute rest period, it was hoped that such a precaution would prevent the subjects from becoming selectively adapted to a particular region in the spectrum.

6.7.5 Statistical analyses

Repeated measures ANOVAs were performed for the two reproductive cycles during 2001 and 2002. An Anderson-Darling test was performed on each data set to assess normality prior to analysis.

6.8 Results

1). Summer and winter 2001

In contrast to Cronly-Dillon and Sharma (1968), both males and females were found to be less sensitive to red light in winter than in summer (Repeated measures ANOVA: $F_{1,18} = 5.55$, $p = 0.03$; $F_{1,20} = 37.43$, $p = 0.0001$, respectively). However, no differences according to sex where found in the red sensitivity of the three-spined stickleback during summer 2001 when all subjects were in reproductive condition (Repeated measures ANOVA: $F_{1,18} = 2.94$, $p = 0.10$), see Fig. 6.3. Similarly, no sex differences were found during winter 2001 when all individuals tested were outside breeding condition (Repeated measures ANOVA: $F_{1,20} = 2.14$, $p = 0.16$).

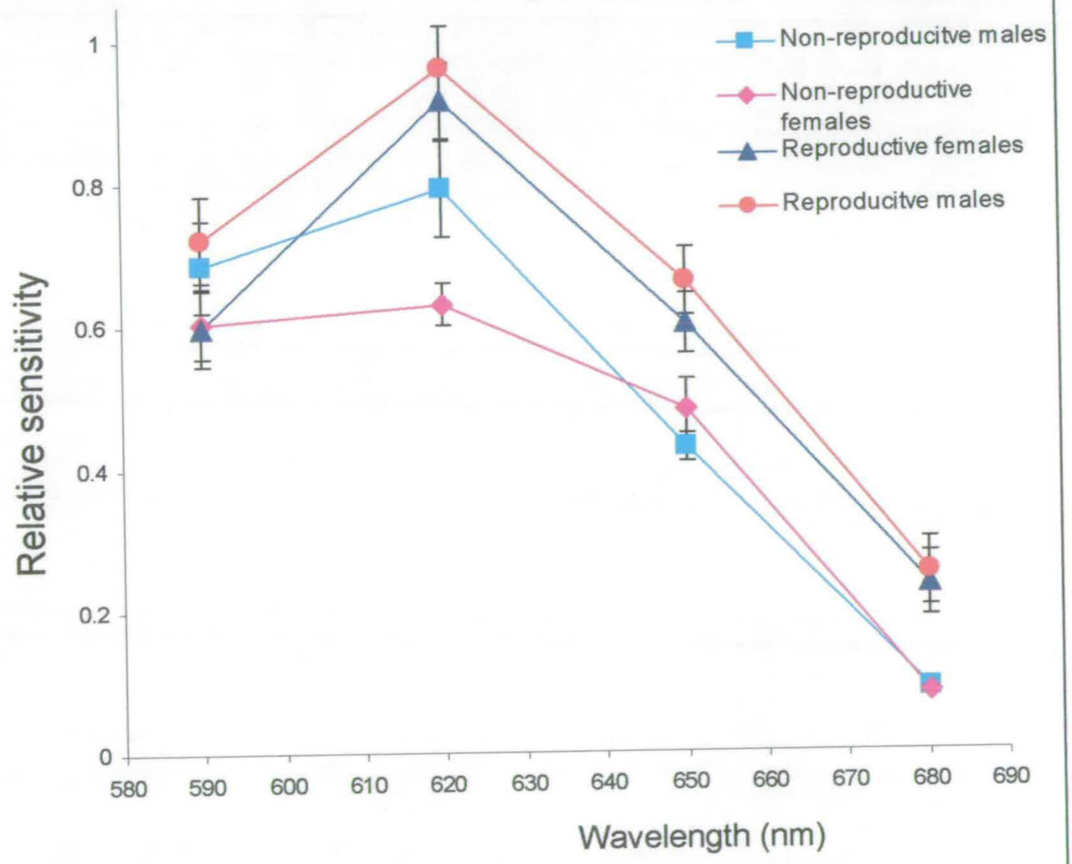


Figure 6.3 The relative spectral sensitivity of the three-spined stickleback to red wavelengths of light according to sex and reproduction condition during 2001. In this trial, the first sample group collected from Balmaha Pond was tested whilst in reproductive condition (summer), and the second sample group tested when the fish were outside breeding condition (winter). The four stimulus wavelengths tested were 590nm, 620nm, 650nm, and 680nm. Vertical bars denote standard error from the mean.

2). Summer and winter 2002

Again, both males and females were found to be less sensitive to red light during the winter than in summer (Repeated measures ANOVA: $F_{1,17} = 11.43$, $p = 0.003$; $F_{1,18} = 4.59$, $p = 0.04$, respectively). No sex differences in the red sensitivity of the three-spined stickleback were revealed for subjects collect in February, when they were not in breeding condition (Repeated measures ANOVA: $F_{1,18} = 0.953$, $p = 0.34$), see Fig. 6.4. Similarly, no sex differences were found during summer 2002 when all individuals tested were in breeding condition (Repeated measures ANOVA: $F_{1,17} = 0.036$, $p = 0.85$).

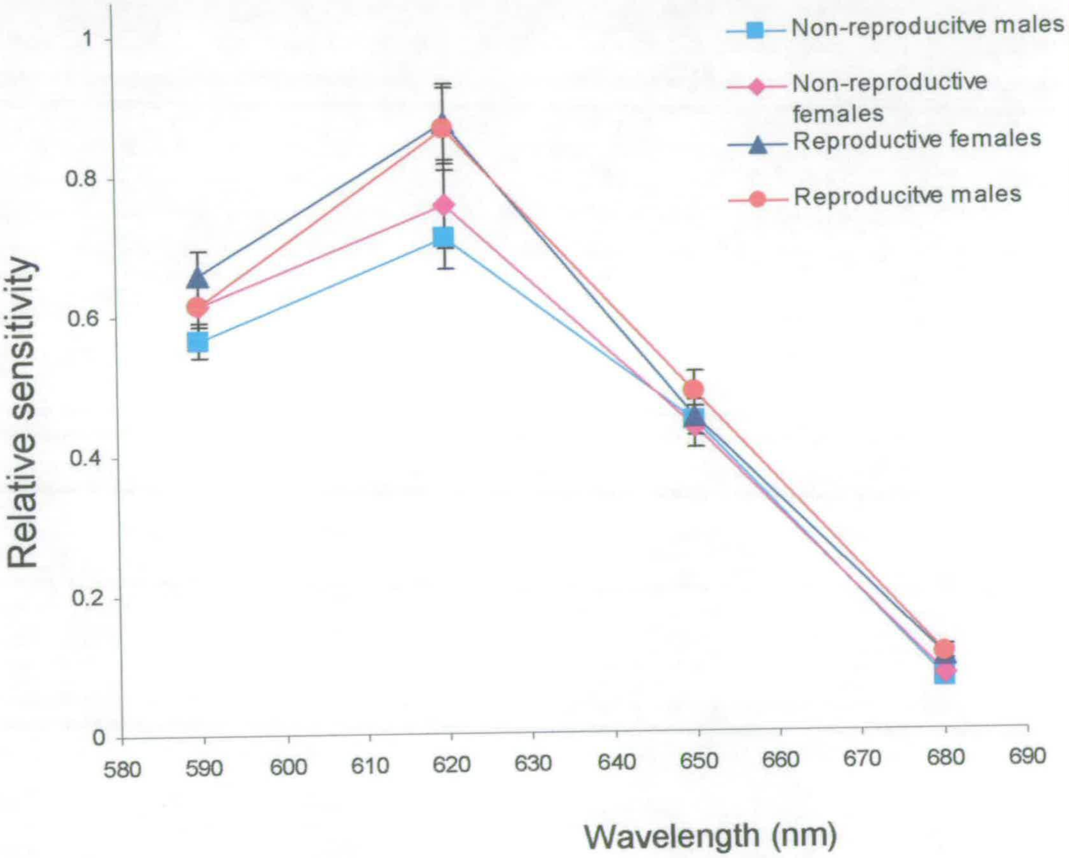


Figure 6.4 The relative spectral sensitivity of the three-spined stickleback to red wavelengths of light according to sex and reproduction condition during 2002. In this trial, the first sample group collected from Balmaha Pond was tested whilst in non-reproductive condition (winter), and the second sample group tested when the fish were found enter breeding condition (summer). Again, the four stimulus wavelengths tested were 590nm, 620nm, 650nm, and 680nm. Vertical bars denote standard error from the mean.

6.9 Discussion

The results presented in this chapter provide evidence that the three-spined sticklebacks living in Balmaha Pond exhibit a seasonal shift in their spectral sensitivity, with their visual system becoming increasingly sensitive to red light during spring and summer. However, in contrast to the findings of Cronly-Dillon and Sharma (1968), this shift was not restricted to females but was found to be a characteristic of all of the individuals sampled. The finding that both males and females were found to exhibit shifts in their sensitivity is important, as seasonal changes in spectral sensitivity which involve both sexes have been widely documented for several other species of freshwater fish (Loew and Dartnall, 1976; Muntz and Wainright, 1978; Muntz and Mouat, 1984). Furthermore, as the increase in red sensitivity revealed in the reproductive cycle of 2001 was reproducible during the first half of 2002, a period in which few changes in the age structure of the population are expected (Wootton and Smith, 2000), it is possible in this instance to rule out the prospect that demographic changes are driving the observed shift in red sensitivity.

Light adaptation

The subject's degree of light adaptation during experimental examination is critical to the production of accurate sensitivity curves. Failure by Cronly-Dillon and Sharma (1968) to keep the background conditions at a constant intensity between experimental periods meant that threshold levels were suppressed during the second test phase and could not, therefore, be compared statistically across season. That light adaptation is critical is also illustrated by evidence suggesting that the dimensionality of colour space can be a function of light intensity. For example, in goldfish, there is evidence to suggest that the red cones in the retina do not contribute to photopic vision when ambient light levels are low (Neumeyer and Arnold, 1989; Neumeyer *et al.*, 1991), and may instead contribute to the output of the rods when light intensities are close to the threshold for photopic vision (Powers & Easter, 1978). With this in mind, it is possible that the observed shift in spectral sensitivity during 2001 could be caused by a progressive reddening of the background lamp, as this will cause spectral sensitivity to appear increasingly suppressed as the experiment progresses. However, as the lamp used in the 2001 cohort was also used in the analysis of the 2002 cohort, such an effect would be expected to reveal a further decrease in the red sensitivity during 2002. This was not the case as both sexes were found to become more sensitive to red light in April 2002, suggesting that the light-adapted state of subjects in experiment 6 was not a confounding factor in the analysis.

Changes in the visual system underlying the observed shift in spectral sensitivity

Experiment 6 depicts a situation where the spectral sensitivity of both males and females change with time. Such seasonal changes in spectral sensitivity are a common feature of the visual ecology of many freshwater fish, and are often mediated by changes in the rhodopsin/porphyropsin ratio or in the opsin moiety (Bridges, 1972; Beatty, 1975; Bowmaker, 1990). While the three-spined stickleback is known to possess a rhodopsin/porphyropsin pair in each class of photoreceptor (Ali and Anctil, 1976), the wavelength intervals at which sensitivity was tested in experiment 6 are too large to discern if the observed changes in spectral sensitivity are driven by a characteristic shift in the wavelength position of the spectral sensitivity curve. Hence, it is not possible to tell if seasonal changes in sensitivity are caused by changes in the rhodopsin/porphyropsin ratio or by a selective sensitisation of the visual system, which would leave the absorption maxima of the sensitivity curve unaltered. If selective sensitization is driving the seasonal changes in sensitivity it is most likely brought about by a sensitization of the red opponent visual pathway or an increase in the absolute amount of red-sensitive pigment in the retina, as both mechanisms would increase red sensitivity without moving the wavelength position of the red peak (Cronly-Dillon and Sharma, 1968).

That the seasonal shift in spectral sensitivity exhibited by the sticklebacks does not involve a change in the wavelength position of the spectral sensitivity curve is noteworthy, as the employment of a psychophysical technique, such as the optomotor response, is critical to its detection. Due to the relative difficulty associated with the execution of psychophysical methods, they have been rarely employed in the examination of seasonal shifts in visual sensitivity, and, hence, it may well be the case that such seasonal shifts in sensitivity are a common feature of the visual ecology many species of freshwater fish. In view of this, it would be of interest to examine the spectral sensitivity of other freshwater fish such as the carp *Cyrprinus carpo,* roach *Rutilus rutilus*, tench *Tinca tinca*, and perch *Perca fluviatilis*, which are not believed to display a seasonal shift in the sensitivity of their visual system (Bowmaker, 1990).

6.9.1 Mechanisms underpinning shifts in spectral sensitivity

Changes in the spectral sensitivity most often observed in those species where spectral shifts are mediated by a variation in the rhodopsin/porphyropsin ratio, have been found to occur naturally at particular times in the life cycle or season. A clearer picture of what might initiate change in spectral sensitivity was obtained by Dartnall *et al.* (1961) who reported that the light regime under which fish were held was critical to a change in the rhodopsin/porphyropsin of the rudd *Scardinius erythrophthalmus*. Subsequent work has revealed that changes in photopigments, including wholesale changes to the photoreceptor types within the retina, are found to alter with changes in photoperiod, light intensity, temperature, and hormone levels (Beatty, 1969; Thorpe, 1971, 1973; McFarland and Allen, 1977; Tsin and Beatty, 1979; Whitmore and Bowmaker, 1989).

Whilst the exact nature of the casual environmental factors pertaining to visual pigment changes is not fully understood, such cues might be implicated in the shift in red-sensitivity in the three-spined stickleback. Indeed, since the period of heightened red-sensitivity exhibited by the stickleback coincides with the onset of breeding activity, it is also possible that such changes are associated with high concentrations of gonadal and gonadotrophic hormones found during this period (Hoar, 1962). In other species of fish, the proportion of pigments has been found to vary with the administration of thyroid hormone (Allen 1971; Wood and Partridge, 1983; Browman and Hawryshyn, 1992), and it would be useful, therefore, to examine if the observed shift in red sensitivity found in the stickleback is maintained.

6.9.2 The functional significance of seasonal shifts in spectral sensitivity

Spring and summer months are associated with a period of transition in the life history, photic environment, and visual behaviour, of many species of freshwater fish. Consequently, this period is also one in which optical tasks a fish performs are found to change greatly. Hence, in order to remain functionally expedient, it might be expected that the visual system of fish will exhibit seasonal plasticity, adapting to changing optical stimuli. Often the visual requirements of the numerous visual stimuli pertinent to a species are, however, contradictory, leaving the causal mechanism for a change in sensitivity ambiguous, and in consequence the responses of the visual system to similar light regimes are not consistent from one species to the next. In view of this, several hypotheses have been suggested as to why fish, such as the three-spined stickleback studied in this thesis, might experience a shift in the sensitivity of their visual system. These hypotheses include: changes to the photic environment, developmental and behavioural changes, signal tuning, and diet.

i). Environmental changes

The photic environment found in freshwater bodies such as Balmaha Pond is a dynamic one, subject to considerable changes in spectral quality and intensity over the course of year. Indeed, the increase in red sensitivity found in experiment 6 arises during a period when Balamha Pond is dominated by longer wavelengths of light (see Chapter 3). Since visual performance is largely dictated by prevailing photic conditions, it is not unreasonable to suppose that the visual system of a species residing in these waters will be adapted to prevailing conditions (Lythgoe, 1979). Even within a species, it might be expected that the extent to which the visual system shifts during summer months will be dependent upon the nature of the photic environment. Muntz and Mouat (1984) measured one such change in the visual sensitivity of three different populations of the trout *Salmo trutta*, to seasonal changes in the photic environment. In all sites, they found the trout retina to be dominated by porphyropsin for the majority of the year, with the less red-sensitive rhodopsin appearing only in the summer. That the population found to have significantly less rhodopsin in the summer was also found to inhabit waters shifted furthest towards the red end of the spectrum is consistent with the notion that pigments may be tuning to match the ambient spectra. However, what is also of interest in this study is that the shift to more blue-sensitive rhodopsin occurred during summer, a period when more red sensitive pigments might be thought to be appropriate, illustrating that the relationship between ambient spectra and spectral sensitivity is not always intuitive, and that other factors may be influencing the shift in visual pigments in this species.

ii). Developmental and behavioural changes

Diadromous species, such as salmonids and eels, exhibit dramatic changes in their visual sensitivity which are thought to be associated with changes in their development (Beatty, 1966, 1975; Pankhurst, 1984). Prior to migration, the visual pigments in these species change, mediated by a change in the A_1/A_2 chromophore ratio, or in the opsin moiety itself, with the retinal pigments shifting to match the new environment. In addition, migrating salmon have also been found to experience a loss in ultraviolet sensitivity when they undergo their first migration (Bowmaker and Knunz, 1987; Hawryshyn *et al.*, 1989). Beaudet *et al.* (1997) also provide evidence that the ultraviolet cones re-appear in the retina of sexually mature salmonids prior to returning to the freshwater spawning grounds. That these hormonally driven changes are anticipated in advance of the migration is evidence of the genetic basis of this pigment shift.

Although more subtle, developmental changes apparent in non-migratory species of freshwater fish are also thought to be responsible for seasonal shifts in spectral sensitivity, and these changes could account for seasonal changes in the stickleback. Such changes are evident during the late juvenile stages of many freshwater fish when individuals begin to alter their foraging behaviour. If the change in this behaviour moves the animal into different waters, or prey on food items presenting a different optical requirement, it is likely that such a change in behaviour may alter the nature of the visual tasks pertinent to the visual system (Lythgoe, 1979). An example can be found in the black bream *Acnathopagrus butcheri* (Shand *et al.*, 2002). At the onset of the late juvenile stage, the visual system of the bream becomes more red-sensitive, and this is a period which coincides with a migration to deeper waters dominated by longer wavelengths of light. These shifts in spectral sensitivity were also evident in laboratory-reared populations and confirm the notion that such changes are developmentally driven.

Seasonal changes in the behaviour of the stickleback population at Balamha Pond may also cause variation in spectral sensitivity. For example, small scale, positional movements within the photic zone cause the visual system to operate in light conditions which differ in terms in spectral quality and intensity, and this may again drive a seasonal shift in spectral sensitivity. Positional movements within the water column are a common feature of the ecology of many fish and are associated with seasonal variation in feeding and antipredator behaviour. These movements need not be large, as small scale vertical migrations to surface waters during summer feeding bouts are likely to be sufficient to cause a change in spectral sensitivity (Muntz and Wainright, 1978). Seasonal changes in behaviour may also alter the time of day at which evolutionary significant visual tasks are performed, and this may also drive seasonal changes in spectral sensitivity. In support, Munz and McFarland found that the visual pigments of many reef fish, both predator and prey species, are tuned to the ambient spectra apparent during crepuscular rather than daytime periods as this time represents a period high in feeding activity and was, thus, key to their survival (McFarland and Munz, 1975a, 1975b; Munz and McFarland, 1975).

iii). Signals and receiver tuning

The sensitization of the three-spined stickleback to red light arises during a period when males develop a red mosaic pattern. Hence, as experiment 6 indicates that both males and females become more red-sensitive during summer months, it could be argued the spectral tuning of the visual pigments to the red mosaic signal might also be adaptive for the male. Male-male interactions are an important part of the breeding behaviour of this territorial species (Nilsson and Nilsson, 2000), and in becoming more sensitive, it is possible that a male is able to track more effectively rival males in breeding condition entering his territory. The importance of the red signal to the male is borne out by experimental evidence suggesting that three-spined stickleback males assess the degree of redness of other rivals prior to the initiation of antagonistic behaviour (Rowland, 1982, 1994; Baube, 1997).

Nevertheless, it should be noted that the ultimate causes for the evolution of signals and receivers are inherently linked, and so must not be regarded in isolation. Indeed, it is likely that a suite of visual tasks drive the evolution of the visual system, affecting the evolution of both signal and receiver, with the outcome of this process representing a functional balance of the visual requirements (Endler, 1993b). Inherent in this balance are three interrelated processes - light transmission properties of the environment (where this is also contingent upon the visual behaviour of the animal); spectral tuning of the visual system; and the matching of the optical properties of the signal to the visual system – and it is these three factors that shape the evolution of signal design (Endler, 1993b; Boughman, 2002). Despite close co-evolution between vision and sexual traits, whether the seasonal shift in sensitivity pre-dated the nuptial signal, which was hence shaped by the shift itself, or evolved in response to the red colouration is not clear.

iv). Diet

In contrast to the above theories, the observed shift in red sensitivity documented in this chapter may not serve any adaptive function *per se*, but might rather be simply a consequence of the seasonal changes in the diet of the three-spined stickleback during the year. Whilst the pathway by which species manufacture the visual pigments from the diet is somewhat uncertain, there is evidence that the visual pigments of freshwater fish are derived from either from pro-vitamin A_1 (a precursor of both retinal and retinol) or retinal obtained directly from the diet (Beatty, 1984). Diet has been found to affect the porphyropsin formation in fish, with the level of 3-

dehydroretinal in the diet critical to its occurrence in the retina (Knowles and Dartnall, 1977). In view of this, it is possible that the increased sensitivity to red light evident in population of sticklebacks at Balmaha Pond may be driven by an increased amount of photopigment in the retina, and this, in turn, may be due to a seasonal change in diet. Such seasonal changes in the diet have been documented for other three-spined stickleback populations, with the abundance and variety of prey species changing over the course of a year (Wootton, 1984).

6.9.3 Could seasonal changes in spectral sensitivity promote speciation in the three-spined stickleback?

Because signals that match the perceptual abilities of the observer are more likely to be detected in a cluttered environment, the extent to which signal and receiver match each other in terms of performance might affect mating success, and this is likely to be true for a species reliant on the visual sense such as the stickleback. Furthermore, if the seasonal shift in red sensitivity in the stickleback varies from population to population, then this variation may reduce the probability of mating between populations, as females may, due to subtle differences in perception, prefer one signal over another (Boughman, 2002). In fact, there is some evidence that this may be the case. Population differences in the seasonal shift in spectral sensitivity have been documented in the Rudd (Muntz and Wainright, 1978), where populations from different waters were found to exhibit considerable divergence in this shift, and it is possible that this is also true for a species such as stickleback which is believed to exhibit population differences in spectral sensitivity (McDonald and Hawryshyn, 1995). In addition, using populations of three-spined sticklebacks originating from waters differing in their spectral content, Boughman (2001) was able to show that female sensitivity to red light varied with photic condition, and that this contributed to a divergence in mate choice preferences across populations. Whether this divergence in preference is based on the true colour of the red patch alone, or due to overall colour contrast with the background or other patches in the mosaic signal of the male is unclear (Ryan, 2001). Nevertheless, if the seasonal changes in spectral sensitivity do contribute to a divergence in signal and signal preference across populations, it is possible that such shifts will ultimately lead to reproductive isolation, contributing to speciation (Ryan, 1990; Boughman, 2001).

Chapter 7

General Conclusions

7. General Conclusions

7.1 Introduction

A variety of techniques have been used in this thesis to develop an understanding of the visual ecology of the three-spined stickleback. The work within the thesis suggests that the three-spined stickleback is capable of ultraviolet photoreception, and is able to respond behaviourally to ultraviolet light under ambient light conditions. It also describes, for the first time, that ultraviolet wavelengths of light are used in the mate-choice decisions of female three-spined sticklebacks. Finally, the thesis provides evidence that the stickleback undergoes a seasonal shift in visual sensitivity, with both males and females becoming more sensitive to red light during summer months.

As detailed discussions of the main findings have been presented at the end of each chapter, it is the aim of this chapter to summarise and conclude on the thesis as a whole. The implications of these findings on this well studied experimental species, as well as possible areas of future study are also discussed.

7.2 Ultraviolet sensitivity in the three-spined stickleback

Transmittance data obtained from the ocular media presented in chapter 2 indicate that the optical components of the stickleback's visual system are consistent with the perception of ultraviolet light. This finding is borne out by electrophysiological data, which revealed that the stickleback is capable of ultraviolet photoreception. Whilst such evidence, although persuasive, is insufficient to prove ultraviolet perception, the fact that fish were subsequently found to be able to track visual landmarks differing only in their ultraviolet content supports the notion that the stickleback is able to see ultraviolet light. In addition, female mate-choice experiments found that females, when assessing the attractiveness of two potential mates, prefer those males who display across the full spectrum rather than those limited to the human-visible range, 400-700nm. Since the red mosaic signal has been found to reliably indicate female preference in this species (Milinski and Bakker, 1990), it is possible that the addition of ultraviolet wavelengths serves to enhance the contrast of this signal.

A survey of the aquatic environment from which subjects were collected confirmed that functional amounts of ultraviolet photons were present in their habitat throughout the year. The fact that ultraviolet light was found to be present during June, a period when short wavelengths were found to be strongly attenuated, is significant as it might be expected that the red shifted environment caused by the breakdown of organic material in the water would present a situation where ultraviolet wavelengths would be poorly represented (Losey *et al.*, 1999). This finding can largely be attributed to the fact that the habitats in which three-spined sticklebacks are found to reside are often shallow, and it is this reduction in path-length that reduces total attenuation. Only in areas of dense vegetation were the intensity levels of ultraviolet light found to approach the possible limit of functional vision.

7.2.1 The implications of ultraviolet sensitivity in the three-spined stickleback

That the ability to perceive ultraviolet wavelengths has been found in a population of sticklebacks originating from a red-shifted habitat lends weight to the argument that ultraviolet sensitivity may be fairly widespread across the *Gasterosteus aculeatus* species complex. Moreover, as ultraviolet wavelengths have been found to play a role in the visual behaviour of this species, such a finding will have implications for the design and execution of behavioural experiments in this species (Bennett *et al.*, 1994). Ideally, behavioural experiments should be conducted under full spectrum conditions, as in arbitrarily omitting the ultraviolet region of the electromagnetic spectrum we are subjecting the stickleback to abnormal illumination conditions, and this may have an impact upon their behaviour. This is undoubtedly true in those instances where colour choice experiments are performed. Similarly, the finding that ultraviolet wavelengths affect the mate choice decisions in the stickleback means that the design of such experiments should be executed under full spectrum conditions. The classification of breeding coloration in the stickleback must also take in account their ultraviolet sensitivity, as in failing to do so such studies will risk misclassifying colour, and so will express colour on a basis inappropriate to the visual system of the stickleback (Endler, 1990, Bennett *et al*, 1994).

7.3 Spectral tuning in the three spined stickleback

The three-spined sticklebacks at Balmaha Pond exhibit a seasonal shift in their spectral sensitivity, becoming more sensitive to red wavelengths during summer months. That this shift was found to coincide with a spectral shift in the transmittance of their habitat lends support to the notion that the visual system responds to a change in photic conditions, however, the precise mechanism that drives this shift remains uncertain. Nevertheless, such a shift in sensitivity may account for the secondary sexual characteristics of the stickleback, where the red mosaic signal has evolved to exploit the sensory bias towards red light during this period (Sargent *et al.*, 1998).

7.4 Future Directions

i) Tetrachromacy

With the existence of a fourth, ultraviolet photoreceptor arises the potential for tetrachromacy in the three-spined stickleback. In determining the dimensionality of colour vision it should, however, be remembered that not all retinal photoreceptors may contribute, and so the number of receptor types in the retina cannot alone determine the dimensionality of colour vision in any given species (Goldsmith, 1994). Hence, to establish the dimensionality of colour vision in the stickleback –

how many receptors contribute to chromatic information - it would be of use to carry out colour mixture experiment which assess the minimum chromatic requirements to match a white light source (Neumeyer, 1992). By doing so it would then be possible to model colour more accurately in this species.

ii) Loss of the ultraviolet photoreceptor

Although ultraviolet sensitivity has been found in an aquatic environment that strongly attenuates shorter wavelengths of light, it would be beneficial to examine the visual system of stickleback populations from more dystrophic environments. In so doing it would be possible to assess if such conditions drive the loss or numerical reduction of ultraviolet receptor class from retinal mosaic. If so, such divergence in the visual system may drive differences in mate-choice behaviour, and it would be of interest to examine if this divergence contributes to reproductive isolation between stickleback populations.

Similarly, it would be of interest to examine anadromous populations of three-spined sticklebacks to ascertain if there are any changes to their visual system prior to migrations into coastal waters. Changes in the visual system have been documented in other anadromous species of fish (Beatty, 1966) and might, therefore, be a feature of the visual ecology of the stickleback. If such a shift in sensitivity were found to exist, it would be useful to compare the spectral sensitivity of these anadromous populations with the exclusively marine and freshwater populations of sticklebacks found in the same waters. If the anticipated shift in spectral sensitivity of the anadromous form matches the spectral sensitivity of these non-migratory populations, then this it would provide strong evidence that the anticipated changes are in response to the change in environment.

iii) Microhabitat and light

Much is known about the visual display behaviour of the stickleback and it would be of interest to quantify the photic conditions of the microhabitat during this behaviour. Time of day of day, the choice of display area, and the nature of the visual background will all affect the visual impact of display, and it is possible that sticklebacks alter their display behaviour in such a manner as to maximise the optical signal. For example, male display behaviour may take place in open water at a time of day when illuminating light is rich in a particular region of the spectrum (Endler, 1991). In so doing, the displaying male may be exploiting the relationship between the receiver, signal and the signalling environment in an attempt to maximise signal detection (Ryan, 1990). In a similar manner, it would also be useful to quantify turbidity during display behaviour and the way this affects reaction distance of female to male displays.

iv). The effect of hormones on spectral sensitivity

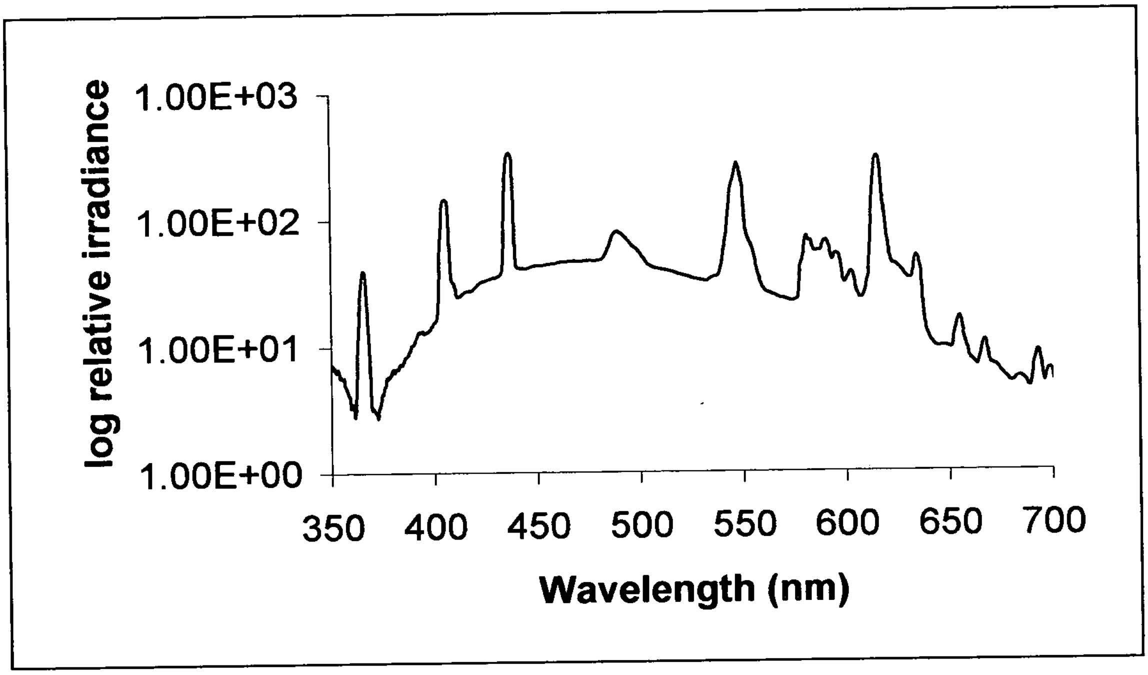
The period of heightened red-sensitivity exhibited by the stickleback coincides with the onset of breeding activity, and it is possible that such changes are associated with high concentrations of gonadal and gonadotrophic hormones found during this period (Baggerman, 1968). In other species of fish, the proportion of pigments has been found to vary with the administration of thyroid hormone, and it would be useful, therefore, to examine to this shift in red sensitivity under controlled condition. If carried out under controlled illumination such a study would be able to determine if the shift in red sensitivity is driven by changes in environmental light or if it is entirely hormonally driven, anticipating the seasonal change in photic conditions.

That many questions remain unanswered means that further investigation of the visual ecology of the three-spined stickleback would be beneficial. Indeed, since we already understand much about the visually mediated behaviour of the stickleback, and since stickleback populations are found to pervade a variety of aquatic habitats, it is likely that in answering these questions we will answer many of the questions pertinent to the field of visual ecology. Moreover, as the emphasis of visual ecology moves away from the inter-species comparisons of the past to answer questions of fitness, it is likely that the three-spined stickleback will gain the prominence it has in other fields.

APPENDIX

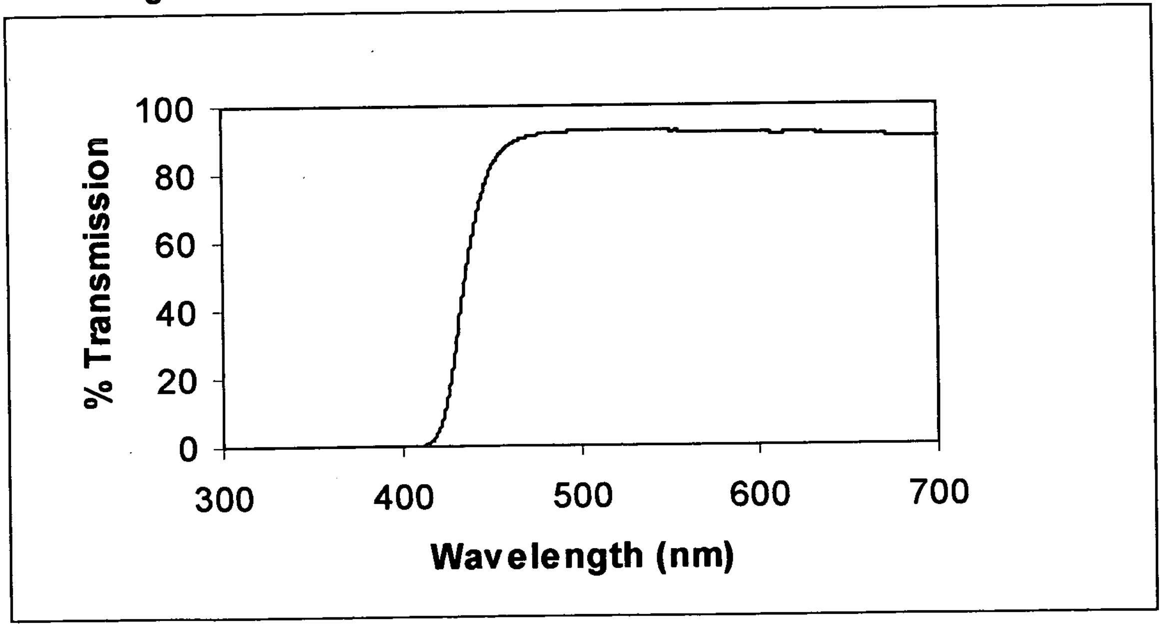
1.1 Relative Irradiance (log quantal flux) of Arcadia fluorescent tubes.

Data collected using an underwater cosine collector placed at a depth of 30cm in the test tank.



1.2 Cut-off filters (Edmund Optics Inc).

Percentage transmission of Long-pass adapting background light

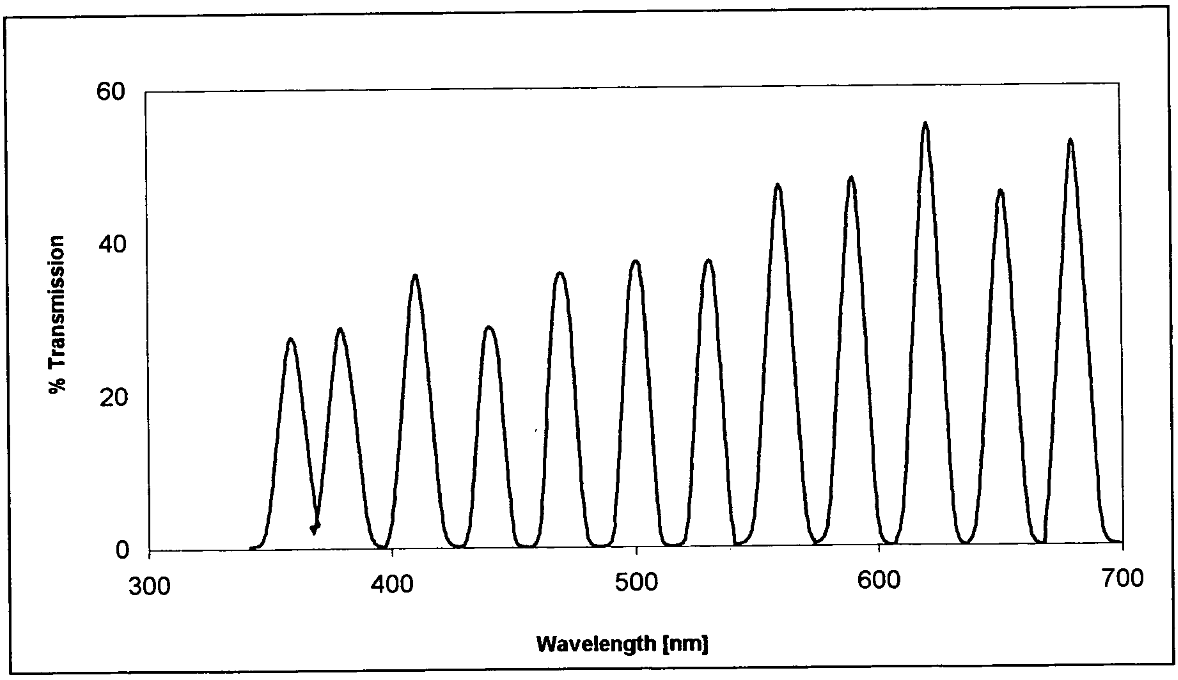


1.3 Narrow Band Interference Filters:

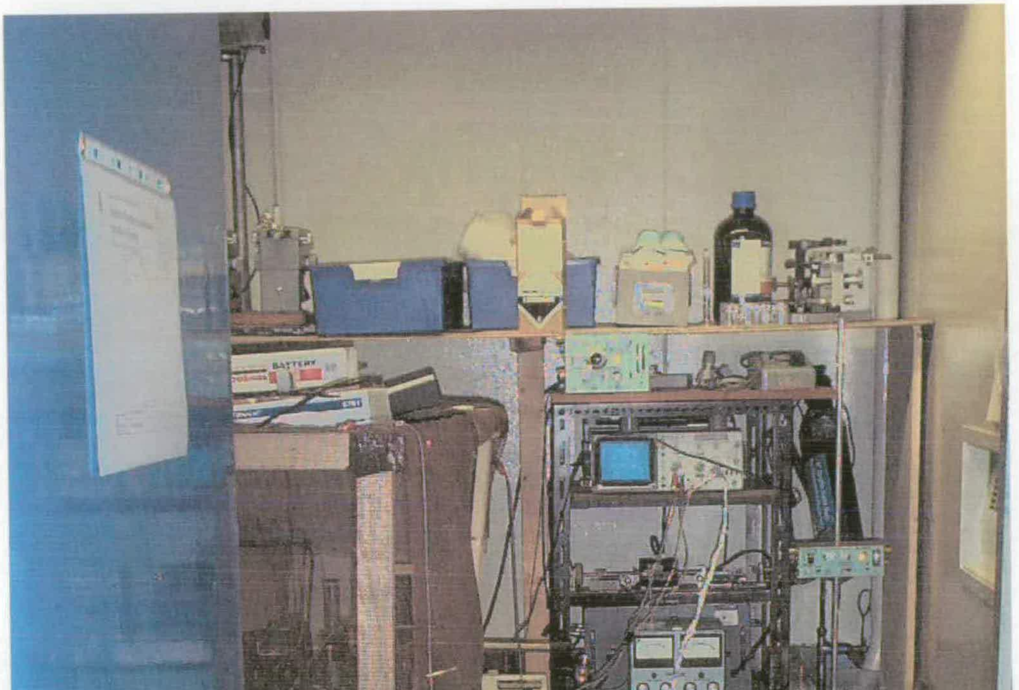
Published performance ratings (Coherent Inc).

CWL (nm)	360	380	410	440	470	510	530	560	590	620	650	680
Effective index n	1.45	1.45	1.45	2.1	2.1	2.1	2.1	2.1	2.1	2.1	2.1	2.1
FWHM (nm)	10.6	11.2	9.7	7.5	7	7.4	8.5	9.4	10	10.6	11.4	11.5
Minimum transmission	38%	30%	45%	45%	45%	50%	50%	50%	50%	50%	50%	50%

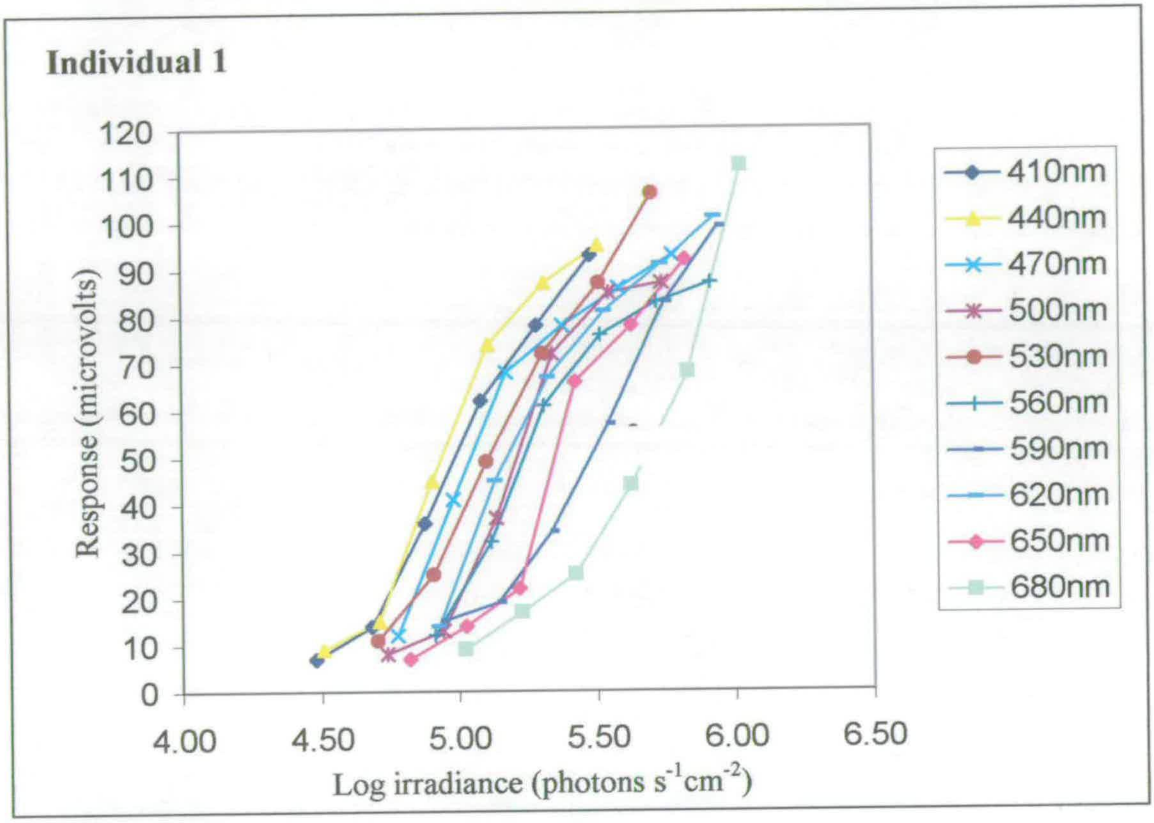
Recorded Transmission Spectra of Interference filters

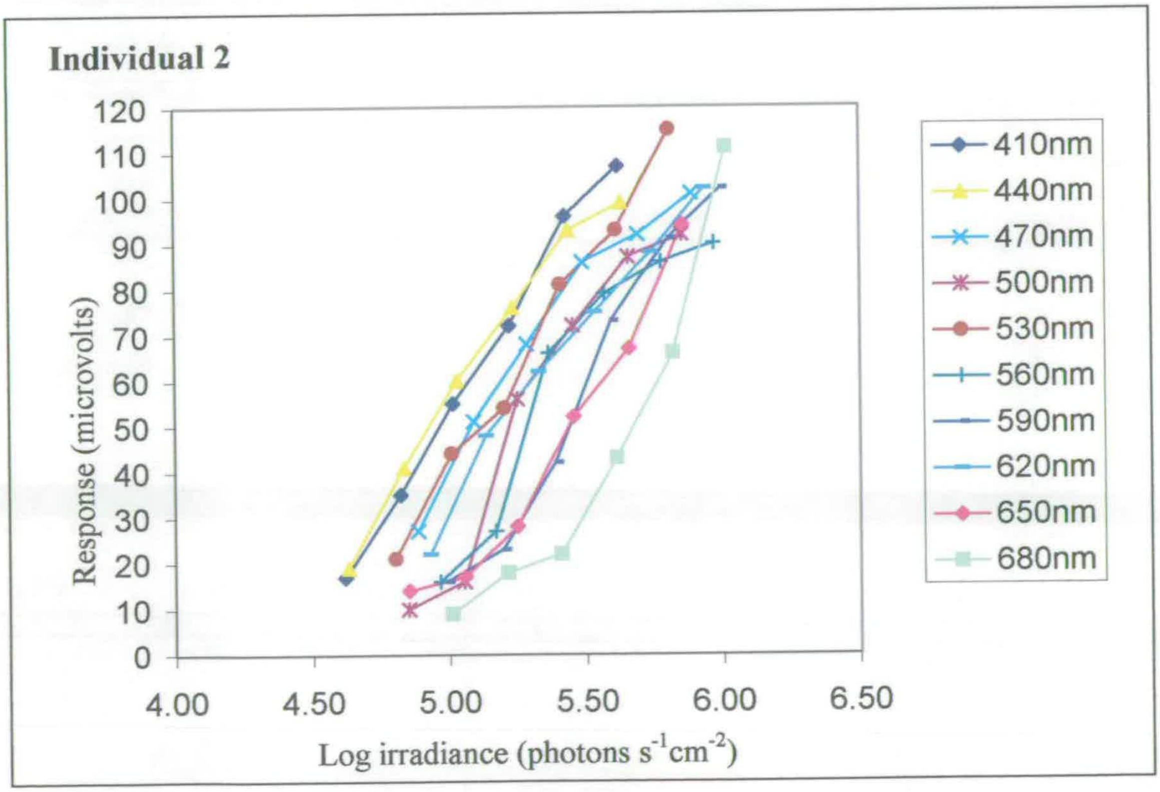


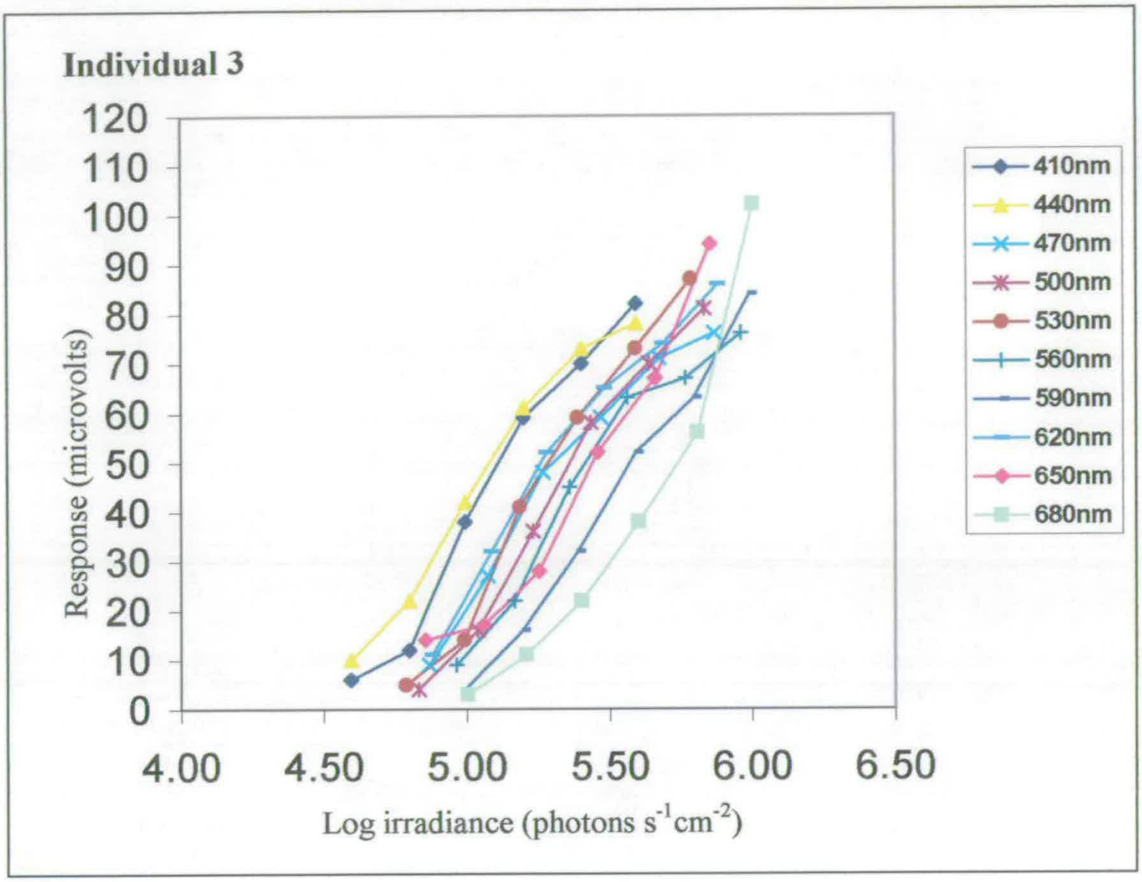
1.4 Electrophysiology rig used in experiment 2b.



1.5 Irradiance response functions for nine individuals obtained over 10 wavelengths in the human-visible spectrum. All responses represent peak *b*-wave amplitudes (in microvolts).






Individual 3
120
110
100
90
80
70
60
50
40
30
20
10
0
Response (microvolts)
4.00
4.50
5.00
5.50
6.00
6.50
Log irradiance (photons s-1cm-2)
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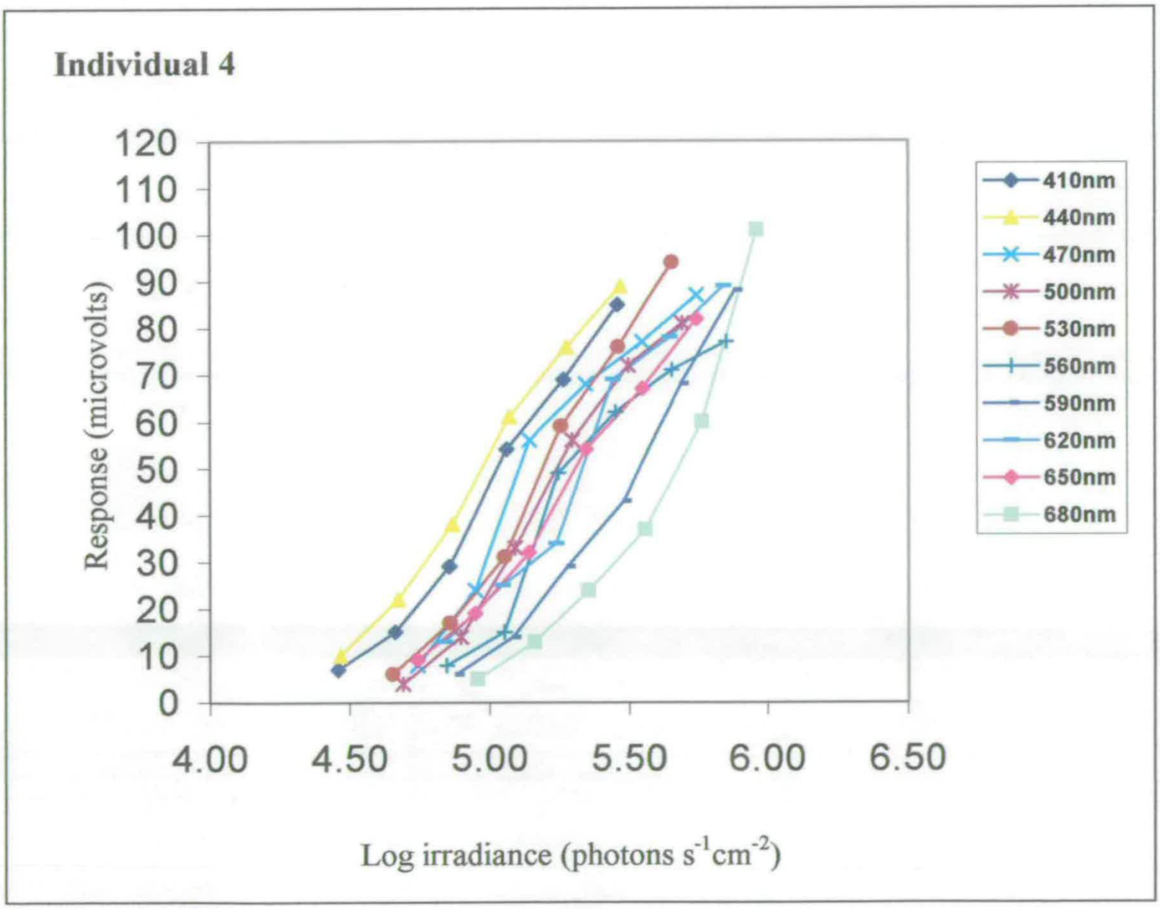
440nm
470nm
500nm
530nm
560nm
590nm
620nm
650nm
680nm


Individual 4
120
110
100
90
80
70
60
50
40
30
20
10
0
Response (microvolts)
4.00
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5.00
5.50
6.00
6.50
Log irradiance (photons s-1cm-2)
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470nm
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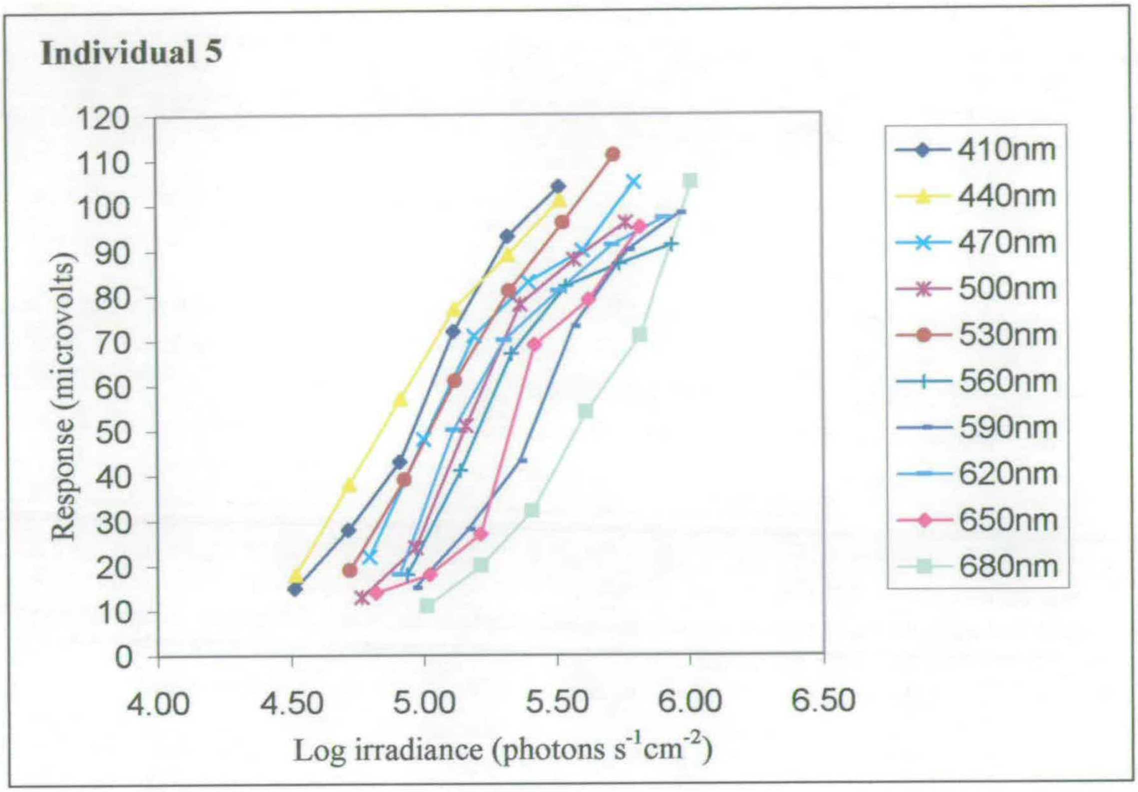
530nm
560nm
590nm
620nm
650nm
680nm


Individual 5
Response (microvolts)
0
10
20
30
40
50
60
70
80
90
100
110
120
4.00
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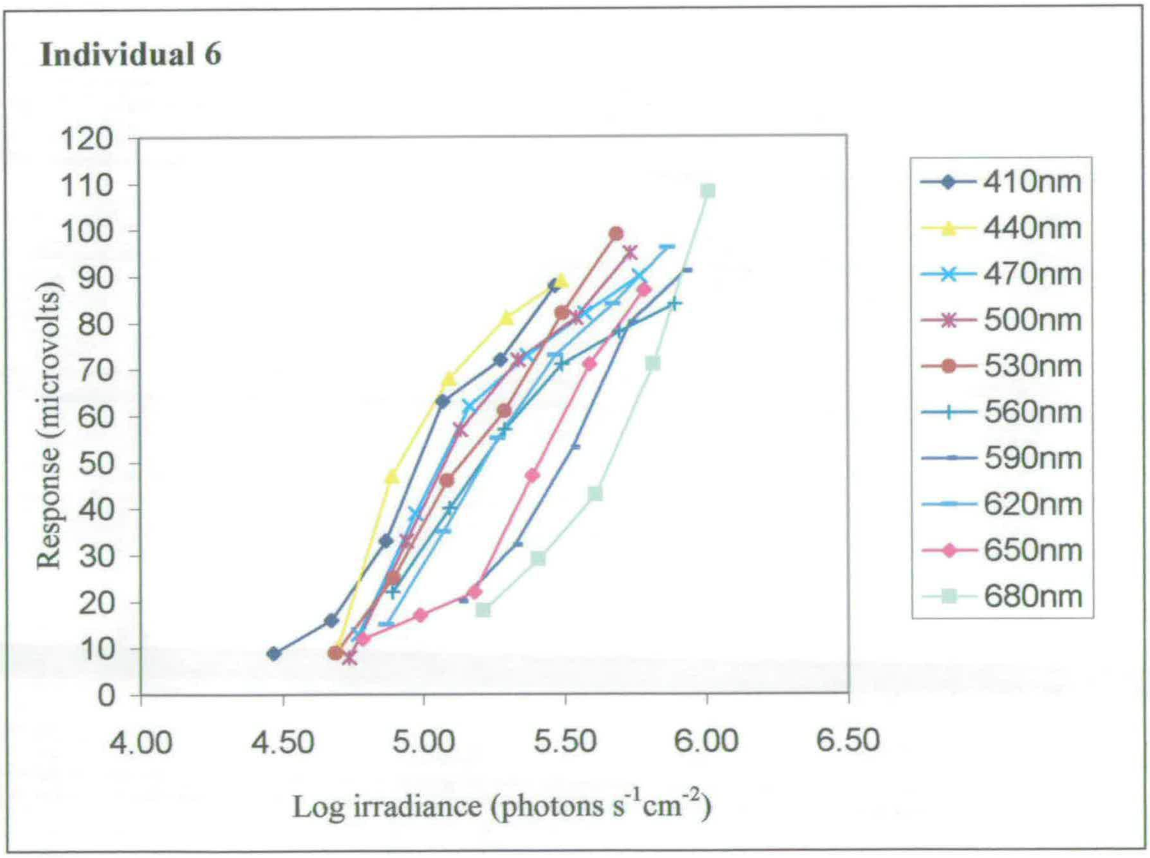
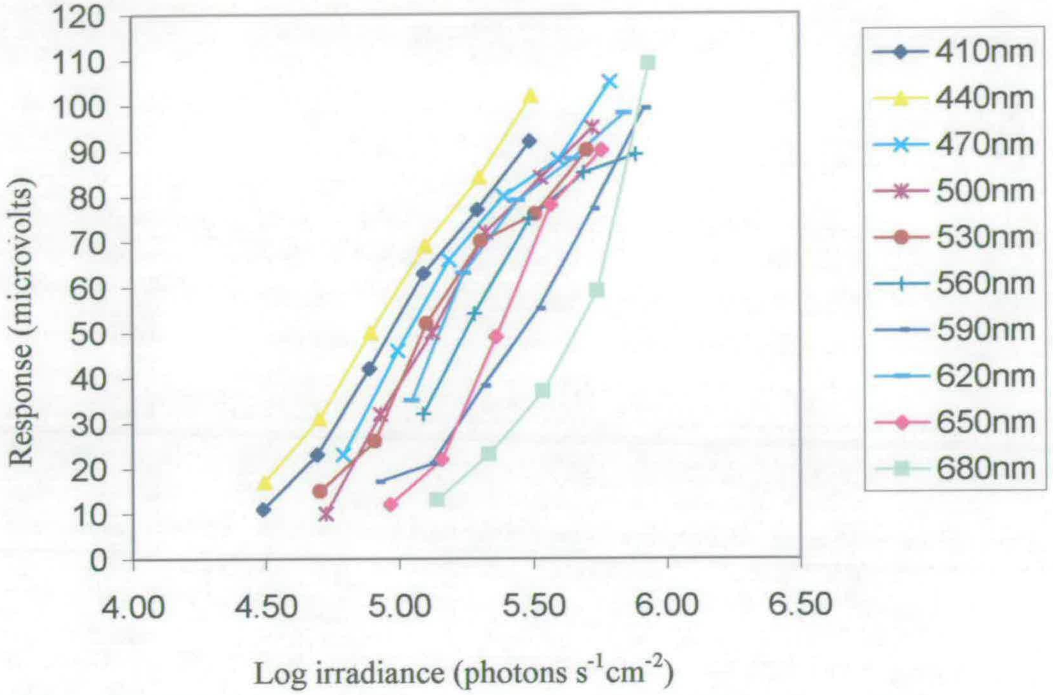
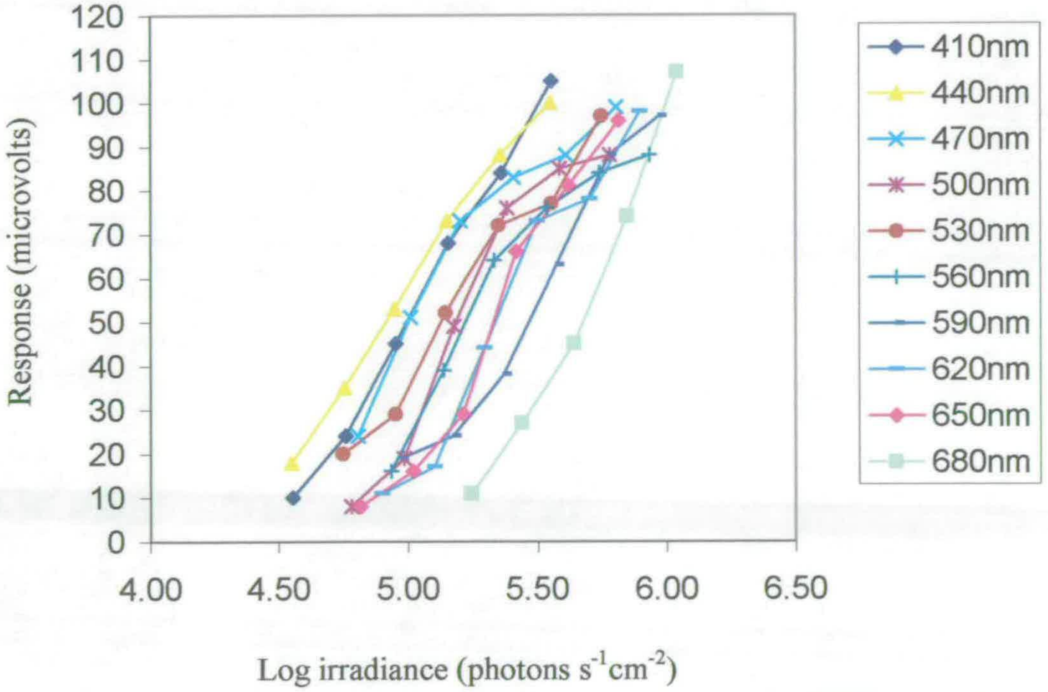
5.00
5.50
6.00
6.50
Log irradiance (photons s-1cm-2)
410nm
440nm
470nm
500nm
530nm
560nm
590nm
620nm
650nm
680nm

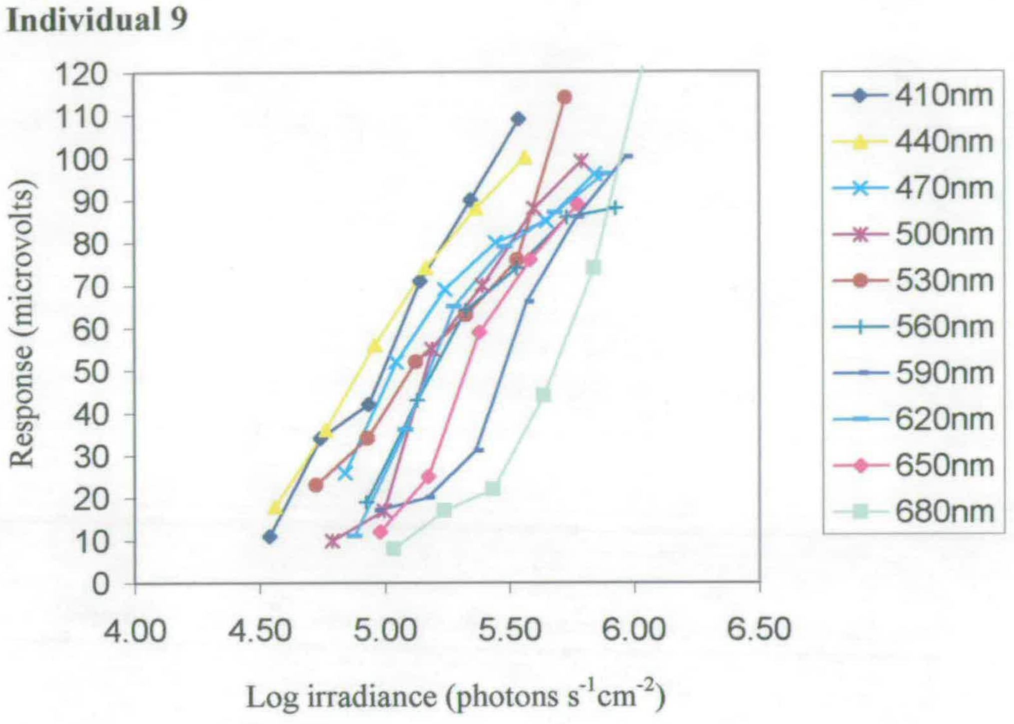

Individual 6
Response (microvolts)
0
10
20
30
40
50
60
70
80
90
100
110
120
4.00
4.50
5.00
5.50
6.00
6.50
Log irradiance (photons s-1cm-2)
410nm
440nm
470nm
500nm
530nm
560nm
590nm
620nm
650nm
680nm

Individual 7



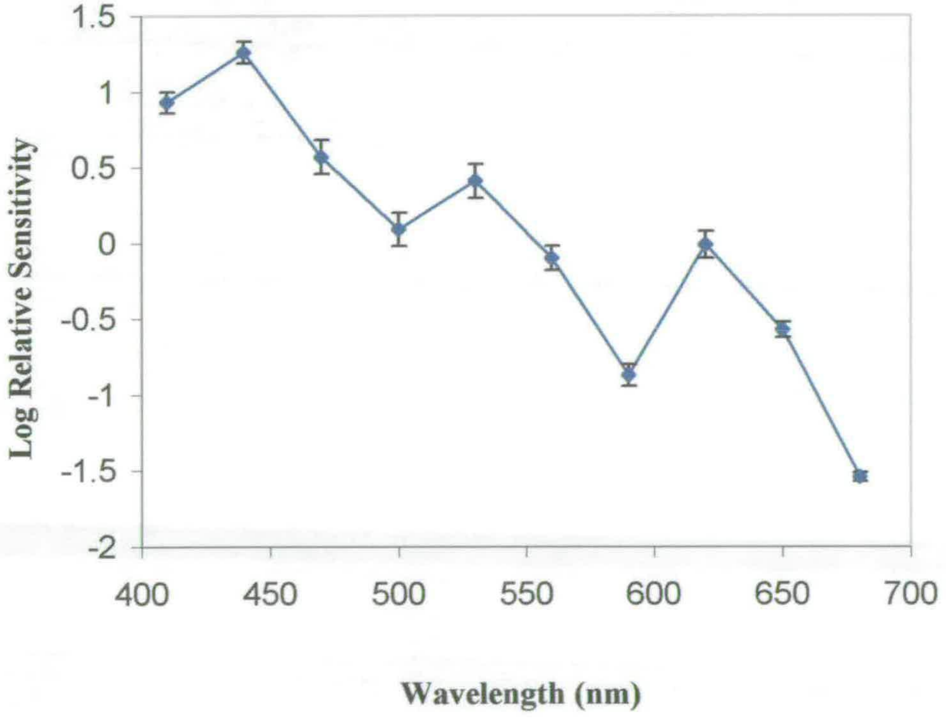
Individual 8





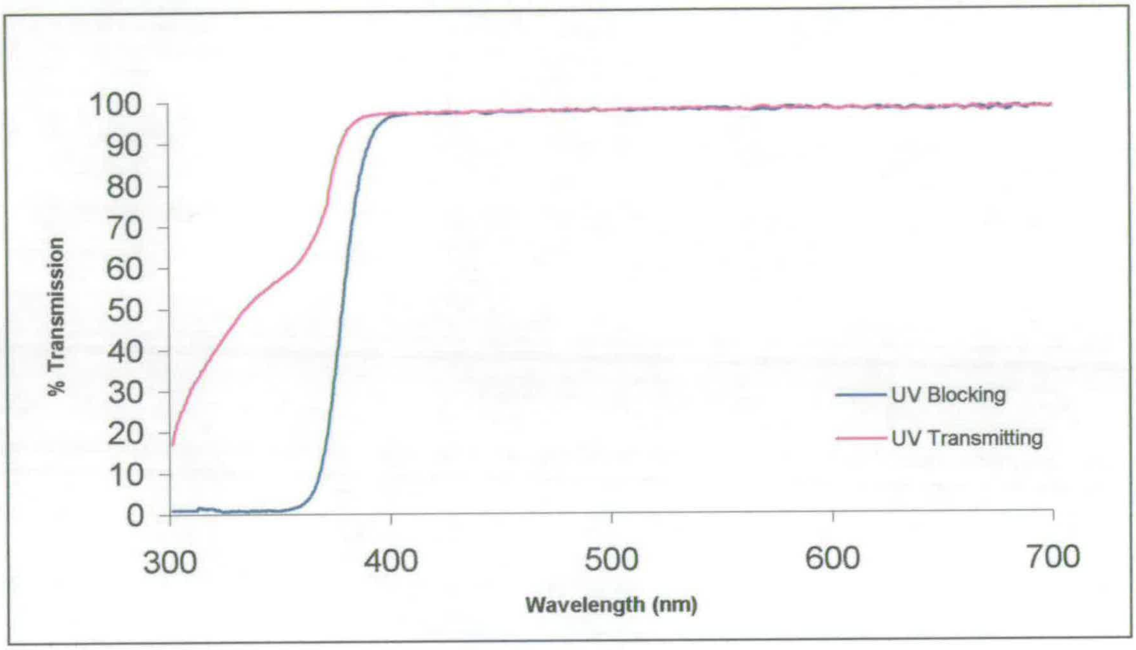
Individual 9

1.6 Spectral Sensitivity of the three-spined stickleback derived using a 40μV response criterion.

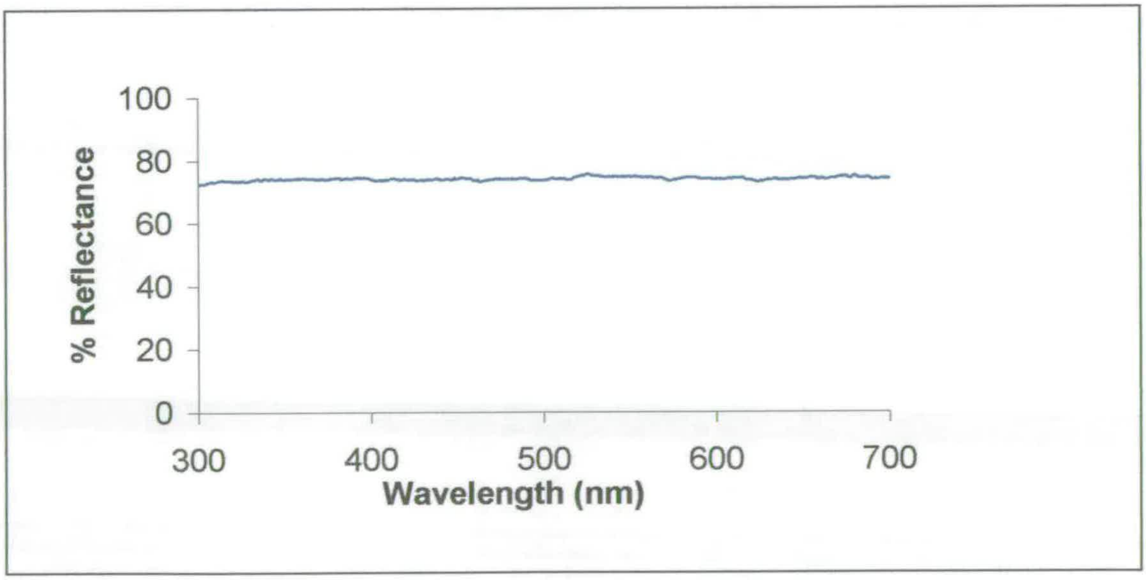


Averaged spectral sensitivity (n=9) of adult three-spine stickleback obtained under broadband background adapting conditions. Error bars represent ±1 standard error of the mean.

1.7 Transmission spectra for Perspex filters (UV transmitting & UV blocking).



1.8 Proportional Reflectance of Aluminium Background



1.9 Foraging performance : GLM Model

Foraging performance during the test trial phase:

Propn. of correct choices = Filter + Holding Tank + Filter*Holding Tank

Foraging performance during the control phase:

Propn. of correct choices = Control Group + Filter + Holding Tank

+ Control Group *Holding Tank + Control Group*Filter + Filter*Holding Tank

+Control Group*Filter*Holding Tank

1.10 Mate Choice in the stickleback: GLM Models

Filter preference:

Propn. of preference Time = Filter + Tank + Trial (Tank) + Filter*Tank

Comparison of Experimental Phases:

Propn. of preference time = Experimental Phase + Tank + Experimental Phase*Tank

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