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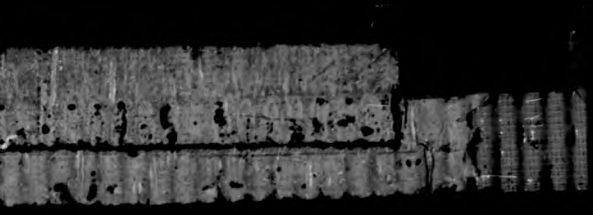

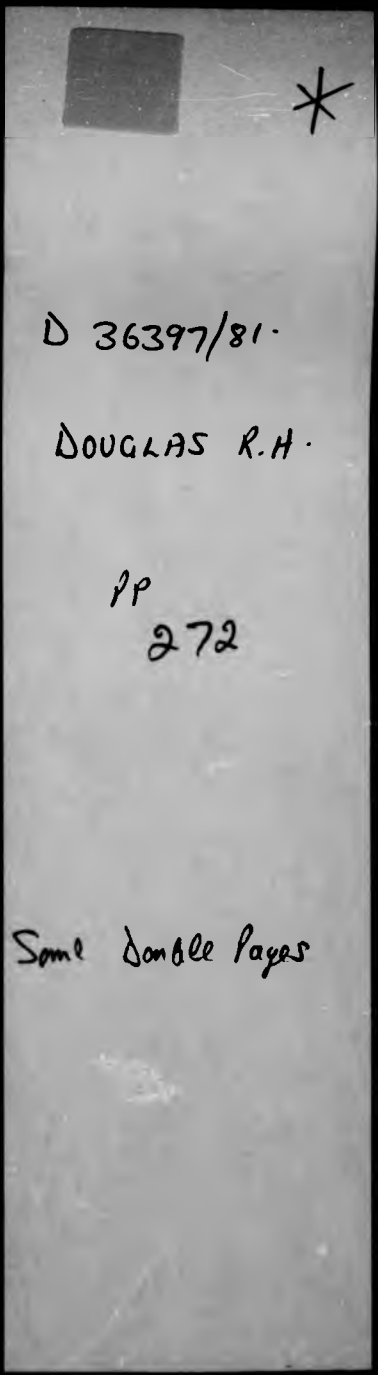

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VISUAL ADAPTATION AND SPECTRAL SENSITIVITY IN RAINBOW TROUT

BY

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I would like to thank sincerely my supervisor, Professor W.R.A. Muntz, for his continual help and encouragement and without whom this study would not have been possible.

Special thanks must also go to Dr L.G Ross for readily supplying much needed technical assistance on numerous occasions.

Finally, I am most grateful to Heather for her unfailing support and great patience during the course of the last three years.

ABSTRACT

The first part of this study is an investigation of the photomechanical movements in the retina of rainbow trout. These movements were followed during natural twilight periods and their rates of light and dark adaptation determined in the laboratory following a sudden change in the adapting illumination. The results are discussed in relation to previous data and form an introduction to the remainder of the study.

The possible existence of an endogenous rhythm of photomechanical movements was subsequently examined in both laboratory and naturally entrained fish. A pattern of retinomotor movements during extended periods of darkness was found that is unique among species so far examined, with peaks of light adaptation coincident with dawn and dusk. It is suggested that such an apparently non-adaptive physiological rhythm is related to trout behavioural patterns and reveals a basic crepuscular controlling mechanism. No endogenous rhythm was observed in constant light.

The function of photomechanical movements was examined in two ways. Firstly, the level of extractable visual pigment was found to remain constant during both dawn and dusk periods, suggesting that part of the function of photomechanical movements is to protect the rod visual pigment. The intensity of bleaching light needed to bleach a criterion amount of visual pigment in the light adapted condition was further found to be thirty-six times as great as in the dark adapted condition, suggesting the pigment epithelium is very effective in its shielding function. Secondly, the close temporal relation observed between sensitivity changes, measured using the ERG b-wave, during both light and dark adaptation and the position

of the retinal elements further suggests that photomechanical movements determine sensitivity. Form changes of the ERG during light and dark adaptation are also briefly discussed.

The second part of this study concerns the spectral sensitivity and response to flicker of the rainbow trout. The scotopic action spectrum, determined using the ERG criterion b-wave technique, agreed well with the absorption spectrum of the extractable visual pigment. The photopic action spectrum, on the other hand, obtained using both the ERG and a behavioural appetitive training technique, was observed to be more complex, showing three distinct maxima. The position of these maxima and general shape of the photopic spectral sensitivity differed depending on the technique used. It is suggested that ERG photopic action spectra are largely determined by inhibitory interactions between cones, while the behavioural spectral sensitivity curve is best explained by independent receptor interaction at most wavelengths, with some inhibition between the red and green receptors.

CONTENTS

INTRODUCTION

This study has two main aims, one relevant to teleosts as a whole, the other more specific to the species studied. The first section (chapters 1 - 4) concerns the photomechanical changes that are known to occcur in most teleost retinas. Although these movements of the retinal epithelial pigment and visual receptors were first described nearly a century ago (1.1), they have been little investigated in recent times and certain aspects of their function are, as yet, unclear. Up to date, for instance, there have been few controlled experiments concerning their adaptive significance, and their exact function has not been determined adequately. Furthermore, exactly how these movements are controlled is uncertain. There have been several reports of photomechanical changes continuing during extended periods of darkness (2.1), indicating some form of central control, but there have also been descriptions of these movements failing to occur in such conditions, which may be indicative of local control. Thus after a general introduction to retinomotor movements and a discussion of some of the problems involved in their study (chapter 1), their endogenous rhythms (chapter 2) and possible function (chapter 3 & 4) are examined with reference to results obtained for rainbow trout.

The second part of the study specifically concerns the rainbow trout. Perhaps surprisingly, very little is known about the visual capabilities of this species, despite the fact that it is both readily available and commercially important, and is thought to be primarily a visual feeder (eg; Adron et al 1973). In an attempt to fill this gap, the study of adaptation in this species, of which the study of photomechanical movements forms a part, was extended to an electroretinographic investigation of its spectral sensitivity and response to flicker, in both

the light and dark adapted condition (chapter 5). As a comparison to this, and as a further step toward understanding the visual mechanisms underlying the spectral response, the sensitivity of the rainbow trout to different wavelengths was also determined by a behavioural two choice method (chapter 6). This forms a useful basis for comparison to other teleosts.

CHAPTER 1. RETINOMOTOR MOVEMENTS - A GENERAL INTRODUCTION

1.1. INTRODUCTION

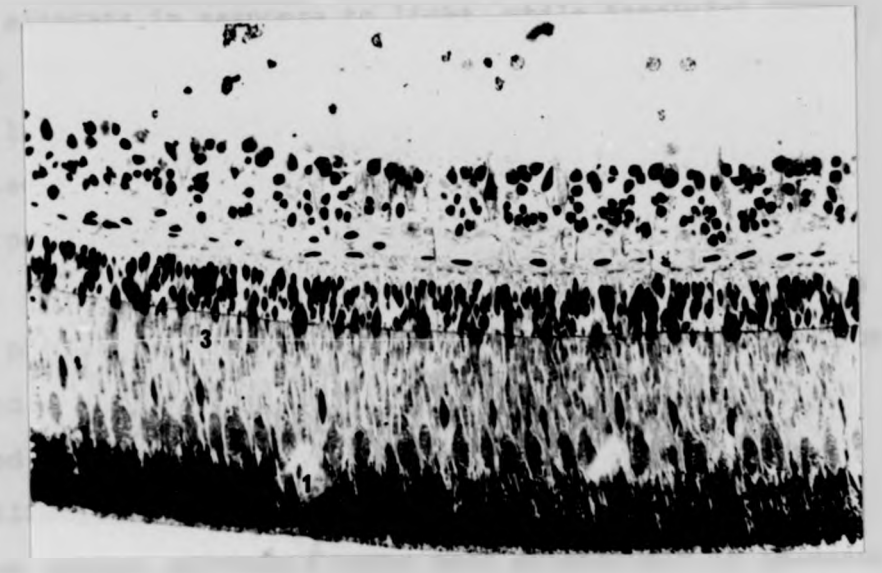
Retinomotor (photomechanical) responses are the movements of the retinal epithelial pigment and the visual receptors in response to ambient lighting conditions. In the dark adapted state, the epithelial pigment aggregates at the back of the eye, the cones expand to take up a position near the lamina basalis in close proximity to the pigment epithelium, while the rods are contracted near the external limiting membrane (e.l.m.) (fig 1.1a, the rods are not visible due to their small size). In response to light the cones contract to take up a position near the e.l.m. completely surrounded by expanded epithelial pigment. The rods, which exchange places with the cones, are buried in the pigment epithelium near the lamina basalis (fig 1.1b).

Individual variations in the positions of the pigment epithelium were noted as early as 1856 by Müller and again by Morano (1872). Czerny (1867) stated that the visual cell layers of light adapted fish were more difficult to separate from the pigment epithelium than those that were dark adapted. He suggested that this might be due to the migration of the epithelial pigment between the receptors in the light. Yet it was not until 1877 that Kühne and Boll independently discovered that light caused the pigment to expand and that darkness caused its aggregation. Englemann (1885) and his student van Genderen Stort (1886 & 1887) were the first to show that the cones also moved in response to light, and that they did so in the same direction as the epithelial pigment. This observation, first made on frogs, was later confirmed in other vertebrate classes including birds, fishes and amphibians.

The situation concerning rod movement was more uncertain

Fig 1.1. Transverse sections of (a) dark adapted and (b) light adapted rainbow trout retinas.

(a)



X 312

(b)

1 - pigment epithelium

2 - cone ellipsoid

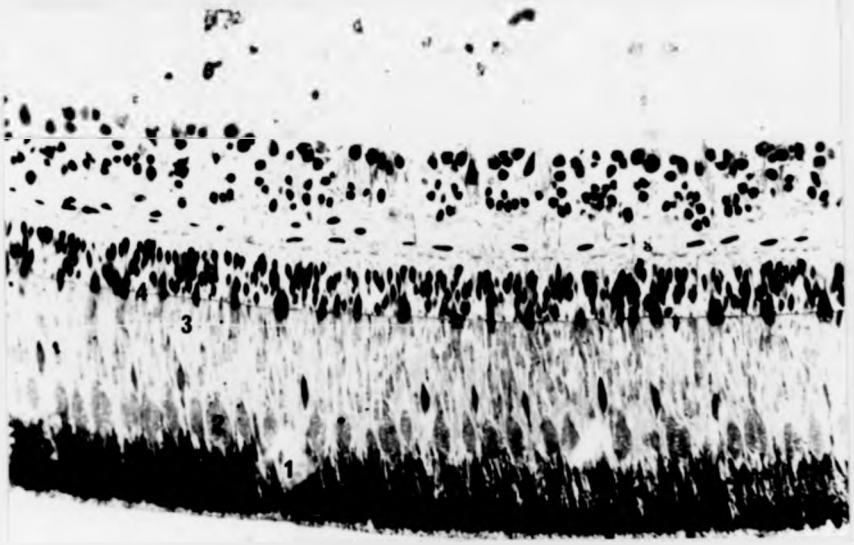
3 - position of rods (not visible)

4 - e.l.m.

X 764

Fig 1.1. Transverse sections of (a) dark adapted and (b) light adapted rainbow trout retinas.

(a)



X 312

(b)

1 - pigment epithelium

2 - cone ellipsoid

3 - position of rods (not visible)

4 - e.l.m.

X 764

for many years. Van Genderen Stort (1886), Garten (1907) and Exner & Januschke (1906), using different species, showed that rods elongate in response to light, while Angelucci (1884), Gradenigro (1885) and Arcoleo (1890) all observed the opposite effect of light in the frog and toad. Lederer (1908) and Hess (1910) tried to resolve the situation in the frog, and showed that the rods, contrary to earlier opinion, also elongated in the light, and it is now generally accepted that in all species that show photomechanical movements, light causes the elongation of rods and dark their contraction. This earlier literature is reviewed in greater depth by Garten (1907), Arey (1915) and Detwiler (1943).

These retinal movements occur most extensively in teleosts and anurans, though they also occur, to varying degrees, in urodles, reptiles and in both nocturnal and diurnal birds. Movements of retinal elements have never been conclusively shown in mammals, although Arey (1915) presents some limited evidence showing that they may occur. Within fish they are almost entirely restricted to the teleosts (Ali & Wagner 1975, for a comprehensive list), although they have been found to a lesser extent in Amia, a holostean (Ali & Anctil 1974). They do not occur in deep - sea teleosts (Ali & Hanyu 1974 and personal observation) or in Dipnoi and Lepidosireniform Brachiopterygii (Pfeiffer 1968). Elasmobranchs are also thought to lack retinomotor movements, and although many species of chondricthian fish do display choroidal pigment movements associated with occlusable tapeta (eg; Denton & Nicol 1964), in the present context these are not considered as retinomotor movements as the epithelial pigment is not involved. It could however be argued that such movements and movements of pigment involved in occlusable corneas of certain fish (eg; Appleby & Muntz 1979), should be included in the general category of photo-

mechanical phenomena, as they are mechanical movements in response to light. In fact, Appleby & Muntz (1979) point out the many similarities that do exist between tapetal, corneal and epithelial pigment migration.

Since the initial intensive study of photomechanical movements, around the turn of the century, little work has been done on the subject. The most extensive recent studies have been those of Ali and his co-workers, but several questions are still left unanswered. Two of these, regarding the function and endogenous rhythms of these movements, will be examined in subsequent chapters. The present chapter is a general introducation to retinomotor movements, using results obtained with the rainbow trout, Salmo gairdneri, a species never before studied in this respect. The speed of these movements in both the laboratory and nature, as well as their thresholds in nature, will be compared to, and interpreted with the help of, published results on other species. Through these results, certain general problems involved in the study of retinal movements will be exemplified and anomalies in the literature discussed. This will then serve as a foundation for the work described in subsequent chapters.

1.2. GENERAL METHODS

The following description of experimental methods applies to all the subsequent work on retinomotor movements reported in this thesis. All fish used were obtained from Howietoun and Northern fisheries, Bannockburn. Sizes used differed depending on the experiment, ranging from 18 cm in electroretinographic recordings to as small as 8 cm in some rhythm experiments.

1.2.1. Dissection and fixation. All dissection and fixation was carried out in dim red light. Fish were removed from the experimental situation and immediately killed by a sharp blow on the head. The eyes were removed from the orbit and the complete eye, punctured at the corneal/scleral junction to facilitate penetration, immersed in Bouin's fixative for at least twenty-four hours. Following fixation the eye was removed under normal lighting conditions, hemisected, and a square of retinal/scleral tissue removed from around the base of the optic nerve (fig 1.2a). This ensured that a similar area of the eye was always sampled from different individuals, which is of importance as different parts of the retina may show differing degrees of migration or pigmentation (eg: Hess 1910, Fujita 1911, Wunder 1925 and Kobayashi 1957), although on superficial examination this did not seem to be the case here.

Subsequently, the tissue was washed in distilled water, dehydrated in D.M.P. (acidified 2.2 dimethoxypropane, Muller & Jacks 1975) and embedded in Emix resin. 2μ- 5μsections were then cut on an LKB pyramitome and stained with 1% Toluidine blue in a 1% Borax solution.

1.2.2. Measurement. One good section was obtained from anywhere within the above mentioned area and examined under a projection light microscope. Distances could then be measured using a ruler, a method more accurate and less tiring than the use of a graticule. At least two sites were sampled, one either side of the optic nerve, with at least five cone measurements and one pigment measurement being made at each site. More readings were made in eyes that were dark adapted[1] or in an intermediate

1 Throughout the following text, the terms light and dark adapted refer only to the photomechanical state of the retina and make no implication as to the other,non-photomechanical, process involved in adaptation.

Fig 1.2. Transverse sections of rainbow trout retinas.

(a) X70 , showing the area around the optic nerve (l) from which measurements were taken in order to determine retinal cone and pigment indices.

(b) X118 , showing variation of epithelial pigment thickness as the retina is mechanically stretched.

(c) X320 , showing variation in epithelial pigment thickness depending on if it is attatched to the choroid.

(d) X315 , showing distortion of the retina near the optic nerve.

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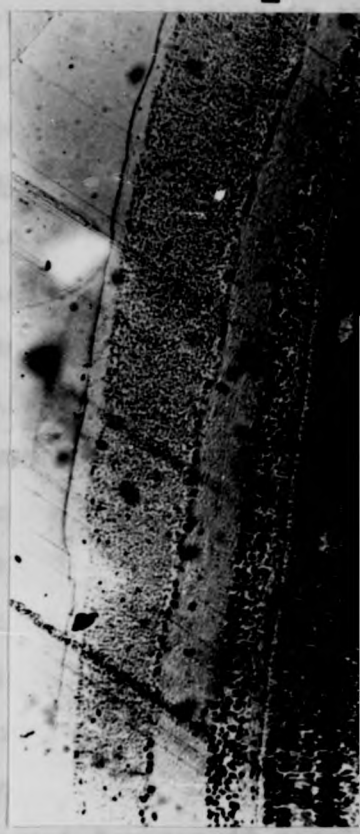

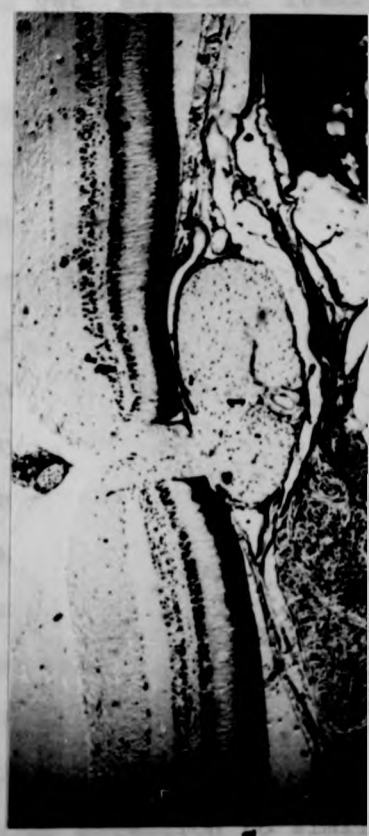

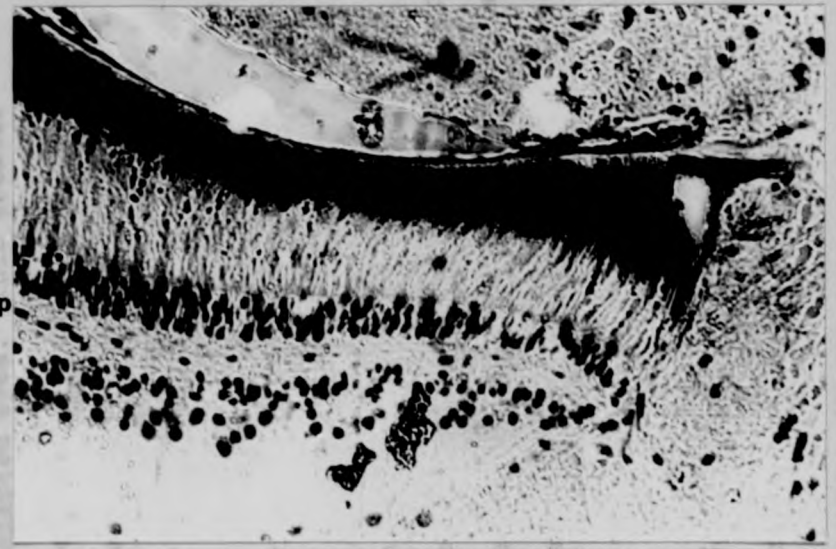
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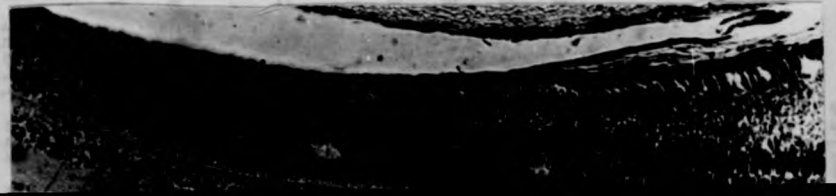
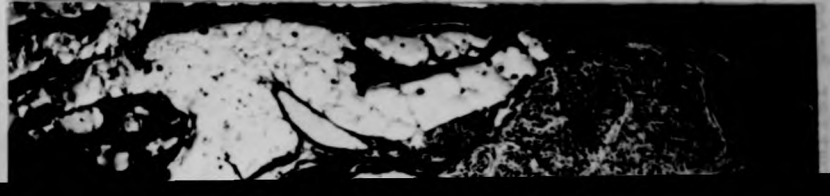




state of adaptation, as cone positions in such sections are more variable than in the light adapted eyes. This greater intra-individual variation of cone position in intermediate states was also noted by Engström & Rosstrop (1963) in the roach, Leucisus rutilus. To compensate for variation between individuals also noted by the above authors, wherever possible at least three fish were sampled for each experimental condition (see individual sections for specific details). Different cone types were not distinguished. By sampling from a large number of fish, and from several locations within each section, differences in retinomotor movements of different cone types that may be present (eg: Walls 1942, Muller 1954,Engström & Rosstrop 1963, Nicol 1965, John et al 1967 and Olla & Marchioni 1968) will be averaged out. All measurements were made with the experimenter ignorant of the exact lighting conditions the eye being measured had been exposed to, thus controlling for any bias in an observer trying to get "expected" results.

Some sections sustained mechanical damage during fixation, which resulted in their being discarded. In the light adapted state the epithelial pigment interdigitates with the cones, thus anchoring them together, but in the dark adapted condition and in intermediate states this is not always the case, resulting in frequent tearing of the pigment epithelium away from the receptors. Although tearing was sometimes seen in light adapted eyes, it was much less frequent. Sometimes tearing was not complete and the pigment epithelium was only stretched. An extreme case of this is shown in fig 1.2b, and a less severe case in fig 1.2c, where the epithelial pigment changed thickness depending on if it was attached to the choroid or not. Therefore care was taken in examining sections to avoid stretched areas, but some mistakes were inevitable. John & Kaminester (1969) drew attention to similar problems and attributed errors on the

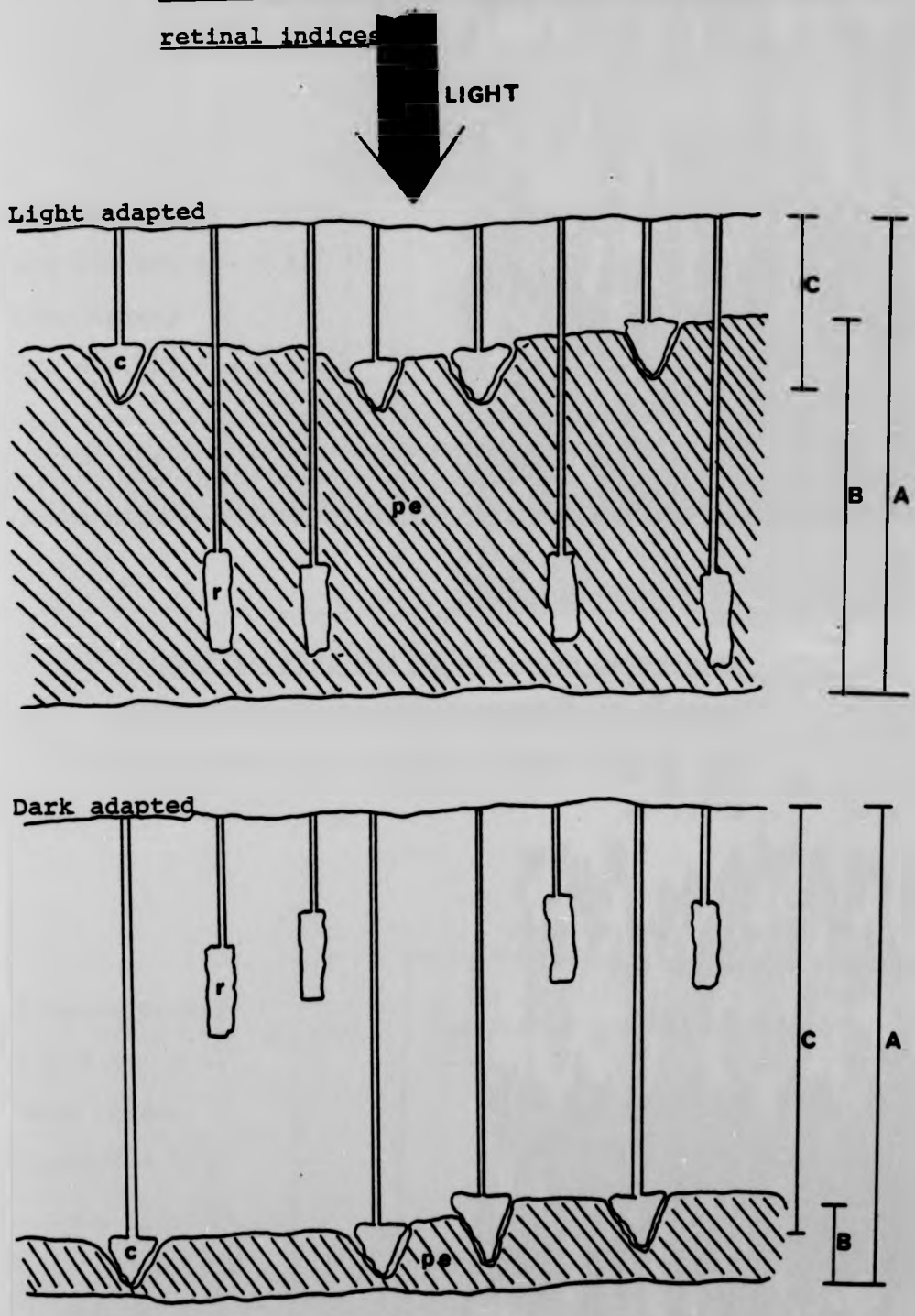
order of 5% to imperfect histological sections. Also measurements were never made in the immediate proximity of the optic nerve, as here receptors and pigment are in abnormal configurations (fig 1.2d). A further source of possible error in measuring the thickness of the epithelial pigment is that it does not advance or withdraw uniformly. The pigment contained in different epithelial processes may migrate to different extents. A subjective determination as to the average extent of migration must therefore be made, which will inevitably lead to some degree of error.

1.2.3 Indices. The state of adaptation of the eye was expressed using both cone and pigment indices. No measurements on rods were made as they could not be distinguished by the present histological procedure. The cone index is taken as the distance between the e.l.m. and the base of the cone ellipsoid (C), divided by the distance between the e.l.m. and the basement membrane (A). Similarly, the pigment index is the distance between the basement membrane and the outermost projection of the pigment (B) divided by A (fig 1.3).

These indices have been extensively used in almost all recent studies on retinomotor movements. Their use is preferred over direct measurement of cone and pigment layers, as they account for oblique sectioning, which ordinary measurement does not. They are also more informative and accurate than the arbitrary categories of adaptation used by earlier authors. However, there are certain problems involved in their use.

The main problem involved in the use of these indices, is that different distributions of epithelial pigment can give the same index value. Fig 1.4 shows two eyes, both obviously in different states of adaptation, yet they both have virtually the same pigment index. In fig 1.4a the pigment is not yet fully expanded as a lot of the pigment is still aggregated at the back of the eye, although strands of pigment have already migrated

Fig I.3. Diagrammatic representation of light and dark adapted retinas showing the parameters measured to obtain retinal indices

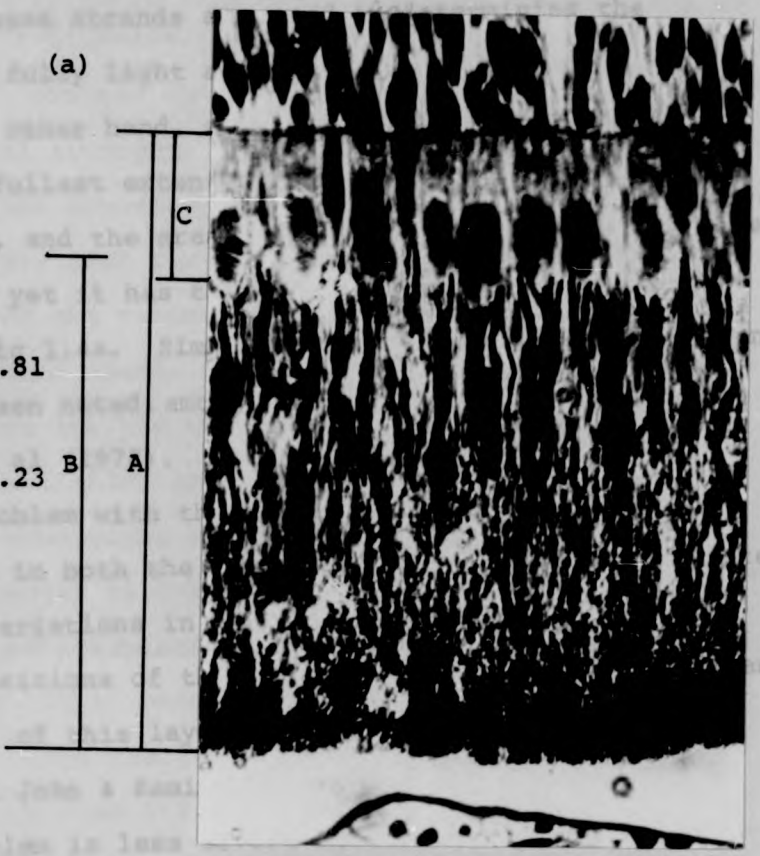


p e - pigment epithelium, r - rods, c - cones

Pigment index (P.I.) = B/A, Cone index = C/A

Fig 1.4. Transverse sections of two rainbow trout retinas in different states of adaptation, yet showing similar pigment and cone indices. X778.

(a)



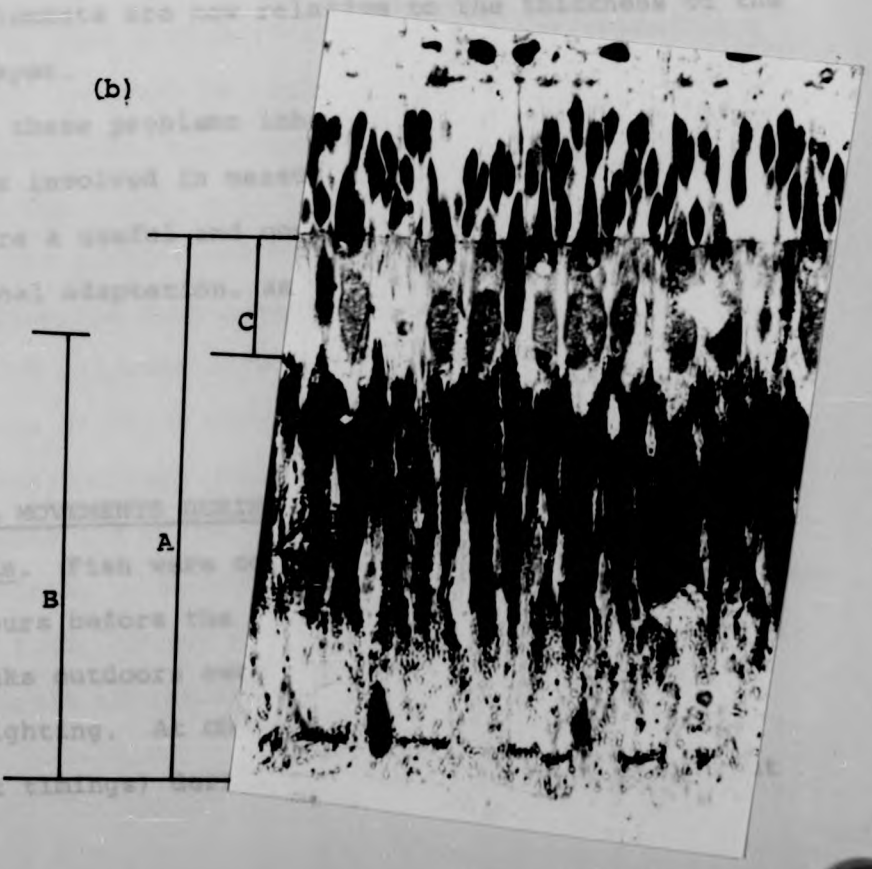
Pigment index;

B/A = 7.9/9.8 = 0.81

Cone index;

C/A = 2.3/9.8 = 0.23

(b)



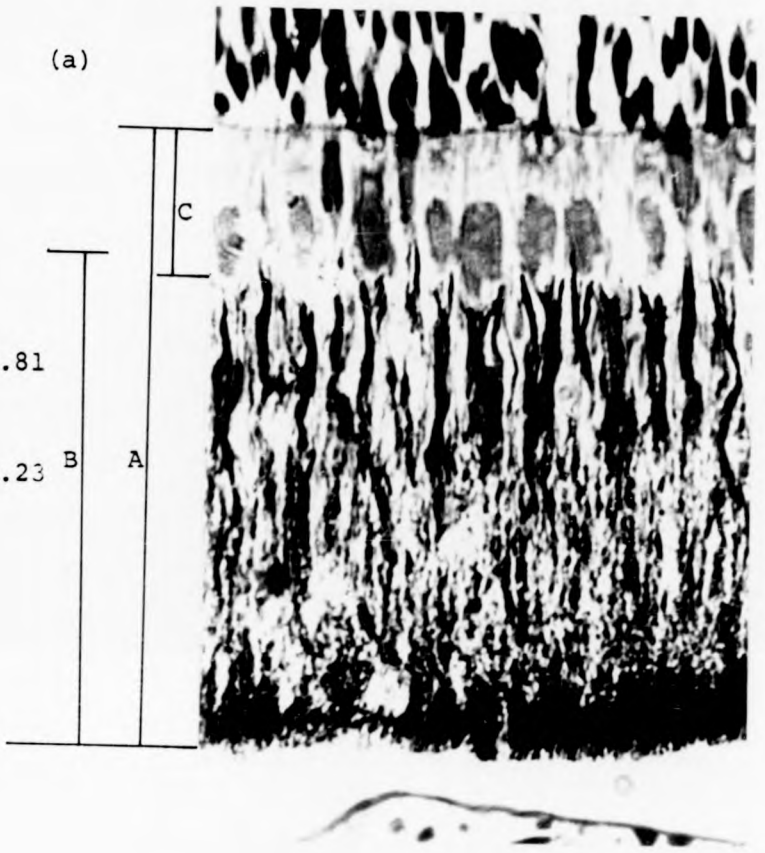
Pigment index;

6.9/8.5 = 0.81

Cone index;

1.8/8.5 = 0.21

(a)



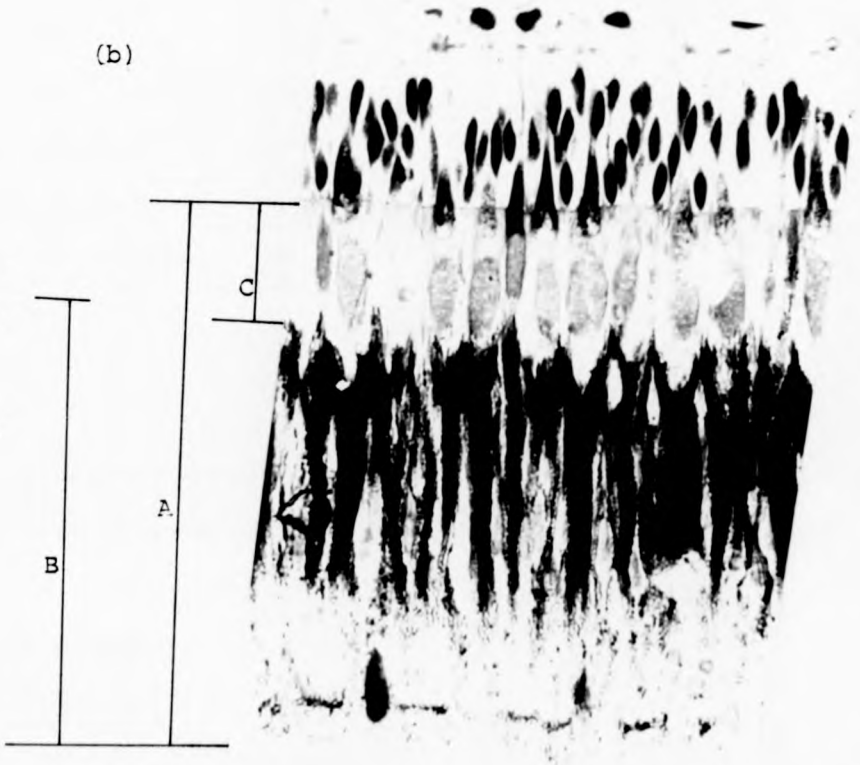
Pigment index;

B/A = 7.9/9.8 = 0.81

Cone index;

C/A = 2.3/9.8 = 0.23

(b)



Pigment index;

6.9/8.5 = 0.81

Cone index;

1.8/8.5 = 0.21

maximally. As these strands are used in determining the pigment index, a fully light adapted value will be obtained. Fig 1.4b, on the other hand, shows the epithelial pigment expanded to its fullest extent, with most of the pigment around the cones, and the area near the lamina basalis completely free of pigment, yet it has the same pigment index as the eye illustrated in fig 1.4a. Similar patterns of epithelial pigment migration have been noted, among others, by von Studnitz (1933) and McFarland et al (1978).

Another problem with these indices is that they give false variations in both the fully light and dark adapted state. This is due to variations in the thickness of the visual cell layer, as the positions of the cones and pigment are independent of the thickness of this layer in these adaptive states (John & Gring 1968 and John & Kaminester 1969). In intermediate states this problem is less severe because the positions of the retinal elements are now relative to the thickness of the visual cell layer.

Despite these problems inherent in the use of indices and the errors involved in measuring, they yield consistent results and are a useful and convenient indication of the state of retinal adaptation, as long as one is aware of their limitations.

1.3. RETINAL MOVEMENTS DURING NATURAL TWILIGHT PERIODS

1.3.1. Methods. Fish were collected from the fish farm at least four hours before the first animals were sampled, and placed in tanks outdoors away from the influence of any artificial lighting. At chosen intervals (see figs 1.5 & 1.6 for exact timings) during the rise and fall of the light

intensity three fish were killed, and one eye immediately immersed in Bouin's fixative. The other was placed in a polypropylene bottle, wrapped in tin foil to keep out the light, and submerged in liquid nitrogen. The extractable visual pigment was removed from the frozen eyes at a later date (chapter 3), while the former eyes were used for the histological determination of the positions of the retinal elements. Three dusk periods were examined in this manner.

The procedure for the one dawn followed was exactly the same. Fish were collected in the afternoon and kept under natural lighting conditions until the following dawn, at which time fish were sampled as during dusk.

The levels of illumination at the times of sampling were obtained from U.S. Naval Natural Illumination charts (1952).

1.3.2. Results. The results for the three dusk and one dawn period are shown in figs 1.5 & 1.6. Each point represents the average index value of several fish (see legends for details).

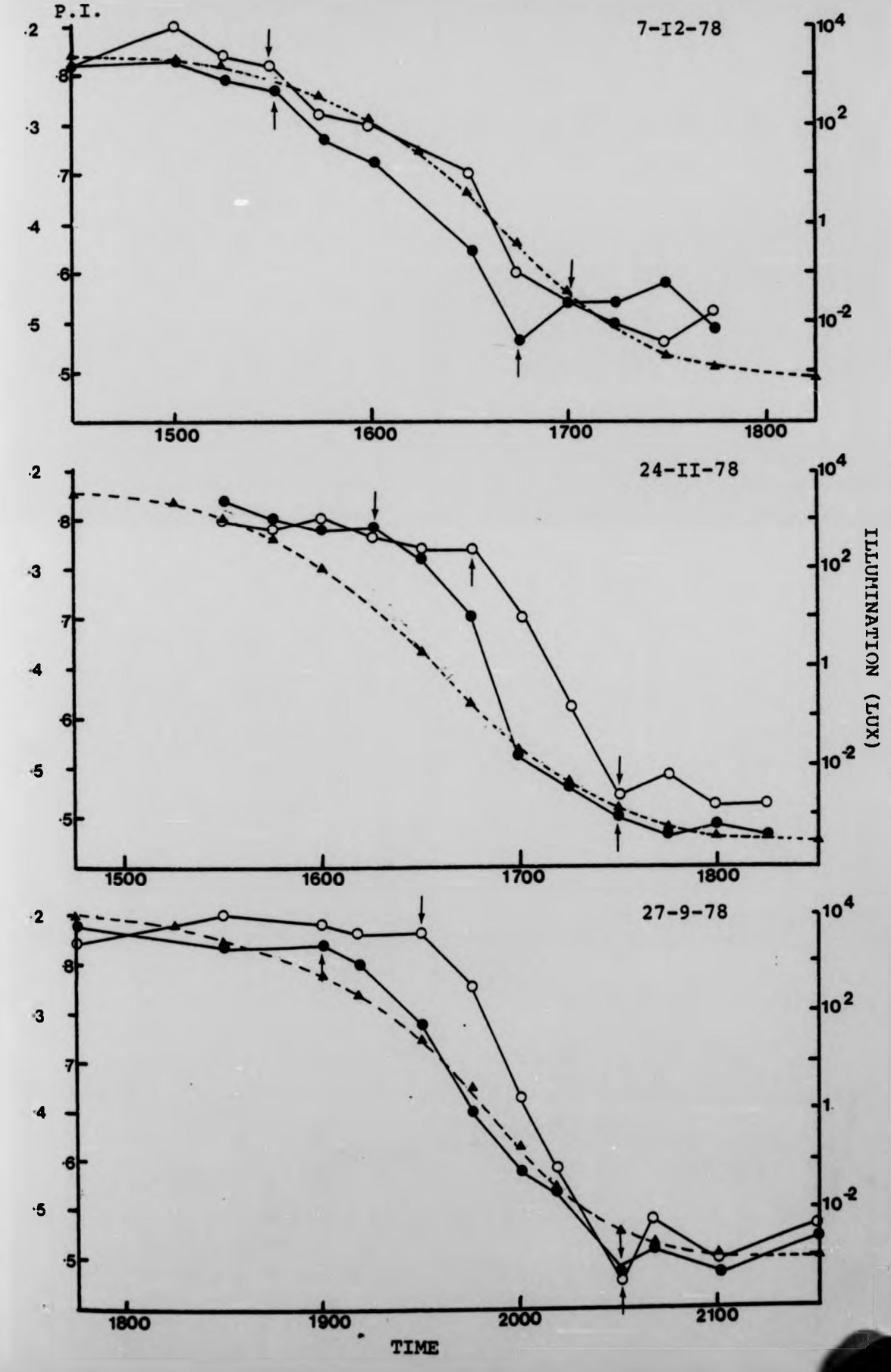
During one dusk period (7-12-78), both the pigment and the cones follow the fall in light intensity together, whilst in the other two cases, the pigment starts to migrate half an hour before the cones, although the cones do eventually catch up with the pigment and both finish dark adaptation together. During the dawn period there is an indication that the cones may be adapting slightly ahead of the pigment.

The points at which migration of the various retinal elements are subjectively judged to begin and end are marked by arrows in figs 1.5 & 1.6. The duration of these migrations and the intensities at which they start and finish are shown in table 1.I and 1.II. These results can only be approximations, as it is difficult to determine objectively exactly when

Fig 1.5. Retinal pigment (●) and cone (○) indices at various times during three dusk periods.

Each point is the average index value from three fish.

(- - -) represents the level of illumination.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P.I.
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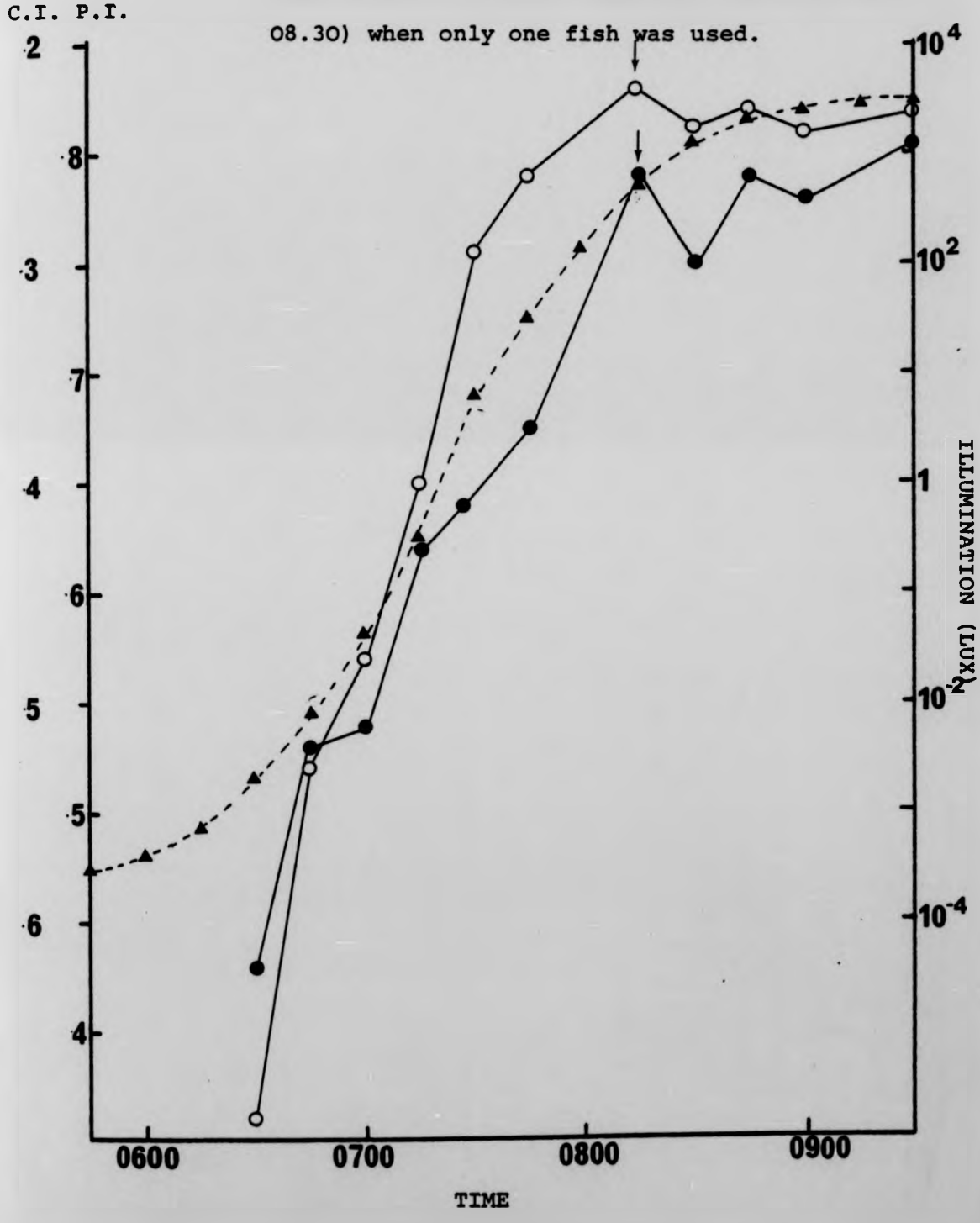
24-II-78
27-9-78
1500
1600
1700
1800
1900
2000
2100
TIME
ILLUMINATION (LUX)

Fig I.6. Retinal pigment (●) and cone (○) indices at various times during dawn (8 - I2 - 78).

Each point is the average index value from two fish, except for three occasions (06.45, 08.I5 & 08.30) when only one fish was used.



(- - -) represents the level of illumination.

Table 1.I. Time taken for retinal cones and epithelial pigment to dark adapt during three dusk periods.

DATE	CONES		PIGMENT	
	TIME	DURATION (mins)	TIME	DURATION (mins)
7-12-78	15.30 - 17.00	90	15.30 - 16.45	75
24-11-78	16.45 - 17.30	45	16.15 - 17.30	75
27-9-78	19.30 - 20.30	60	19.00 - 20.30	90

Table 1.II Level of illumination at which photomechanical movements begin and end during three dusk periods and one dawn.

PIGMENT EPITHELIUM

DATE	START OF MIGRATION (lux)	END OF MIGRATION (lux)
Dusk 7-12-78	10^3	6×10^{-1}
Dusk 24-11-78	40	2×10^{-3}
Dusk 27-9-78	8×10^2	8×10^{-3}
Dawn 8-12-78	-	6×10^2

CONES

Dusk 7-12-78	10^3	6×10^{-2}
Dusk 24-11-78	3×10^{-1}	2×10^{-3}
Dusk 27-9-78	50	8×10^{-3}
Dawn 8-12-78	-	6×10^2

migration begins and ends. No threshold intensity for the start of dawn movement is given as it seems migration began immediately at, or before, the onset of the experiment. Dawn pigment and cone movements are complete at an intensity (6×10^2 lux) approximately equal to that at which dusk pigment movements begin (4×10^1 - 10^3 lux). The large variation of the threshold intensities for the start of dusk cone migration prevents any general comparisons.

1.4. TIME TO LIGHT AND DARK ADAPT IN THE LABORATORY

1.4.1. Methods. To determine the time taken to light adapt, forty -three fish were dark adapted overnight (15 hours) in the laboratory. Then five fish, at least one from each of the tanks used for the dark adaptation, were killed in the dark to give a dark adapted baseline value (time 0) and fish sampled at various intervals after this, following the switching on of the tank lights (650 lux). As killing and fixation take a finite time, expecially if more than one fish is involved in a sample, and as fish were sampled frequently over the first ten minutes of adaptation, two experiments were carried out with staggered sampling times.

Conversely, thirty fish were light adapted for five and a half hours (650 lux) and two fish sampled at the end of this time (time 0) to act as a light adapted baseline. Fish were then sampled at intervals after switching the lights off.

Precise details as to how many fish were killed at each time are given in the legends of figs 1.7 & 1.8. The water temperature in both experiments was 16 - 17°C.

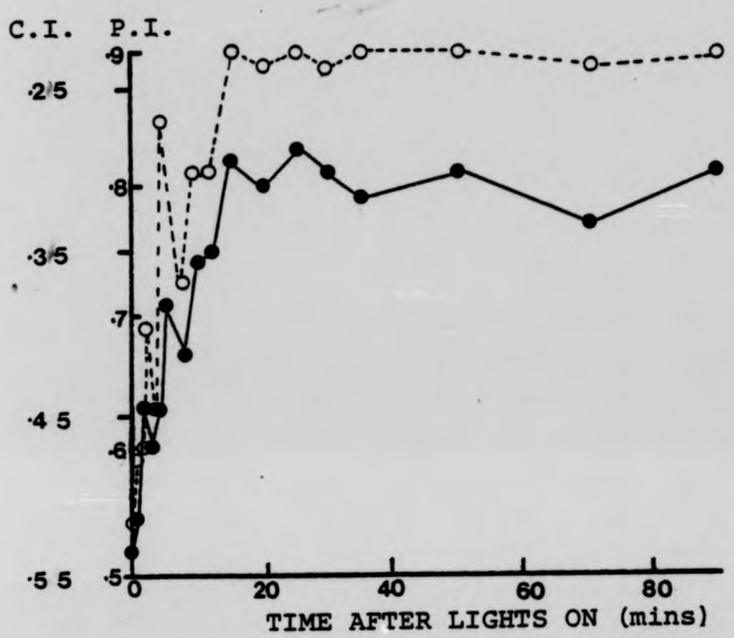
1.4.2 Results. Typical light adapted indices found in nature are approximately 0.8 for the pigment and 0.25 for the cones,

Fig 1.7. Retinal pigment (—) and cone (---) indices at various times after the lights were turned on in the laboratory.

Each point at 1,2,3,4,8 & 12 minutes is the index value of one fish,points at 15,20,25,30,40,50,60 & 90 are the average index value of three fish and points at 5 & 10 are the average index value of four fish.

Fig 1.8. Retinal pigment (—) and cone (---) indices at various times after the lights were turned off in the laboratory.

Each point is the average index value of two fish.


C.I.
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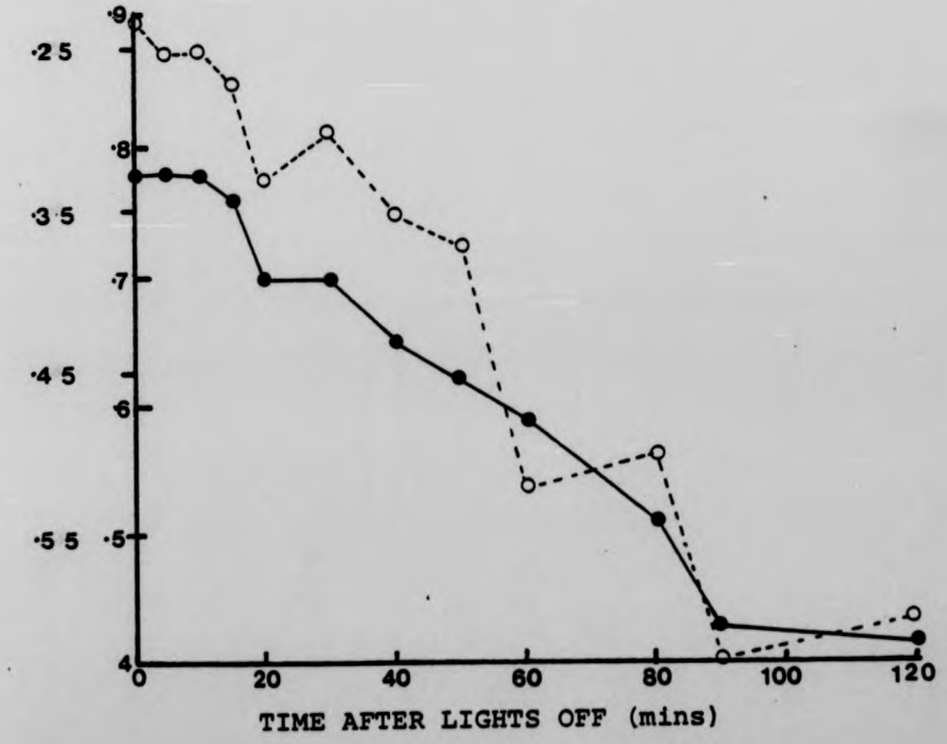
·55
·5
0
20
40
60
80
TIME AFTER LIGHTS ON (mins)


·9
·25
·8
·35
·7
·45
·6
·55
·5
4
0
20
40
60
80
100
120
TIME AFTER LIGHTS OFF (mins)

with corresponding dark adapted values of 0.55 and 0.5 (1.3.2). In the laboratory both the pigment and the cones reach a steady state at these light adapted values after only fifteen minutes adaptation (fig 1.7). Dark adaptation, on the other hand, takes very much longer (fig 1.8). Both the pigment and cones reached a steady level ninety minutes after the lights were extinguished, but this level is at indices of 0.43 (P.I.) and 0.62 (C.I.), which are more dark adapted than the natural values obtained in section 1.3.2.

There is no evidence of cones and pigment moving at different rates, or of one starting migration before the other. Light adaptation starts immediately, whereas there is a delay of ten to fifteen minutes before the beginning of dark adaptation.

1.5. DISCUSSION.

1.5.1. Variability of the photomechanical response. From the results presented above it can be seen that rainbow trout have retinomotor movements that are comparable to those of most other teleosts. Yet a detailed comparison of the rates and thresholds of these movements to those obtained in previous studies on various species is not a simple matter, since they are influenced by a great many factors. Temperature, for instance, has long been known to affect the position of the retinal elements (Angellucci 1884, Gradenigo 1885, Herzog 1905, Fujita 1911, Arey 1916, Wunder 1925, Arey & Jennings 1943, Detwiler 1943, and Ali 1960 & 1961b). Most recently Ali (1964a) showed that Salmo salar light adapted more quickly at 20°C than at 5°C. Similarly, the light intensity used to light adapt a fish, and the intensity and duration of light that a fish is exposed to before dark adaptation, can also have a profound effect on the rate (eg: Ali 1962 and Kobayashi 1957). A further factor that

increases the difficulty of comparisons between studies is differences in subjective judgement among authors as to when adaptation is complete, a fact recognised early on in the study of retinomotor movements by both Arey (1916) and von Studnitz (1933).

The often large discrepancies between studies, however, are unlikely always to be explained by such differences in temperature, lighting condition and subjective judgement, and much of the observed difference between adaptation rates is probably real age and species variation, attributable to differences in habitat and/or mode of life of the individuals (Ali 1975 for review). Wunder (1924), Brett & Ali (1958) and Ali (1959 & 1961), for instance, have all shown retinomotor movements to vary a great deal within a single study depending on both age and species.

1.5.2. Adaptation rates in the laboratory. Despite these difficulties, a comparison between the present results on the rainbow trout and published data on other species, especially salmonids, may be useful, as several general trends observed in previous studies are confirmed here. Light adaptation in the laboratory, for example, is seen to be faster than dark adaptation, as has been found in almost all other studies. A latent period before dark, but not before light, adaptation was also observed, as it was in four species of pacific salmon (Brett & Ali 1958, Ali 1959), although the latencies observed in Ali's studies were generally longer, probably because of the lower light intensities used for pre-adaptation in the present study (650 lux as compared to 4300 & 68000 lux). This observation agrees well with the fact that Ali et al (1961) found no latent period in the atlantic salmon when an intensity of only 270 lux was used.

When making more specific comparisons with the actual

TABLE I.III Time taken for the epithelial pigment and cones to light and dark adapt in various salmonids in the laboratory.

SPECIES	Time taken to light adapt (mins) Pigment	Cones	Time taken to dark adapt (mins) Pigment	Cones	ILLUMINATION (lux)	Reference
SOCKEYE SALMON						
emerged fry	25	20	30	35		
late fry	10	15	45	40		
smolt	20	15	40	50		
COHO SALMON						
emerged fry	15	25	40	40		
late fry	20	10	35	35		
smolt	20	15	40	40		
PINK SALMON						
emerged fry	25	25	45	35	4.3×10^3	Ali (1959)
late fry	20	20	30	35		
CHUM SALMON						
emerged fry	10	20	30	20		
late fry	20	10	40	35		
ATLANTIC SALMON	40	45	>70	>70	10^4	
Yearlings	35	55	45	25	1	Ali (1962)
ATLANTIC SALMON	15 - 20	35	45	70	1.6×10^3 (20°C)	
	45	55	>14 hrs	>14 hrs	(5°C)	Ali (1964)
ATLANTIC SALMON juvenile	60	45	70	70	2.5×10^2	Ali et al (1961)
SOCKEYE SALMON fingerlings	20 - 25	20 - 25	60	55 - 60	6.8×10^4	Brett & Ali (1958)
Rainbow trout	15	15	90	90	6.5×10^2	Present study

rates of adaptation found in other laboratory studies (Table 1.III) one runs into more serious difficulties, and the situation is far less clear cut. Complete dark adaptation seems to take longer in most other studies with only atlantic salmon yearlings pre-adapted to 10^4 lux (Ali 1962) having a comparable rate. One could argue that the intensity used for pre-adaptation in the present study (650 lux) is greater than that used on Salmo salar (Ali et al 1961) and Salmo salar yearlings which were pre-adapted to one lux (Ali 1962), thus ensuring a longer duration of dark adaptation, but this would not explain the faster rate of sockeye salmon fingerlings dark adaptation after pre-adaptation to 68000 lux (Brett & Ali 1958). Similarly, the four species of pacific salmon (Ali 1959) all show faster rates of adaptation although the intensity of pre-adaptation was very much higher (4300 lux). As pacific salmon show a tendency towards longer dark adaptation with age (Ali 1959) and as the rainbow trout used were older than the fish in the above studies, the longer dark adaptation could be explained in terms of age differences. The 20°C used by Ali (1964a) is higher than the temperature in this study, so a comparison in this case is not possible. Thus only a rather tenuous comparison can be made to previous rates of dark adaptation. It would be even harder to establish a credible relationship between previous work and the present rates of light adaptation.

The foregoing discussion indicates that meaningful comparisons of rates of adaptation between studies, on the same or different species, are almost impossible due to the many variables involved, and that if comparisons are made, the intensity and duration of exposure to light, the temperature, age and species must all be considered. Even this does not eliminate variation due to differences in subjective criteria

among authors, although the use of indices goes some way toward eliminating this error.

1.5.3. Experiments using natural illumination. Laboratory studies on rates of adaptation are also of limited value because fish in their natural habitat are never exposed to such abrupt changes in illumination as occur in the laboratory. Therefore, as pointed out by Nicol (1963), it is probably more worthwhile to find out the speed of migration of the retinal elements under the conditions of changing light intensities encountered in nature. This can be achieved by sampling fish throughout the natural twilight periods, as was done in section 1.3. Comparisons between such studies should be biologically more meaningful, but differences in species, age, temperature and time of year must still be considered. Of these, location and time of year are especially important as they determine the rate of change of the natural level of illumination.

Rates of cone migration observed over natural dusk periods in this study (table 1.I) vary a great deal (40 - 90 minutes) over the three periods examined, whilst the pigment adapts in 75 to 90 minutes on each occasion. This rate of pigment migration compares quite favourably to the duration of pigment migration in sockeye and coho smolt and pink salmon fry (75,75 and 90 minutes respectively) in nature (Brett & Ali 1958). Agreement between cone rates is not as good, due to the large variation of rates in rainbow trout.

The other aspect of retinomotor movements that has frequently been investigated is their thresholds. Again such studies can be of two kinds, involving either natural light changes or laboratory illumination. Laboratory studies entail keeping fish at different constant intensities for a certain

period of time and then observing the positions of their retinal elements. Results from several such studies are summarised in table 1.IV. As can be seen, thresholds differ a great deal between species. Such large variations in the laboratory were noted as early as 1925 by both Wunder and von Frisch. These differences can be either true species differences, reflecting the visual capability of each species, or they may be false variations caused by any of the above mentioned differences in experimental conditions. In view of such large differences among laboratory thresholds, comparisons with the present field studies are only of limited value. On the whole, retinal movements in all of these laboratory studies started at much lower levels of illumination (10^{1} - 10^{-2} lux) than those of the rainbow trout (10^{3} - 10^{-1} lux).

Comparisons between retinomotor rates and thresholds determined in the laboratory and those obtained in nature are only of limited value for another reason. The latent period observed during dark adaptation in the laboratory makes it difficult to determine from field records just when the process started. A latency period might thus explain why Blaxter & Jones (1967) observed that the retinas of herring kept in the laboratory at a constant intensity responded at about one log unit of intensity higher than those from fish sampled during a natural dusk (see below). The retinomotor movements in nature are probably triggered by one intensity, but due to a latency period do not seem to respond until a later, lower, level of illumination. Ali & Hoar (1959) also noted discrepancies between field and laboratory studies.

A comparison with thresholds obtained in other field studies may however be more valid. Such studies have been carried out by both Blaxter and by Ali, with their various

TABLE I.IV. Levels of illumination over which the retina changes from light to dark adaptation in various laboratory studies.

Species	Range (Lux)		Reference
	Pigment	Cones	
Anchovy - Engraulis encrasicholus	10^{0}-10^{-1}		Protasov et al (1960)
Sea bream - Sargus annularis	10^{-1}-10^{-2}		Protasov et al (1960)
Smelt - Atheria mochon pontica	10^{-1}-10^{0}		Protasov et al (1960)
Cod - Gadus morhua	10^{-2}-10^{-3}		Protasov (1964)*
Myoxocephalus quadricornis	10^{-2}		Protasov (1964)*
Catfish - Anarhichas lupus	10^{-2}-10^{-3}		Protasov (1964)*
long rough dab - Hippoglossoides platessoides	10^{-2}-10^{-3}		Protasov (1964)*
Plaice-Pleuronectes platessa	10^{-2}		Protasov (1964)*
Capelin - Mallotus villosus	10^{-2}-10^{-3}		Protasov (1964)*
Coal fish - Pollachius virens	10^{-2}-10^{-3}		Protasov (1964)*
Roach - Leucisus rutilus		2.7×10^{-3}-10^{-5}	Engström & Rosstrop (1965)
Silver mackerel - Trachurus japonicus	$5\text{-}3\times10^{-2}$	10^{0}-8×10^{-3}	Kobayashi (1957)
Goldfish - Carassius auratus	>25-.008	23- <.008	Kobayashi (1957)
Loach - Misgurunus augvillicaudatus	.07 - .002	.08 - .002	Kobayashi (1957)
Pacific salmon Oncorhynchus spp alevins - sockeye	10^{1}-10^{-1}	10^{+2}-10^{0}	Ali (1959)
alevins - chum	10^{0}-10^{-1}	10^{0}-10^{-2}	Ali (1959)

Table I.IV. cont

Species	Range (Lux)		Reference
	Pigment	Cones	
late fry - chum	10^{1}-10^{0}	10^{1}-10^{0}	Ali (1959)
pink	10^{1}-10^{-1}	10^{1}-10^{0}	Ali (1959)
coho	10^{1}-10^{-2}	10^{0}-10^{-2}	Ali (1959)
sockeye	10^{+2}-10^{-1}	10^{1}-10^{-1}	Ali (1959)
smolts - coho	10^{1}-10^{-1}	10^{0}-10^{-2}	Ali (1959)
sockeye	10^{+2}-10^{-3}	10^{0}-10^{-1}	Ali (1959)
Atlantic salmon - salmo salar	10^{-2}-10^{-3}	10^{-1}-10^{-3}	Ali (1961)
Herring	10^{1} -10^{-2}	10^{1} -10^{-2}	Blaxter & Jones (1967)

* Author did not specify if laboratory or field study
- if author did not state pigment or cones the results are put in the pigment column.

TABLE I.V Levels of illumination over which the retina changes from light to dark adaptation in various studies using natural illumination

Herring - Clupea harengus	10^{0}-10^{-2}	10^{0}-10^{0-2}	Blaxter & Jones (1967)
stickleback	10^{1}-10^{-1}		Blaxter & Staines (1970)
sole	10^{1}-10^{-1}		Blaxter & Staines (1970)
salmon	10^{-2}		Blaxter & Staines (1970)
Plaice - Pleuronectes platessa	10^{0}-10^{-2}		Blaxter (1968 a)
Pacific salmon sockeye smolt	4.52×10^{2} -10^{0}	2.37×10^{2}-10^{0}	Brett & Ali (1958)
coho smolt	2.37×10^{2} -3.2×10^{-1}	1.6×10^{2}-10^{-1}	Brett & Ali (1958)
pink fry	1.4×10^{2} -10^{-3}	3.2×10^{1}-10^{-3}	Ali & Hoar (1959)

co-workers, by sampling at known intensities over the natural twilight period (table 1.V). The results obtained by Ali for the start of pigment migration on other salmonids agree, in general, quite closely with those for rainbow trout, and migration is complete at around the same intensity as in the pink salmon. The large variation of cone thresholds found in this study prohibits any real comparisons.

Both laboratory experiments and experiments using the natural dusk period have shown cones to start dark adaptation at lower levels of illumination than the pigment epithelium in several species of teleost (Brett & Ali 1958, Ali 1959 and Blaxter & Jones 1967). This agrees well with the cones starting migration after the pigment at dusk in the present study. As the cones finish migrating at the same time as the pigment this implies a faster rate of cone migration, which was not supported by the laboratory studies presented here. Faster rates of cone migration in the laboratory have, however, been noted by several authors (eg: Fujita 1911, von Studnitz 1933 and Ali et al 1961).

Cones beginning migration after the pigment at dusk, as well as starting migration before the pigment at dawn were also noted by McFarland et al (1979) in the French grunt, Haemulon flavolineatum. The authors interpreted this as being a mechanism that allows both photopic and scotopic vision over twilight periods, a time at which the fish undergo their daily behavioural migrations. Munz & McFarland (1973) consider twilight to be visually the most difficult period, as it involves the transition between photopic and scotopic vision, and suggest this is why there is a "quiet period" on coral reefs, when neither nocturnal or diurnal species are active. Differential thresholds for cone and pigment migration may go some way

towards overcoming this visual problem. At dusk the pigment withdraws before the cones while the light level is still relatively high, exposing both rods and cones to the light during twilight. Conversely, cones move before the pigment in the morning, thus exposing themselves, along with the already exposed rods, to the light. Therefore both rods and cones are exposed and may be utilised over the twilight periods, ensuring maximal safety for the grunts during their behavioural migration. A similar interpretation can be applied to the present results (figs 1.5 & 1.6), since trout are a crepuscular species active at twilight (see chapter 2).

It would be interesting to carry out a comparison between crepuscular and non-crepuscular fish to see if there is a difference in the behaviour of their retinal elements, although there appears little reason why all species should not have what seems such a highly adaptive system.

The system will not work, however, unless the withdrawl of pigment epithelium is slow enough at dusk to ensure that the rod visual pigment is sufficiently protected from bleaching by bright light, and yet fast enough to allow rods to be exposed at twilight. This was untested by McFarland et al (1979), but in a subsequent section (chapter 3), it is shown that the rod visual pigment is indeed sufficiently protected during dusk.

Brett & Ali (1958), Ali (1959) and Ali & Hoar (1959) use a theory directly contrary to that outlined above to explain the twilight downstream migration of several species of juvenile pacific salmon. These authors suggest that a period of night (twilight) blindness, caused by the eyes being in a semi-adapted state, results in a failure of rheotactic reponses, causing the fish to be passively swept downstream.

There is in fact a great deal of evidence showing that visual stimuli are involved in the rheotactic response (Arnold 1974, for review), but other factors such as tactile cues, lateral line responses, velocity gradients, turbulence, acceleration, rotation and electrical stimuli, are all known to be involved as well. Arnold (1974) summarises the evidence pertaining to juvenile salmon migration and concludes that it may well be a passive downstream migration, owing to the loss of visual reference points. Such a theory is supported by the observations that shining lights at migrating fish stops their migration, as does moonlight, and by the association of daytime migration with turbid water.

Such a period of night blindness is, however, the exact reverse of what has been proposed for both the grunt and rainbow trout, and one could, using the argument outlined above, postulate that during downstream migration the fish are in fact capable of quite good vision. There are thus two contrasting theories regarding the visual capabilities of fish during twilight, and at present there is no real evidence to support either one. From an adaptive standpoint, however, it would seem preferable to reject a theory invoking failure of visual mechanisms, as it is very unlikely that natural selection would select a system that is seemingly so totally unsuited for such an important visual period. Disbelief in such a mal-adapted theory has been expressed by several authors on similar grounds (eg: Blaxter 1970 and Blaxter & Jones 1967).

CHAPTER 2 ENDOGENOUS RHYTHMS OF RETINOMOTOR MOVEMENTS

2.1 INTRODUCTION

During an unsuccessful investigation into the spectral sensitivity of photomechanical movements in rainbow trout, several eyes appeared fully light adapted, although the fish had been subjected to darkness for several hours. It was thought that these inconsistencies might be due to an over-riding internal rhythm, causing fish placed in darkness during normal daylight hours to remain light adapted. The possible existence of such a rhythm was therefore investigated in greater detail.

Fish kept in the light are always found to be fully light adapted, no matter what the time of day, but some form of persistent endogenous retinomotor rhythm during extended periods of darkness has been reported in six species of fish and is indicated in a further two. As such internal rhythms have been reported before "just another study" on a different species may not seem worthwhile, but the present results are worth describing for several reasons. Firstly, Ali (1959 & 1961) and Ali & Wagner (1977) have reported that retinomotor rhythms do not occur in several species of salmonid, and salmonids are therefore widely quoted as not possessing an endogenous photo-mechanical rhythm. This is not substantiated by the present results. Secondly, the form of the rhythm found in rainbow trout is totally different from any pattern previously reported. Finally, it is felt that earlier studies, although adequate to demonstrate a rhythm, may not be detailed enough to reveal the exact pattern of such movement.

Welsh & Osborn (1937) were the first to report a retinomotor rhythm in fish. They noted that the eyes of *Ameiurus nebulosus*, kept in constant darkness, were more dark adapted when killed

at night than when killed during the day (fig 2.1a). This difference was only very slight on the second day. The authors state that such rhythms are widespread among other fish species, but furnish no further details. Similarly Arey & Mundt (1941) sampled Ameiurus, kept in constant darkness, at noon and midnight and found cones to be more dark adapted at midnight than at noon for the four days of the experiment. The rods and epithelial pigment showed no evidence of a rhythm under these conditions.

Wigger (1941) is often quoted as having shown a persistent rhythm in the goldfish, Carassius auratus, but this is not the case. The fish were fully dark adapted four hours after sunset. The retinal elements then started to migrate toward their light adapted positions, until after twelve hours in the dark they took up a position intermediate between light and dark adaptation. This position was then maintained for the remaining fifty hours of the experiment (fig 2.1b). It is interesting to note that the most light adapted fish in this experiment were sampled at sunrise. John, Segall & Zawatsky (1967) found evidence contrary to this, reporting a persistent rhythm of goldfish single and double cones in continual darkness. Fish kept on an approximately twelve hour light/dark cycle in the laboratory for forty-five days and then put in continual darkness, displayed this rhythm for all three days of the experiment (fig 2.1c). A similar rhythm was displayed by fish that had been exposed to the natural light/dark cycle (fig 2.1d).

John & Haut (1964) reported a rhythm in the black banded tetra, Astyanax mexicanus, by dark and light adapting two fish at noon and midnight. Fish light adapted at these times were both fully light adapted, indicating the absence of a rhythm in the light, but fish dark adapted at midnight were found to be more dark adapted than those dark adapted at noon. This

study was extended by John & Kaminester (1969), who followed the movements of twin and single cones and epithelial pigment during the first day in darkness by sampling every hour for twenty-four hours, commencing the morning after the beginning of darkness (fig 2.2a). These movements were then followed in less detail for six cycles in constant darkness, by sampling at twelve hour intervals beginning at 01.00 on the first night (fig 2.2b). During the first day, the morning migration started one hour before sunrise, as did the evening migration one hour before sunset, and in both cases finished three to four hours after commencing. This rhythm persisted for all six days of the experiment in an undiminished form.

Olla & Marchioni (1968) kept bluefish, Pomatomus saltatrix, on an artificial light/dark cycle for seven days. On the eighth day the lights remained off, and fish were sampled every three hours for thirty-six hours (fig 2.1e). The shift of cones from the dark to a light adapted position was found to be a gradual one, while the pigment remained in a dark adapted position throughout the experiment.

John & Gring (1968) investigated both bluegills, Lepomis macrochirus, previously exposed to the natural light/dark cycle and brought into the laboratory at sunset (fig 2.1f), and fish exposed to an artificial light/dark cycle for twenty-four days (fig 2.1g). In both cases the pigment and cones showed rhythmic movements when kept in darkness, but even during the first day the eyes were not fully light adapted and the rhythm became even more rudimentary as the experiment progressed. It is again interesting to note that the most light adapted cones occured at sunrise and sunset.

A persistent rhythm has also been reported in Nannacara anomala (Wagner, unpublished observation quoted in Wagner & Ali

Fig 2.1. Cone positions in the retinas of fish kept in continual darkness - summary of previous experiments.

(a) Welsh & Osborn (1937) - the first two points are the average values of 9 fish while the last two are the average of 10 fish.

(b) Wigger (1941) - Each point is the average of two fish. The fish were put into darkness at 18.00 and sampled every two hours for the first thirty hours and then less frequently until fifty hours after the onset of darkness.

(c) John et al (1967) - Each point is the average of two fish previously adapted to an artificial light/dark cycle (lights on at 06.00 and off at 19.10).

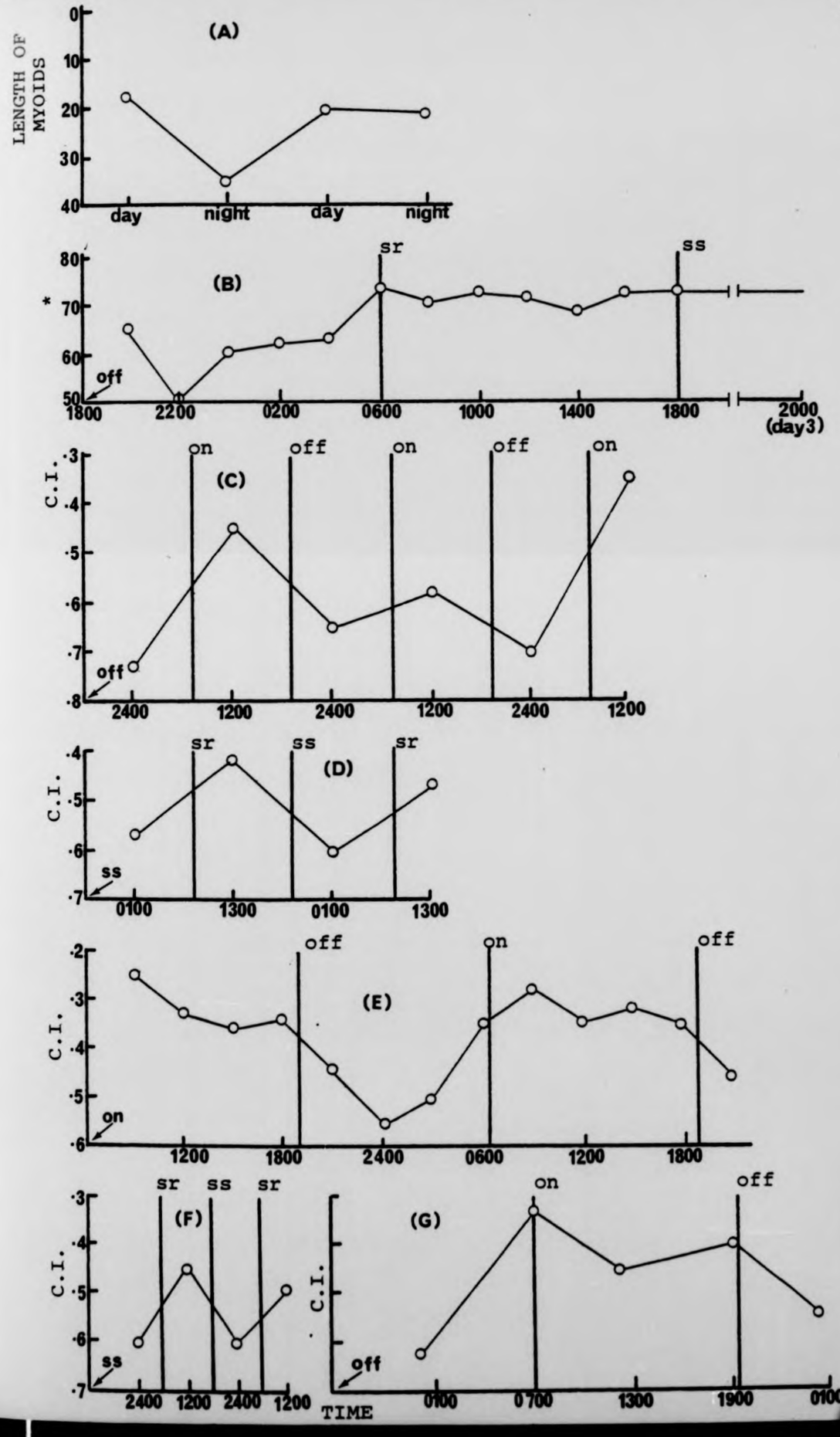
(d) John et al (1967) - Each point is the average of two fish entrained to the natural light/dark cycle and put into darkness at sunset.

(e) Olla & Marchioni (1968) - Each point is the average of two fish previously adapted to an artificial light/dark cycle (lights on at 06.30 and off at 19.30).

(f) John & Gring (1968) - Each point is the average of 10 fish entrained to the natural light/dark cycle and put into darkness at sunset.

(g) John & Gring (1968) - Each point is the average of 10 fish previously entrained to an artificial light/dark cycle (lights on at 07.05 and off at 19.20).

* $\frac{\text{distance between lamina basalis and the cone center}}{\text{distance between e.l.m. and lamina basalis.}}$


LENGTH OF MYOIDS
(A)
0
10
20
30
40
day
night
day
night
(B)
sr
ss
80
70
60
50
*
off
1800
2200
0200
0600
1000
1400
1800
2000
(day3)
(C)
C.I.
·3
·4
·5
·6
·7
·8
on
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on
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off
2400
1200
2400
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2400
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(D)
sr
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sr
·4
·5
·6
·7
0100
1300
0100
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off
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0100
TIME

1977) and an internal rhythm may be the cause of "irregularities" in the behaviour of cones in the common sole, Solea solea,and the merry sole, Microstomus kitt, when subjected to darkness during daylight hours (Nicol 1965). Unfortunately Nicol did no controlled experiments to see if this was the case.

On the above evidence, it is tempting to extrapolate these results and conclude that some fish, in conditions of continual darkness, exhibit retinomotor movements such as those found in fish experiencing natural lighting conditions (fig 2.5). However, such a conclusion, based on so few data points, may not be valid, as will be shown below. Most of these studies involved sampling every twelve hours and at the best every three hours (Olla & Marchioni 1968). This infrequent sampling may lead to the true pattern of movement being obscured. The demonstration that fish sampled at noon are more light adapted than those sampled at midnight, although indicative of some form of rhythm, gives no information about, for example, the position of the retinal elements at dawn and dusk. The one study exempt from this criticism is that of John & Kaminester (1969), who sampled every hour throughout the first day and used this as a basis for interpreting the results of a longer term study involving less frequent sampling.

Several reported demonstrations of a lack of such rhythms are also unsatisfactory for similar reasons. Ali & Anctil (1977), for example, light adapted the walleye and sauger by night and dark adapted them by day, and found this to reveal no evidence for an endogenous rhythm. Similarly, Ali & Pickford (1979) found that killifish dark adapted for twenty-four hours, when killed during the day were dark adapted,thus providing "little support for a possible light independent circadian rhythm". As will be demonstrated below, such statements are again not

valid, based on what are largely incidental observations.

Although some form of retinomotor rhythm has been demonstrated in several species, as yet they have always been reported as being absent in salmonids. Ali (1959) found there to be no rhythm of pigment or cone movements in either continual light or darkness, in various developmental stages of three species of pacific salmon. Similarly, Ali (1961) found no pigment or cone rhythms in juvenile Salmo salar in constant light or of cones rhythms in constant darkness, although there was some very inconclusive evidence of a diurnal pigment rhythm during the first twenty-four hours in the dark (fig 2.3). Finally, Wagner & Ali (1977) were unable to show any rhythmic movements of retinal elements in the brook trout, Salvelinus fontinalis, in either continual light or darkness, after two weeks adaptation to a twelve hour light/dark cycle (fig 2.3). These observations lead Ali to the conclusion that the salmonids are "an arhythmic group".

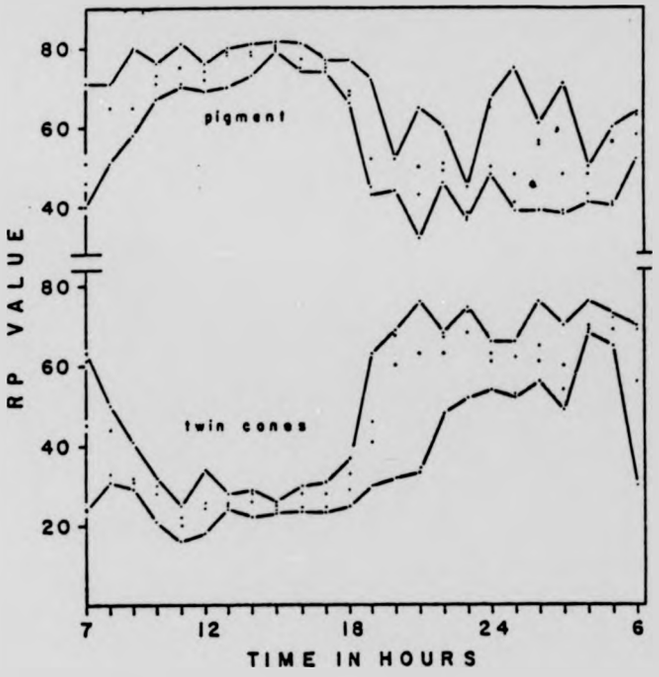
2.2 METHODS

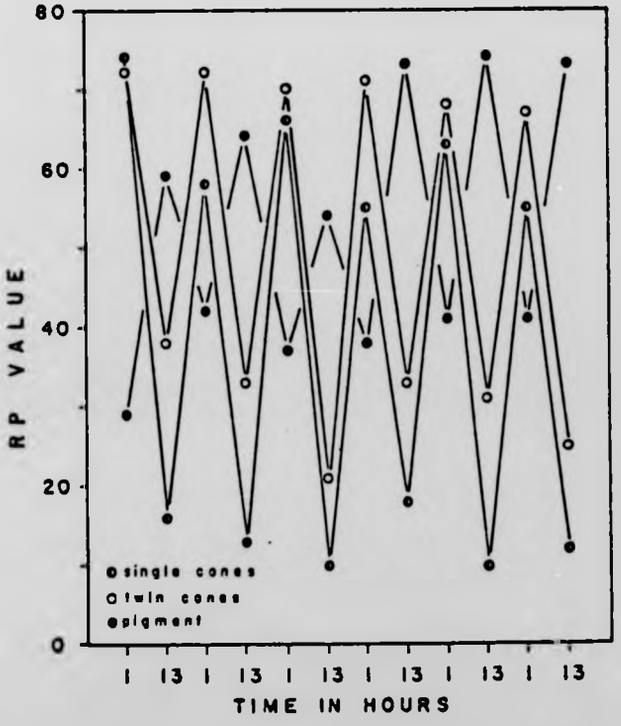
The existence of a retinomotor rhythm was initially investigated using fish adapted to an artificial twelve hour light/dark cycle in the laboratory for varying periods of time (exps. 1 - 5). Subsequently, fish taken directly from natural light conditions were used (exps. 6 & 7).

On arrival in the laboratory, fish used in experiments 1 - 5 were kept for several weeks in an aquarium, and fed daily on trout pellets. For experimental adaptation the fish were transferred into laboratory tanks and supplied with running water (16^{o}C - 17.5^{o}C). These tanks were covered by a lid containing fluorescent light (650 lux at water surface) which,

Fig 2.2a. Rhythms of twin cones and retinal pigment in Astyanax mexicanus through one 24-hr period in darkness beginning with the first subjective day. Time in hourly intervals at 10 min after the hour. Subjective sunrise, 07.10; subjective sunset, 19.10 hr. Points represent individual fish. The RP value is position of visual components relative to thickness of visual cell layer. (from John & Kaminester 1969)

Fig 2.2b. Free-running retinomotor rhythm of Astyanax mexicanus in 6 days of constant darkness. Time represents 01.00 hr and 13.00 hr. The RP values are means of two fish, excepting third and fourth samples at 01.00 hr, which are each represented by one fish. (from John & Kaminester 1969)


80
60
40
pigment
RP VALUE
80
60
40
20
twin cones
7
12
18
24
6
TIME IN HOURS


80
60
40
20
0
RP VALUE
single cones
twin cones
pigment
1
13
1
13
1
13
1
13
1
13
1
13
TIME IN HOURS

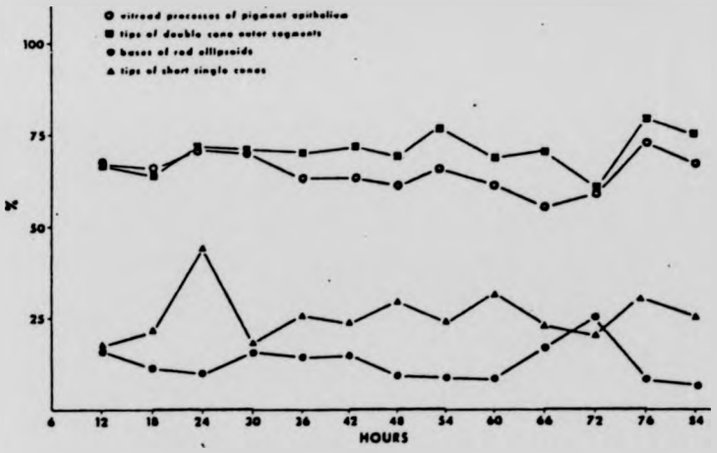


Fig 2.3a. Retinomotor movements during 72 h of constant darkness in Salvelinus fontinalis. The distance between e.l.m (0) and Bruch's membrane (100) is given as a percentage. The thin and thick bars on the abscissa indicate the virtual cycle of illumination. (from Wagner & Ali 1977)

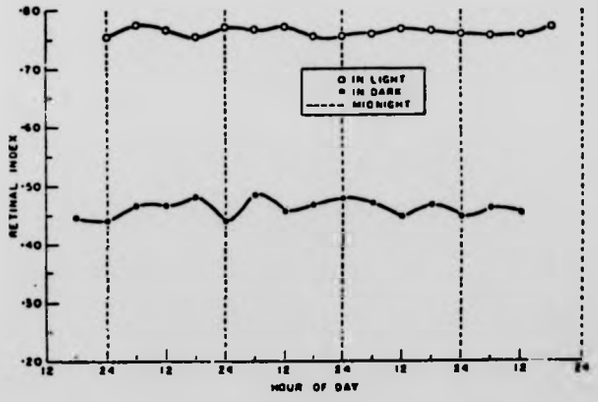


Fig 2.3b. Retinal index of atlantic salmon eyes when subjected to continual darkness. (from Ali 1961)

coupled to a timer, supplied the twelve hour light/dark cycle (09.00 h - 21.00 h or 07.00 h - 19.00 h).

The following table summarises the experiments carried out:

1. 16 fish, artificially adapted for 15 days - sampled during an L/D cycle.
2. 32 fish, artificially adapted for 25 days - sampled in constant light
3. 8 fish, artificially adapted for 7 days - sampled in constant dark
4. 20 fish, artificially adapted for 14 days - sampled in constant dark
5. 20 fish, artificially adapted for 29 days - sampled in constant dark
6. 54 fish, naturally adapted - sampled in constant dark (24 hrs)
7. 199 fish naturally adapted - sampled in constant dark (98 hrs)

In experiment 1 fish were sampled during an actual laboratory light/dark cycle. Subsequently, in experiments 3 - 5 the lights were permanently turned off at "dusk" of the last day of adaptation, and fish sampled beginning around the following "dawn". Conversely, in experiment 2 the lights were left on after the last day of entrainment and fish sampled throughout the following night.

For experiments 6 & 7, fish, which had been exposed to the natural light/dark cycle for at least a year, were collected from the fish farm on the day before the experiment, put in a large tank outside away from any artificial lighting,and kept there to dark adapt during dusk. Fish were sampled throughout the fall in illumination to ensure that they were dark adapting normally and that factors such as stress from the journey had

Fig 2.4. Positions of retinal pigment (●) and cones (○) in various lighting conditions following laboratory adaptation to a 12 hr light/dark cycle.

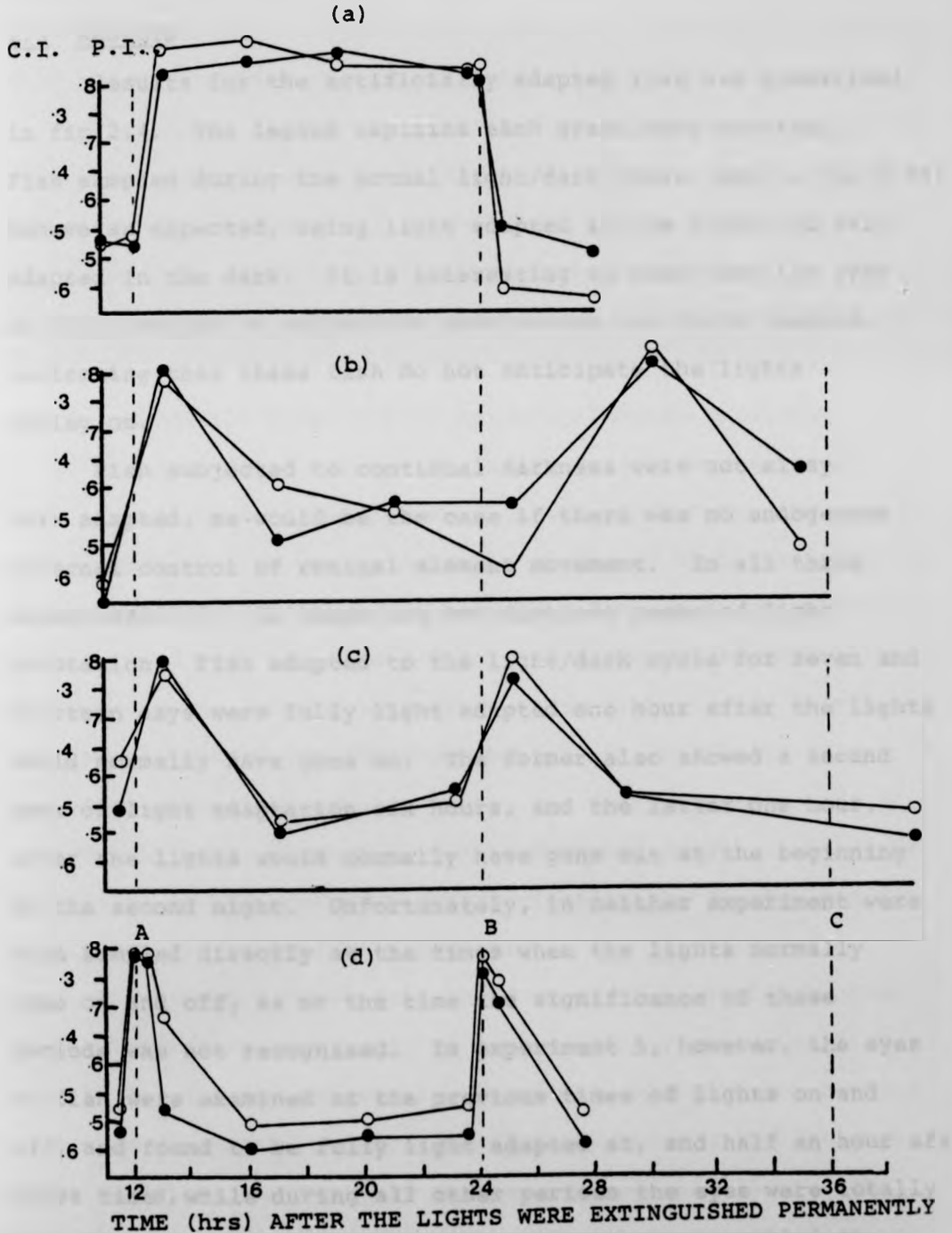
(a) Experiment 1. Each point is the average index value of two fish.

(b) Experiment 3. Each point represents one fish, except 25 hours after the lights were turned off which is the average of two fish.

(c) Experiment 4. Each point is the average of three fish, except the last point which is the average of two

(d) Experiment 5. All points are the average index value of two fish.

A is the time at which the lights would come on had the cycle been maintained. B & C mark the times at which subsequent changes in illumination would have taken place.


(a)
C.I. P.I.
(b)
(c)
A
B
C
(d)
12
16
20
24
28
32
36
TIME (hrs) AFTER THE LIGHTS WERE EXTINGUISHED PERMANENTLY

in no way affected their retinomotor movements. At 22.00 h the fish were transferred, in total darkness, to blacked out tanks in the laboratory and sampled at intervals thereafter.

2.3 RESULTS

Results for the artificially adapted fish are summarised in fig 2.4. The legend explains each graph more precisely. Fish sampled during the actual light/dark cycle (exp 1, fig 2.4a) behave as expected, being light adapted in the light and dark adapted in the dark. It is interesting to note that the eyes of fish sampled at subjective sunrise are not light adapted, indicating that these fish do not anticipate the lights coming on.

Fish subjected to continual darkness were not always dark adapted, as would be the case if there was no endogenous internal control of retinal element movement. In all three experiments (3 - 5) there are two distinct peaks of light adaptation. Fish adapted to the light/dark cycle for seven and fourteen days were fully light adapted one hour after the lights would normally have gone on. The former also showed a second peak of light adaptation six hours, and the latter one hour, after the lights would normally have gone out at the beginning of the second night. Unfortunately, in neither experiment were fish sampled directly at the times when the lights normally came on and off, as at the time the significance of these periods was not recognised. In experiment 5, however, the eyes of fish were examined at the previous times of lights on and off, and found to be fully light adapted at, and half an hour after, these times, while during all other periods the eyes were totally dark adapted. It is possible that had fish been sampled at

these times in experiment 4, they too would have been light adapted.

A superficial examination of fig 2.4b - d would seem to indicate that the dusk peaks become more synchronised with the actual time the lights would have gone off the longer the period of entrainment to the light/dark cycle, but there are not enough data points to allow one to state this with any certainty.

The abrupt changes in illumination experienced by the fish in the laboratory are very different to the more gradual changes experienced in nature. It is therefore possible that these two peaks, seen in laboratory entrained fish, are an artifact of this sudden change. To ensure that this was not the case fish entrained to a natural light/dark cycle were used (exp 6 & 7). Fig 2.5 shows the behaviour of retinal elements throughout a natural twenty-four hour period, using data from chapter 1. This is analogous to experiment 1 in the laboratory. The two experiments (6 & 7) using naturally entrained fish were done six months apart, at times when the declination of the sun was zero. This ensured that the time and rate of change of natural illumination was the same in both cases, thus facilitating direct comparison between the two experiments. In both cases (figs 2.6 & 2.7), the fish sampled during the dusk preceeding the first experimental day of complete darkness showed normal retinomotor adaptation to the ambient light, indicating the journey had not significantly affected this process.

Experiment 6 shows that the dawn and dusk peaks observed in the laboratory also occur in such naturally entrained fish (fig 2.6), with the retinomotor movements following the outside change in illumination exactly during the first dawn, behaving identically to fish actually exposed to such conditions. The second peak occurs about two hours after the level of illumin-

Fig 2.5. Retinal pigment (●) and cone (○) indices throughout consecutive dawn and dusk periods.

As fish were not sampled throughout the twenty-four hour period the indices are extrapolated (–·–·) using average values for fully light and dark adapted eyes from chapter 1. Each point during dawn is the average of two fish, while each point during dusk is the average of three fish.

(- - -) represents the level of illumination.

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point

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Fig 2.6. Retinal pigment (●) and cone (○) indices in naturally entrained fish during 24 hours darkness (exp 6). Each point during the natural dusk represents one fish while all others are the average of three fish, except at 18.00,19.00,19.30 & 21.00 when only two fish gave suitable sections. The illumination (---) is the level of illumination outdoors during the period when the fish were in darkness in the laboratory.

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ation started to fall in nature. Experiment 7 investigated the longevity of this rhythm (fig 2.7). Unfortunately, two fish were sampled on only five occasions during the first day in darkness, as it was anticipated the results would be the same as in experiment 6. The times sampled should have shown the peaks observed in the previous experiment, but failed to do so. Two very distinct peaks of light adaptation did however occur on the second day in darkness, indicating that the pattern is present. From this and from the previous experiments, it seems reasonable to suppose that the peaks were there on the first day of experiment 7, but were "missed" due to infrequent sampling. After the second day in darkness the pattern became more erratic.

In order to investigate the cause of this irregularity on the third and fourth days, the eyes in experiments 6 & 7 were assigned to one of three categories of adaptation depending on their indices (table 2.I). It can be seen that the number of fish whose cones and pigment are in an intermediate state of adaptation tends to increase with time in the dark, while the number of light adapted eyes shows no systematic variation. The number of eyes whose pigment is fully dark adapted also shows a tendency to decrease, but this trend is less pronounced for cones. On the whole, it would seem that the longer the fish stay in darkness, the more eyes are found in an intermediate state of adaptation at the expense of dark adapted eyes.

Fig 2.8, which gives the results of experiment 2, shows that the retinal elements are in a light adapted condition when kept in continual light no matter what the time of day, indicating the absence of a retinal rhythm in the light. Any variation is well within the limits of experimental error.

Fig 2.7. Retinal pigment (●) and cone (O) indices in naturally entrained fish during 98 hours darkness (exp 7).

Each point during the natural dusk represents one fish, each point during the first day is the average index value of two fish while all other points are the average values of three fish, except 10.00 & 19.00 on day two and 01.00. 06.00 & 18.00 on day four which are the average of two fish. The illumination (~--) is the level of illumination outdoors during the period when the fish were in darkness in the laboratory. (A) Represents the period of natural illumination (dusk) the fish were exposed to before being placed in darkness.

Fig 2.7. Retinal pigment (●) and cone (O) indices in naturally entrained fish during 98 hours darkness (exp 7).

Each point during the natural dusk represents one fish, each point during the first day is the average index value of two fish while all other points are the average values of three fish, except 10.00 & 19.00 on day two and 01.00. 06.00 & 18.00 on day four which are the average of two fish. The illumination (---) is the level of illumination outdoors during the period when the fish were in darkness in the laboratory. (A) Represents the period of natural illumination (dusk) the fish were exposed to before being placed in darkness.

C.I. P.I.
(A)
CONTINUAL DARKNESS
ILLUMINATION (lux)
DAY 1
DAY 2
DAY 3
DAY 4
time

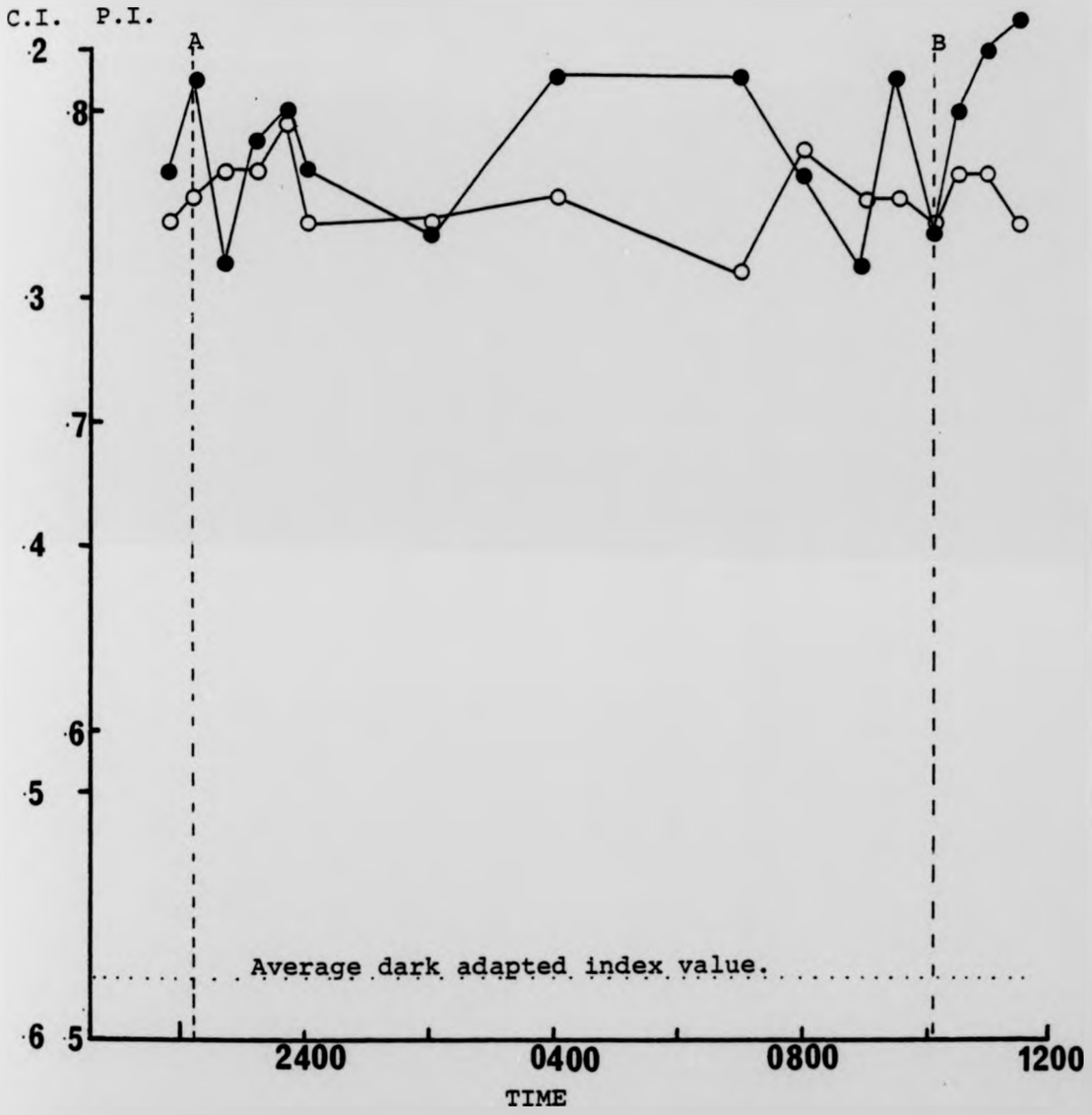

Fig 2.8. Retinal pigment (●) and cone (○) indices of fish kept in continual light after twenty-five days adaptation to an artificial light/dark cycle (exp 2). Each point is the average index value of two fish, except those at 09.30-11.30 which represent only one fish. A & B are the previous times of lights off and on respectively.

TABLE 2.I (a) INDEX VALUES REPRESENTING STATES OF ADAPTATION

Adaptational state	pigment index	cone index
light adapted	>.75	<.3
intermediate	.6 - .75	.3 - .45
dark adapted	<.6	>.45

(b) PROPORTION OF ADAPTATIONAL STATES DURING EXPS 6 & 7 - pigment

experiment	day	% eyes light adapted	% intermediate	% dark adapted
6	1	14	31	55
7	2	11	22	67
7	3	20	37	43
7	4	9	61	30

(c) PROPORTION OF ADAPTATIONAL STATES DURING EXPS 6 & 7 - cones

experiment	day	% eyes light adapted	% intermediate	% dark adapted
6	1	39	29	32
7	2	11	22	67
7	3	26	33	41
7	4	14	48	38

2.4 DISCUSSION

The results show that rainbow trout possess a unique pattern of retinal movements over extended periods of darkness, having two distinct peaks of light adaptation coincident with dawn and dusk. This rhythm lasts for at least two days and probably longer. If it were possible to follow the retinal movements of an individual over an extended period of time, it is likely that one would see the two peaks gradually losing synchrony with the natural changes in illumination. As there is no reason to suppose different fish will lose synchrony together, these peaks will be obscured by averaging the results of different fish. Over the first two days, light adapted eyes were only observed in individuals sampled at the times when the peaks of light adaptation occurred, but on subsequent days light adapted fish were found evenly distributed throughout the day. Individual fish might thus still be exhibiting the rhythm.

From fig 2.7 one can see that the "average state of adaptation" rises over the four days of the experiment. This is partially caused by the loss of synchrony mentioned above, but is also due to the greater number of fish whose eyes are in an intermediate state of adaptation (table 2.I). Such an increase in the number of semi-adapted, at the expense of dark adapted, eyes indicates that this may be the relaxed state of the retina. The interesting consequence of this is that it implies the more complete dark adaptation during the first days in darkness, in naturally and laboratory entrained fish, is an active process.

Several workers have kept fish in darkness for several days in order to "break any endogenous rhythm" and thus ensure total dark adaptation. It now seems, at least in the case of the trout, that this would be counterproductive and that to

ensure totally dark adapted eyes during daylight hours, the fish should be subjected to darkness for only two or three hours. As suggested by Blaxter & Jones (1967), the best way to get around this problem is to use fish that are naturally adapted to the desired condition. Engström & Rosstrop (1963), for instance, kept Leuciscus rutilus in darkness for four days to break any rhythm, but as John & Kaminester (1969) pointed out, the retina that they considered as dark adapted looks more like the noon dark adapted retina of goldfish than a nocturnally dark adapted retina. These eyes had therefore probably also "relaxed" to an intermediate position. Similarly, John & Kaminester (1969) found maximal dark adaptation to occur only during the first night in darkness.

A comparison with the literature (2.1) shows that the rhythm presented here is unique among those reported so far. The only results even remotely similar are those of John & Gring (1968), who noted greatest light adaptation at sunrise and sunset (fig 2.1g), and Wigger (1941), where light adaptation was again greatest at sunrise (fig 2.1b). However, it is quite probable that such a two peaked rhythm is not restricted to rainbow trout, and that is has been missed in other species due to infrequent sampling. As John et al (1967) point out, "the shape of the curve is a function of the sampling interval." If this kind of rhythm is looked for in future studies it may well be found to be quite widespread, even if restricted to certain types of fishes (see below).

All previously reported retinomotor rhythms are assumed, in general terms, to be similar to the kind of rhythm revealed by frequent sampling in Astyanax (John & Kaminester 1969), a pattern similar to the one found in nature (fig 2.5). All these studies have revealed some degree of light adaptation in the

middle of the day, a situation very different to the present study. As John & Kaminester (1969) consider even the relatively small differences in the rhythms of Astyanax, Carassius and Lepomis to be attributable to species differences, it is unlikely that the unique pattern seen in the rainbow trout is attributable to anything else.

If true species variation does exist, an explanation for these differences must be sought. Several studies have established a correlation between the position of the retinal elements and certain behavioural phenomena (Olla & Marchioni 1968, John & Gring 1968, John & Haut 1964 and McFarland et al 1979), so that John & Gring (1968) state, "It would not be surprising to find that behavioural rhythms and retinomotor rhythms are commonly correlated in fishes." Thus an explanation of the observed species differences in retinomotor rhythms may be found by examining the behavioural rhythms of the various species.

Most work on rhythmic phenomena in fishes has concentrated on activity patterns, whilst the rhythms of other functions, which may well be associated with activity, such as, feeding, sound production, hormone levels, oxygen consumption and metabolic activity, have been investigated to a lesser degree. Species are therefore normally classified into one of three categories, based on their activity rhythms; day or light active (diurnal), night or dark active (nocturnal), or active at twilight (crepuscular), although as will be shown below, classification by such criteria is not always this simple. The term diurnal has often been wrongly used to describe a behavioural cycle of twenty-four hour period (eg: Woodhead 1966), when strictly it only applies to daylight activities. To designate a twenty-four hour rhythm the correct notation of circadian should be used, and throughout the following discussion diurnal refers only to cycles maximal during daylight hours.

The photomechanical movements of the rainbow trout show a crepuscular rhythm and it is possible that this is related to a behavioural rhythm of the same type. Many species of fish have been shown to have crepuscular behavioural rhythms, both in the laboratory and in the wild, using such diverse techniques as gill netting, radio and ultrasonic telemetry, direct observation, activity paddles, photocells and a wide variety of other devices that monitor activity levels in the laboratory (eg: Jones 1956 Phoxinus phoxinus, Alabaster & Robertson 1961 Rutilus rutilus, Abramis brama & Perca fluviatilis, Siegmund 1969 Perca fluviatilis & Tinca tinca, Carlander & Cleary 1959 Stizostedion vitreum, Gibson 1973 Pleuronectus platessa, Stickney 1972 Clupea harengus harengus, Hasler & Bardach 1949 Perca flavescens, Gibson 1969 Blennius gattorugine & Blennius sanguinolentus, Darnell & Meierotto 1965 Ictalurus melas, Wikgren 1955 Lota vulgaris, Bohn & Winn 1966 Anguilla rostrata). More comprehensive reviews of fishes having crepuscular behavioural and physiological rhythms can be found in Woodhead (1966), Blaxter (1970), Winberg (1960) and Swift (1962). Such two peaked rhythms are so widespread that Aschoff (1957 &1966) considered them as the most basic and common behavioural pattern in many animals, and it is therefore not surprising to find examples of a similar pattern in salmonids.

Unfortunately, a search of the literature revealed no detailed studies of Salmo gairdneri behavioural rhythms. The three published studies on rainbow trout telemetry (Frank 1968 and Nomura et al 1969 & 1972) are preliminary studies monitoring the ECG. Two make no reference to circadian variations, while Nomura et al (1969) do so, but no satisfactory conclusions can be drawn as sampling only occured every two hours. The most conclusive data come from a laboratory study by Landless (1976), who demonstrated that rainbow trout, trained to demand feed,

showed a correlation between feeding peaks and dusk. The same author also concludes that a dawn peak may have been present "but not clear because of variations in light intensities overnight which allowed more or less nocturnal feeding, which in turn affected the degree of satiation at dawn".

Studies on other salmonid species, especially the brown trout, Salmo trutta, however, are numerous. Some of the most conclusive work, and arguably most relevant to the natural situation, is that using ultrasonic telemetry on wild brown trout in Airthrey loch, at the University of Stirling. These studies often revealed crepuscular peaks of activity (Holliday et al 1974, Tytler et al 1977, Young et al 1972), heart rate (Priede & Young 1977) and feeding (Oswald 1978), with the peaks occurring near, or coinciding with, dawn and dusk. Similarly, van Someren (1940) observed an "evening rise" in brown trout that occured at the same time after sunset every day. Swift (1962 & 1964) demonstrated a peak of activity at dawn in both artificially and naturally fed brown trout, held in cages on lake Windemere. Chaston (1969) also noted peaks of activity at dawn and dusk, as did Bachman et al (1979) using a shuttlebox.

In other salmonds, Hoar, for example, (1942) showed peaks of feeding at dawn and dusk in Salmo salar parr and smolt and Salvelinus fontinalis smolt, as did Kalleberg (1958) in juvenile Salmo salar. Richardson & McCleave (1974) and Varanelli & McCleave (1974) found juvenile Salmo salar to be either nocturnal, diurnal or active at the light/dark transition. Ali (1964b) investigated oxygen consumption, locomotor and feeding activity, also in Salmo salar, but the results of this study are very hard to interpret, although there is a general indication that the fish are day active.

Although,in general,salmonids show crepuscular activity

patterns, the results of Richardson & McCleave (1974) and Varanelli & McCleave (1974) demonstrate that the activity of different individuals of one species can show different patterns. It is therefore often difficult to categorise a species as either diurnal, nocturnal or crepuscular, a problem that is compounded by the fact that other factors, such as time of year, location and the method of measurement can influence the rhythmic pattern observed (see below). These observations moved Eriksson (1978) to say, "The reporters do not even always agree in such an apparently evident thing as the phasing of activity patterns; to look in the literature to find out whether a particular fish species is nocturnal or diurnal might be a hazardous idea".

It has been shown for many groups of animals, including fish, that one of the reasons why it is impossible to generalise about activity patterns is that they change throughout the year. This was first recognised in fish by Wikgren (1953) in the lamprey, Petromyzon fluviatilis. Subsequently, Müller (1969) working on Salmo trutta in three different locations at various latitudes (66°N, 55°N & 47°N), showed that at two locations the activity pattern changed from diurnal in winter to nocturnal in the summer, through an aperiodic phase during the changeover. The phase shift in the third location showed a different pattern. Similar phase changes for S. trutta and S. salar have been reported by Eriksson (1973 & 1978) in extreme arctic regions, except in these studies at the changeover from nocturnal to diurnal the fish were crepuscular. These changes can be explained in terms of a basic two peak system that merges in the extreme arctic daylengths. Therefore, in winter, with very short daylengths (ca three hours of light), the peaks merge to produce a day active pattern, in spring the day-length increases allowing the peaks to separate, thus

revealing the true crepuscular pattern. In summer, with very short periods of darkness, the peaks once again fuse to give a nocturnal pattern. Similar changes will occur to a lesser extent in less extreme latitudes. Such a basic crepuscular pattern may be explained in terms of two separate oscillators coupled to dawn and sunset (Pittendrigh 1953). Changes in behavioural patterns with time of year have also been noted by Kalleberg (1958, S. salar), Chaston (1969, S. trutta), Hasler & Bardach (1949, Perca fluviatilis), Siegmund (1969, Perca fluviatilis, Tinca tinca & Scardinius erythropthalamus) and Staples (1978, Philypnodon breviceps). Eriksson (1978) further noted that when Richardson & McCleave's data for S. salar are rearranged with respect to the time of year, different patterns of activity are seen to occur at different times of year, with the change-over fish once again being crepuscular.

The evidence presented above substantiates the statement made by Bachman et al (1979); "Notwithstanding considerable variation among individual experiments, the overall pattern emerging from the above studies is that activity peaks (in brown trout) tend to be associated with dawn and/or dusk, indicative of a crepuscular pattern." This basic crepuscular pattern demonstrated in S. trutta, correlates well with the endogenous crepuscular retinomotor rhythm found in the present study for S. gairdneri. It would of course be preferable to have similarly extensive behavioural data for S. gairdneri, but a correlation to S. trutta activity patterns may nevertheless be valid as the two species are closely related. If so, it would support the idea that retinomotor and behavioural rhythms are associated.

However, such a two peaked retinomotor rhythm appears to be non-adaptive. It would seem reasonable that at dawn the

retinal elements should move to their light adapted position, and also that they should return to a dark adapted position after encountering darkness, but to become light adapted again at dusk, when normally the elements would be dark adapting, has no obvious function. Such a seemingly non-adaptive pattern can however be understood if one takes the viewpoint of Schwassman (1971), who considers overt rhythms to be no more than the hands of an internal clock: "Overt rhythms must not be confused with the endogenous timing system itself; they are merely proof of its existence ..... Therefore, an adaptive significance of all the separate manifestations of the circadian clock may not always be obvious or may be difficult to recognise ..... in the past too much emphasis was placed on finding some adaptive value in any and all of the overt rhythmic activities of organisms. These are to be looked upon as manifestations of a circadian organisation,and the many overt periodicities by themselves may or may not be of adaptive significance." That is to say, the twin peaks shown by the retinomotor movements of the rainbow trout may be no more than the side effects of the fish's basic crepuscular organisation.

Such a behavioural link with retinomotor movements may be of great benefit in nature, especially if the mechanism outlined in chapter one is operating. Thus by ensuring that the retinal elements move, the crepuscularly active trout will have both its rods and cones exposed, ensuring maximal use of receptors at dawn and dusk, the times at which the fish is most active. Normally at dawn the receptors are initially dark adapted, as is the case in continual darkness. The movements are then triggered by the internal clock and the retinal elements change position. In nature this would be adaptive, but in the experimentally induced continual darkness the elements are

in an inappropriate position for the lighting conditions, and therefore return to the dark adapted position. At dusk, in nature, these movements are once again set in motion, adapting the fish to twilight. The internal mechanism in the artificial continual darkness still continues to trigger a "change" in the position of the retinal elements, thus causing another peak of light adaptation, which due to the maintained darkness will once again be only transitory. The fact that in other studies the retinal elements are maintained in the light adapted position throughout the day while in continual darkness, may be due to a different, non-crepuscular, underlying timing mechanism. It would be interesting to see if other crepuscular fish show similar bimodal retinomotor peaks when maintained in darkness, and if such peaks ever occur in non-crepuscular species.

The control of photomechanical changes, at least in rainbow trout, can thus be thought of as consisting of two components: An endogenous component, that causes the bimodal pattern in maintained darkness, and a direct effect of light, that maintains light adaptation throughout a normal day. Such central control has been indicated, for instance, by the demonstration of an effect of hypophysectomy on photomechanical changes (Ali & Pickford 1979), while a local effect of light has been demonstrated using small spot stimuli by Easter & Macy (1978) and with unilateral illumination by Ali (1964c). A direct stimulating effect of light explains why the fish remain light adapted and do not show any rhythm in continuous light (fig 2.8). This can be regarded as "positive masking" by light of the endogenous rhythm (Schwassman pers com).

The failure of Ali (1959 & 1961) and Wagner & Ali (1977) to find any sort of real rhythm in other salmonids may be due to true species differences, but it is also possible that it is

due to time and place of sampling (see above), age of fish, individual variation or the experimental situation. Differences in behavioural rhythms within a species due to individual variation unrelated to time of year have been noted, among others, by Spoor (1946) and Spencer (1939) in Carassius auratus. The best example of the experimental design affecting the pattern comes from the work of Jones (1956) on the minnow, Phoxinus phoxinus. In open aquaria the fish were day active, but if a brick was put in the aquarium, to provide a simple shelter, the pattern of activity was reversed. Different recording techniques will also account for some variation, since "every registration technique has something in common with a distorting mirror" (Verheijen & DeGroot 1967). Behavioural patterns differing with age have been demonstrated by Staples (1978) in Philynodon breviceps and Darnell & Meierotto (1965) in Ictalurus melas. Woodhead (1966) states that ontogenetic differences probably also occur in marine fishes, but offers no proof. There are thus basically two causes of variation within a species; artificial variation due to the experimental system and real variation due to location, time of year and age of fish. In the light of such factors it is not surprising that when rhythms are compared they show certain differences.

CHAPTER 3 RETINOMOTOR FUNCTION I - EFFECT OF BLEACHING ON VISUAL PIGMENT LEVELS IN LIGHT AND DARK ADAPTED RETINAS.

3.1 INTRODUCTION

Ever since the discovery of photomechanical movements, people have attempted to find an adaptive significance for them. The great abundance of such theories around the turn of the century led Arey (1915) to state that "Many such explanations reveal the resourcefulness of the human mind rather than the ingenuity of nature." These early explanations have been summarised by Arey (1915), and later summaries by Detwiler (1943) and Ali (1971 & 1975) have very little to add, giving a good indication of the lack of progress made in this area of research. Most of these early ideas can now be discarded and only the two major contending hypotheses will be discussed here.

Exner & Januschke (1906) and Herzog (1905) saw retinomotor movements as a means of adapting the eye to photopic and scotopic vision, or as Herzog (1905) put it, to switch the cone system on and off. During daylight, when the level of illumination is high, the cones are contracted and are thus the first receptors to intercept the light, while the rods are buried in the expanded pigment epithelium, and so protected from overstimulation by the high light levels. Conversely, at night, the cones, which are non-functional at such low levels of illumination, expand, accompanied by epithelial retraction, leaving the contracted rods free to receive the low intensity illumination unimpeded. The obvious advantage of such a system is that the receptors involved in vision at any one time are the ones primarily stimulated by the incident light. An additional advantage is that the rod visual pigment is protected from bleaching by the high levels of illumination during the day. Thus the rods,

having large amounts of visual pigment, will be fully functional at dusk, and the system therefore guarantees maximal sensitivity at all times.

The theory of Herzog (1905) and Exner & Januschke (1906) assumes that the cones only begin to migrate in levels of illumination at which they become functional and that at all other intensities they remain fully elongated. Garten (1907) and subsequent authors (eg: Arey 1915 and Detwiler 1943), assumed that in"very dim bright" (Arey 1915 and Detwiler 1943), or at twilight (Garten 1907), only the rods are utilised and that therefore the cones, according to the above theory, should remain elongated. Garten (1907) observed, however, that the cones of Abramis brama had already started to migrate at these low levels of illumination and concluded that as the cones started to contract at an intensity at which he assumed only the rods were involved in vision the theory of Herzog (1905) and Exner & Januschke (1906) could not be correct. This objection, however, is unlikely to be valid because, as outlined in chapter one, at twilight it is probably not only the rods that are involved in vision but the cones as well.

This seemingly erroneous criticism of Garten's led him to propose an alternative. He suggested that the ellipsoids of cone outer segments in teleosts, and the oildroplets in the cones of amphibians, reptiles and birds, would cause a lot of light to be refracted out of the visual cell. The pigment epithelium, interdigitating between the cones when light adapted, would serve to stop this stray light from stimulating neighbouring visual cells. This will serve to increase acuity, and the underlying rods will also be protected, minimising the bleaching of rhodopsin. Conversely, the retraction of the pigment epithelium and expansion of the cones during dark adaptation, coupled with the contraction

of the rods, ensures maximal use of the low level of illumination by the rods. As the rods are no longer isolated from one another, any light escaping will stimulate adjacent receptors, thus serving to further increase sensitivity. At intermediate levels of stimulation neither rods nor cones are isolated, and both will be stimulated by stray light. Garten (1907) assumes that at this intensity sensitivity, and not acuity, is still at a premium.

Garten's theory is often incorrectly applied to rods. Arey (1915), for instance, says "the expanded pigment surrounding the outer members of the visual cells serves to absorb all light which escapes from the rods by refraction and thereby makes possible an independent stimulation of individual cells." Yet the importance of Garten's idea is in its relation to the cones and not the rods, as the rods are either buried in the epithelial pigment when light adapted and therefore cannot be stimulated, or completely free of pigment epithelium when dark adapted. The situation for isolating rods from one another thus never arises. In fact, stimulation of neighbouring rods when dark adapted, as pointed out by Garten, is desirable as it increases sensitivity, whereas isolation between cones is important during high intensity cone vision, when acuity and not sensitivity is needed.

This theory is also often supposedly supported by evidence concerning the optical properties of rods. For instance, Ali (1975) states that, "in pure rod retinas whose rods are slender (eg; deep-sea fishes) R.E.P. is sparse." As Garten noted that in long slender rods total internal reflexion could prevent light escaping, Ali wrongly implies that the pigment epithelium is sparse because the rods are slender. This is not necessarily so. Deep-sea fishes have retinas specialised to maximise sensitivity, and to decrease sensitivity in such retinas by the

presence of pigment would be counterproductive. Sensitivity is in fact the cause for the long rods. The pigment is absent because it would serve no function as the light levels are too low to necessitate rod protection, and cone isolation is irrelevant as there are no cones. Even assuming that individual rods were stimulated without interference from neighbouring rods, any information so gained at the receptor level would soon be lost due to the high degree of summation between rods.

Despite these various theoretical speculations, the precise function of the pigment epithelium in retinomotor movements is still uncertain, and all workers since Arey (1915) have noted that the diversity of retinal responses is so large that a single theory, that can account for all the observed species differences, is unlikely to exist (Arey 1915, Detwiler 1943 & Ali 1975). It must be remembered, however, that such statements are largely based on early work, a lot of which was not properly controlled, and much of the observed diversity is probably apparent rather than real.

At least a partial solution to this problem comes from the recognition that the two major contending theories are not really mutually exclusive. In both theories the rods are protected from overstimulation when light adapted, and on dark adaptation the rods are positioned so as to make maximal use of the impinging illumination. The cone isolation proposed by Garten is not excluded by anything said by Exner & Januschke (1906) and Herzog (1905). The only real conflicting statement between the theories concerns the position at twilight. Garten (1907) proposes that both the rods and cones are active at these intermediate levels of illumination, while Exner & Januschke (1906) and Herzog (1905) adhere to a theory of either rod or cone vision.

It is in fact theoretically possible to propose a function for the photomechanical movements observed in teleosts using both of the above theories, that can account for all the observed retinomotor changes. The basis of such a theory is that at all times best use is made of the receptors. At high levels of illumination the cones are contracted and are thus ideally positioned to receive the light, the expanded pigment epithelium serving to protect the rods from overstimulation, and isolate the cones from one another, thus improving acuity. At intermediate light levels neither the rods nor cones are protected or isolated, thus making maximal use of both receptors at twilight, which may be beneficial at such an important time (chapter 1). While at low light levels the rods are positioned so as to receive the impinging illumination unimpeded by the cones, and sensitivity is maximised by the lack of interdigitating pigment, allowing stimulation of neighbouring receptors by refracted light. Therefore, evidence such as presented by Ali (1975) in favour of Garten's (1907) idea, that in the light adapted state the cones in pure cone retinas of some animals are isolated from one another, in no way contradicts the above theory for a duplex retina.

Theoretical hypotheses should be backed up by experimental evidence, yet up until the present time retinomotor function in vertebrates is an area where there have been very few critical experiments. Proof for one theory or another has come mainly from theoretical speculation or incidental observations. This should in no way detract from the value of such studies, but there is an obvious need for controlled experiments. The following two chapters (3 & 4) outline experiments carried out to investigate one of the possible functions of the epithelial pigment, namely that it serves to protect the rod visual pigment

from bleaching when the retina is light adapted. Support for this has been obtained using two different techniques. In this chapter the effect of bleaching on the extractable rod visual pigment levels in both light and dark adapted retinas is examined, thus determining if expanded pigment epithelium protects the rods from being bleached. Chapter 4 examines the relationship between the position of the retinal elements and retinal sensitivity, as measured by the b-wave.

In the present chapter, three questions will be asked: Is the rod visual pigment protected in nature? Is the pigment epithelium responsible for this protection? To what quantitative extent does the pigment epithelium shield the rods? An answer to the first question was obtained by following the movements of the retinal elements through the twilight periods, as in chapter 1, and relating this to extractable visual pigment levels. If the epithelial pigment serves to protect the rod visual pigment, one would expect the amount of visual pigment to remain constant throughout the day and night. If, on the other hand, it does not, one would expect the pigment levels to be low during the daytime and only rise over the dusk period, as the light level gets low enough to allow regeneration. The highest levels of visual pigment would only be observed in fish during the night. Conversely, during dawn the amount of pigment should fall as the light intensity rises if the rods are unprotected.

The second experiment indicates that it is the covering of the rods by the pigment epithelium that is responsible for this protection, by showing that dark adapted eyes, whose rods are fully exposed, are more susceptible to visual pigment bleaching than are light adapted eyes, which have their rods totally covered by the pigment cells. This was a pilot study for the final experiment, which tried to determine how efficient

the pigment epithelium is in its protective role. Experiment three was similar to one carried out by Back, Donner & Reuter (1965) on the common frog, Rana temporaria, the aim of which was to find the intensities of bleaching lights that will bleach a criterion amount of visual pigment in both the light and dark adapted state. The relative difference between these two intensities serves as a measure of the protective ability of the epithelial cells.

3.2 METHOD OF PIGMENT EXTRACTION

It is usual when extracting visual pigment to separate the retina from the rest of the eye and use it alone for extraction . This was avoided in the present study as pigment was extracted from light as well as dark adapted eyes, whereas former workers have usually only extracted from dark adapted eyes. In the dark adapted condition the rods are easily separable from the epithelial pigment as this is aggregated at the back of the eye. When light adapted, however, the pigment completely surrounds the rods, and any attempt to separate the retina from the pigment epithelium results in the loss of a substantial proportion of the rods.

The eyes were hemisected under dim red light and the front half and lens discarded. The remainder of the eye was then washed five times by placing it in a centrifuge tube containing 5 ml of McIlvanies buffer (pH 4.8) and thoroughly macerating it with a spatula. Following each wash the eyes were centrifuged for thirty minutes at 5000 r.p.m., the supernatant was pipetted off and discarded, and a fresh 5 ml added. These washes resulted in a clear supernatant which was discarded, and 0.5 ml of 3% digitonin solution was added to the residue. After stirring

this was then left to extract overnight at room temperature.

The following morning, after a further thirty minutes centrifuging at 5000 r.p.m., the supernatant containing the pigment was pipetted off and ultracentrifuged for thirty minutes at 17000 r.p.m. with 0.05 ml saturated sodium borate and 0.5 ml of 0.2 ml hydroxylamine (pH 7). The absorption spectrum of the resultant supernatant before and after a ten minute tungsten light bleach was determined on a Cecil CE 505 double beam UV spectrophotometer, and a difference spectrum constructed.

All eyes within an experiment were treated in exactly the same manner so as to avoid errors in the measured amounts of visual pigment of different eyes due to differences in technique. All eyes were, for instance, macerated for exactly one minute. The total amount of pigment in an extract was represented by the maximum density change between the bleached and unbleached pigment. If one assumes the retina is a hemisphere, by measuring the diameter of each eye before extraction the total surface area of the retina can be calculated. In this way variations in the amount of pigment due solely to the size of the eye can be corrected for.

3.3 EXTRACTABLE VISUAL PIGMENT LEVELS DURING TWILIGHT

3.3.1 Methods. The procedure in the field for this experiment has been outline in section 1.3.1. For the extraction of visual pigment all three eyes from one sample were pooled and the resulting maximum pigment density divided by the sum of the surface areas. This figure was taken as representing the relative concentration of pigment in that sample.

3.3.2 Results. The amount of visual pigment at different times

during dusk was determined on two occasions. Fig 3.1 shows the average values obtained at equal light intensities, on both days. Not all values of the amount of visual pigment extracted (table 3.I) are included in fig 3.1, as the same intensities were not sampled in both experiments. The extraction results are plotted above histological results at the times corresponding to these intensities. No actual times can be plotted on the abscissa as the same intensity occurred at different times on the two occasions, although the intensities at which these eyes were sampled can be ascertained from fig 3.1 and table 3.I.

Results for a single dawn are shown in fig 3.2. The large amount of variation in the visual pigment content is due to the fact that on three occasions only one fish was sampled and at the others only two, owing to the death of several fish overnight, caused by a failure in the water supply.

In figs 3.1 & 3.2,(1) represents the amount of visual pigment in dark adapted eyes following ten minutes exposure to full daylight, and (2) is the amount of pigment in fully dark adapted eyes. A more detailed description of this is given in the following section. During neither dawn nor dusk did any group of eyes contain as little visual pigment as bleached dark adapted eyes (1).

3.3.3 Discussion. The amount of visual pigment present in dark adapted eyes that have been subjected to ten minutes of daylight (1), is the amount that would be present in the eyes of those fish exposed to the highest levels of illumination if the rods were not in some way protected. As this low level of pigment was not reached during any stage of either dawn or dusk, the pigment must in some way be shielded from the incident light.Neither did the level of visual pigment increase during dusk or decrease

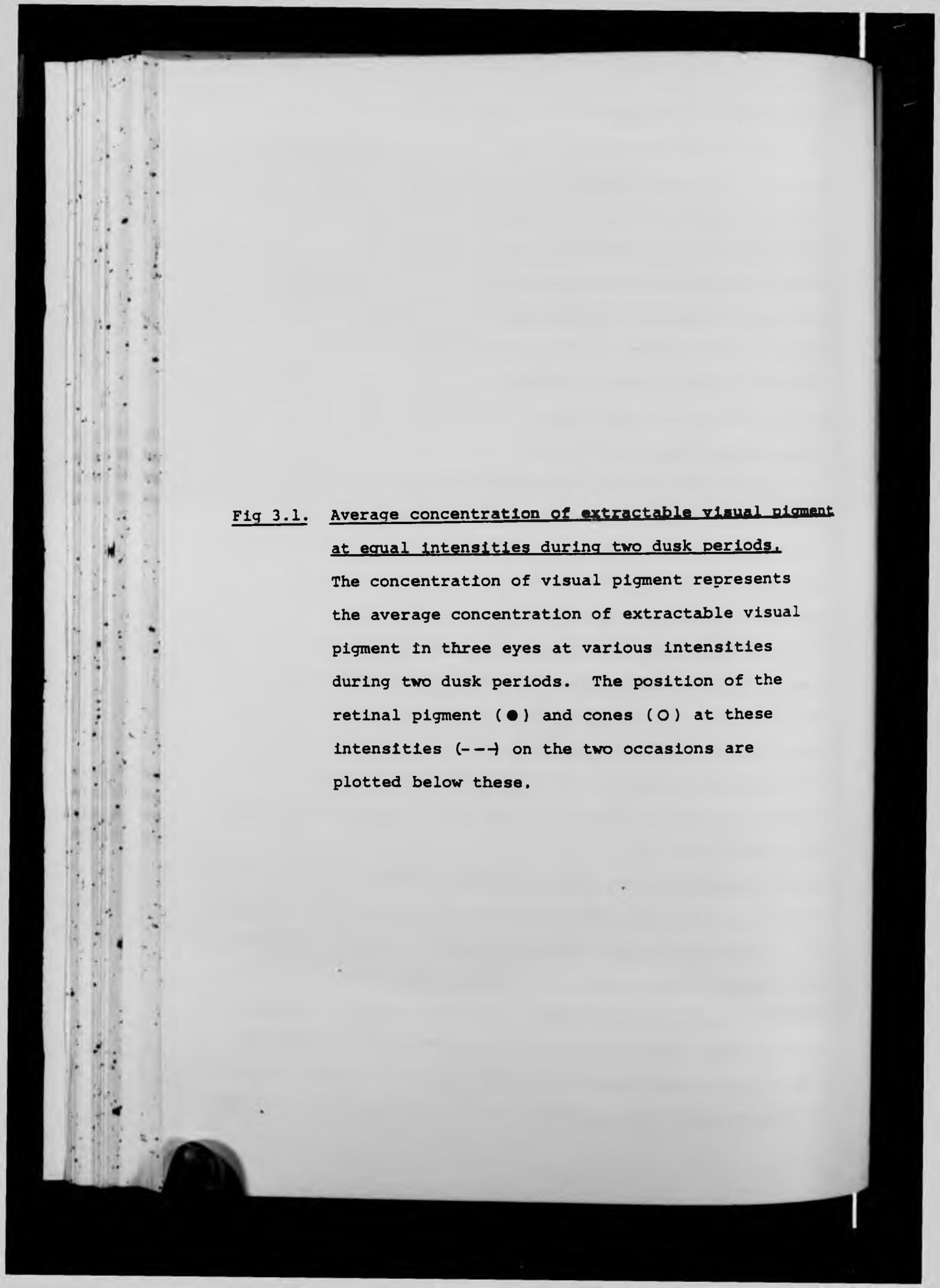

Fig 3.1. Average concentration of extractable visual pigment at equal intensities during two dusk periods.

The concentration of visual pigment represents the average concentration of extractable visual pigment in three eyes at various intensities during two dusk periods. The position of the retinal pigment (●) and cones (○) at these intensities (- - -) on the two occasions are plotted below these.

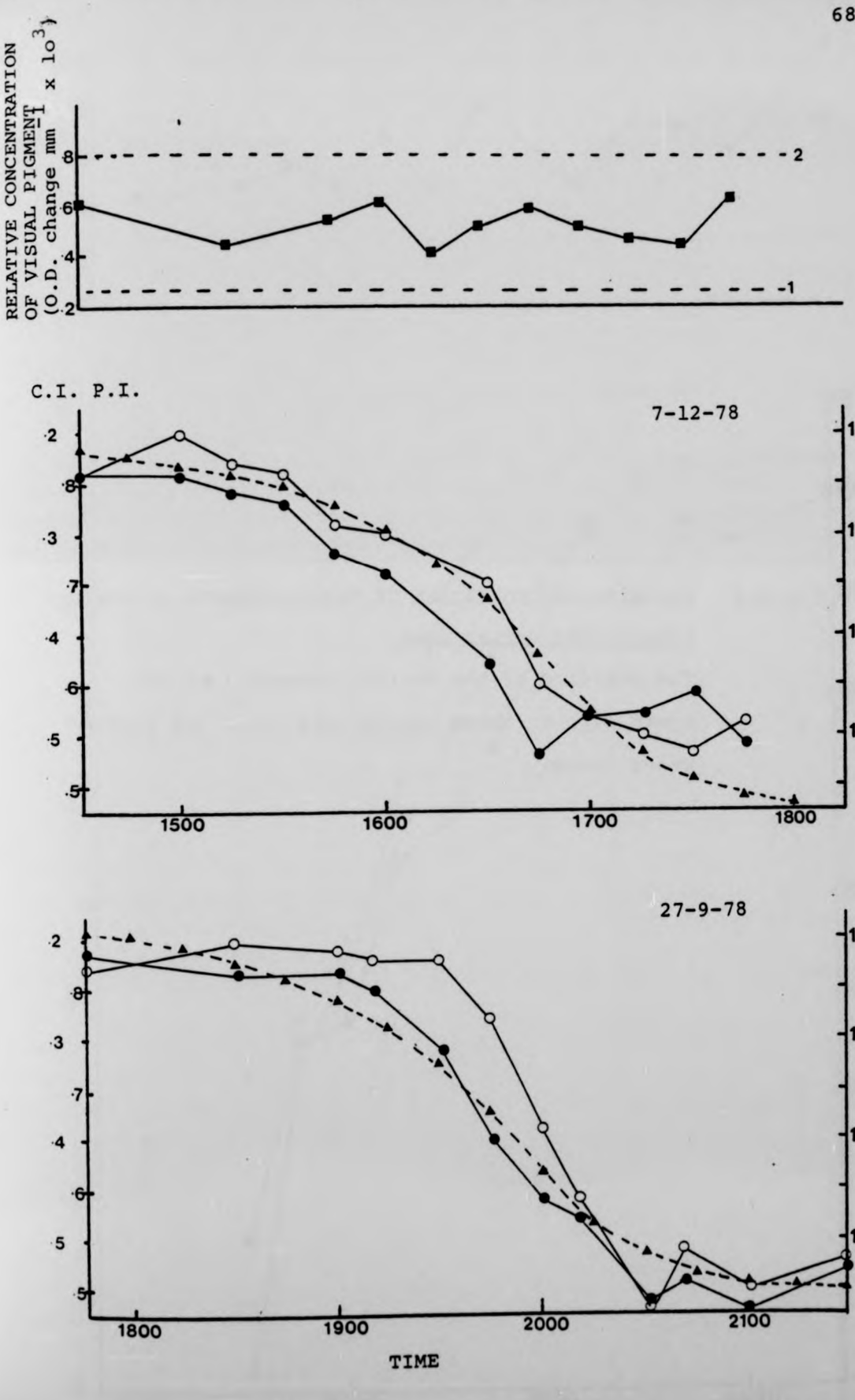

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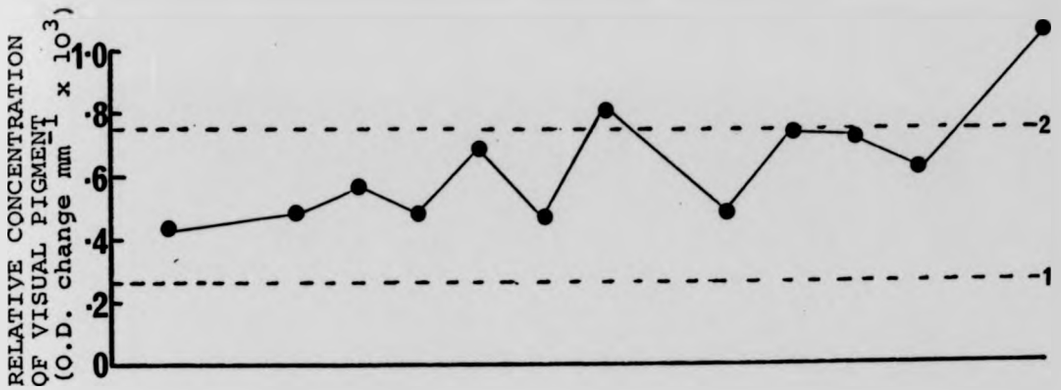
Fig 3.2. Relative concentration of visual pigment at various intensities during dawn.

The position of the retinal pigment (●) and cones (O) at these intensities (- - -) are plotted below these.



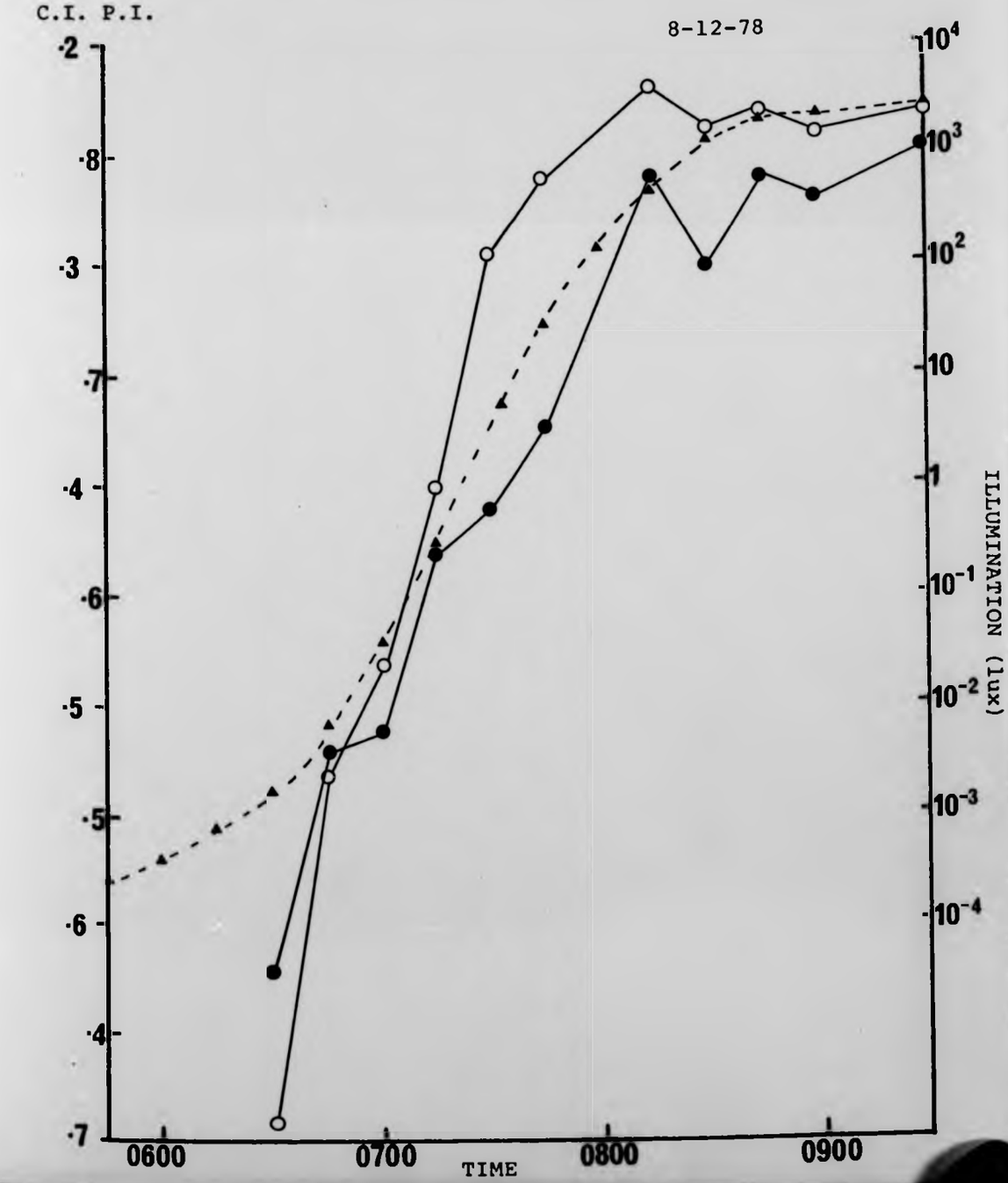


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TABLE 3.I Relative concentration of extractable visual pigment at various intensities during two dusk periods.

The concentration of visual pigment is given as optical density change per $mm^2 \times 10^3$.

Date:	17 - 12 - 78	27 - 9 - 78		Average Concentration of visual pigment
Light Intensity (Lux)	Concentration of visual pigment	Light Intensity (Lux)	Concentration of visual pigment	
7×10^3	.421	1.5×10^4	.824	.623
3.5×10^3	.397			
2.5×10^3	.275	4.75×10^3	.626	.451
10^3	.373			
6×10^2	.544	8×10^2	.566	.555
1.7×10^2	.499	3.8×10^2	.725	.612
4.5×10^1	.531	6×10^1	.304	.418
8	.556	5	.488	.522
6×10^{-1}	.489	3.5×10^{-1}	.692	.591
6×10^{-2}	.510	5×10^{-2}	.528	.519
1.2×10^{-2}	.366	7.5×10^{-3}	.583	.475
3×10^{-3}	.268	2.5×10^{-3}	.636	.452
1×10^{-3}	.508	1×10^{-3}	.762	.635
		6×10^{-4}	.721	

during dawn, as would be expected if the high levels of illumination experienced during the day in any way bleached the pigment. The level of visual pigment during dusk was relatively constant, while the increase in pigment levels during dawn is in fact in the opposite direction to that expected if the pigment were being bleached.

At no time during dusk was the level of visual pigment as high as that in fully dark adapted eyes in the laboratory (2). This may be due to differences in the techniques of extraction, as in the field eyes were frozen before extraction while in the laboratory the pigment was extracted immediately following the death of the fish. It could also be argued that at no time during dusk did the eyes have a full complement of visual pigment, and that the pigment was only fully regenerated after several hours in the dark, at which time no fish were sampled. That this is not the case is indicated by the level of pigment at the beginning of dawn (fig 3.2), before the intensity of illumination has started to rise. These eyes have been in the dark overnight and should thus contain the maximum amount of visual pigment.

This lack of systematic variation and the failure of the pigment level to reach as low a level as that of bleached eyes during either dawn or dusk suggests that the visual pigment is shielded from bleaching during periods of high illumination. Such protection ensures that the fish go into the dusk period with eyes that are fully functional for low light intensity vision, which will save both the time and metabolic energy needed for pigment regeneration.

The theory proposed by McFarland et al (1979), outlined in chapter 1, that differential migration of cones and epithelial pigment ensures both rods and cones are functional at twilight,

is therefore feasible. The above authors state that, "although untested this mechanism might minimise excess bleaching of rod visual pigment and yet allow sufficient movement of the pigment epithelium .....". That bleaching of rod visual pigment is minimised is confirmed here for the rainbow trout.

3.4 EXTRACTABLE VISUAL PIGMENT LEVELS IN LIGHT ADAPTED, DARK ADAPTED AND BLEACHED EYES.

3.4.1 Methods. Three groups of three fish were exposed to different conditions of illumination:

Group (1) - 3½ hours in direct sunlight (ca 5 x 10^4 lux).

Group (2) - 3½ hours in darkness over the same period as group (1).

Group (3) - as group (2) followed by ten minutes exposure to sunlight.

At the end of each treatment, one eye from each fish was fixed in Bouin's fixative for histological examination while the visual pigment was extracted from the other. This experiment was carried out twice (6.10.78 & 19.10.78) at exactly the same time of day (10.00 - 13.30).

3.4.2 Results. The amounts of extractable visual pigment in the three groups of fish are shown in Table 3.II. The fish that had been in the dark for the whole time contained most pigment, although fish that had been in the light had on average only 18% less. Fish that had been dark adapted and then exposed to light, on the other hand, had considerably less pigment, with only 36% as much as fully dark adapted eyes.

The histological results confirmed that fish kept in the light and dark only, are respectively totally light and dark adapted in terms of photomechanical changes. Group (3) eyes, which were only in the light for ten minutes, were nearly totally light adapted, with average pigment and cone indices of 0.73

TABLE 3.II Visual pigment levels in light adapted (group 1), dark adapted (group 2) and bleached (group 3) eyes.

TREATMENT OF FISH	CONCENTRATION OF VISUAL PIGMENT IN EXP. 1	CONCENTRATION OF VISUAL PIGMENT IN EXP. 2	AVERAGE CONCENTRATION OF VISUAL PIGMENT
LIGHT ADAPTED	0.6	0.61	0.605
DARK ADAPTED	0.624	0.852	0.738
DARK ADAPTED FOLLOWED BY 10 MINUTES LIGHT	0.261	0.265	0.263

The concentration of visual pigment is given as optical density change per $mm^2 \times 10^3$.

and 0.27, although, when the fish were first exposed to the light they were dark adapted, as they came from the same tank and were taken out the same time as fish in group (2). This is to be expected because in the laboratory, under weaker light adaptation, it takes only fifteen minutes to light adapt (1.4).

3.4.3 Discussion. These results give further evidence that the visual pigment is protected from bleaching in the light adapted condition, and indicate that the epithelial pigment is responsible for this shielding. If the rod visual pigment is protected the fully light adapted eyes should have a large amount of visual pigment, group (1), as should those of group (2) that have been in the dark, while fish from group (3), whose fully exposed rods were subjected to full daylight illumination, should have very little visual pigment. These expectations were fully confirmed. If, on the other hand, the rods were not protected, groups (1) and (3) should have equally low amounts of visual pigment, with only fully dark adapted eyes having a full pigment complement.

3.5 DEGREE OF ROD PROTECTION AFFORDED BY THE EPITHELIAL PIGMENT

3.5.1 Methods. Pilot experiments showed that the total amount of pigment extracted from the two eyes of a given individual differed by only 13% in both the light and dark adapted state (table 3.III). It was therefore possible to measure the effect of various intensities of bleaching light on the amount of visual pigment in single light and dark adapted eyes, and express this as a percentage of the total amount of visual pigment in the unbleached eye of the same individual.

Following either two hours light or dark adaptation the fish were killed, and their eyes hemisected after removal from

TABLE 3.III. Variation in the extractable visual pigment content of the two eyes from the same individual

CONDITION	EYE A Maximum optical density change	EYE B Maximum optical density change	B as % of A	average B as % of A	average variation
	.1079	.0994	92		
Light	.1105	.1072	97		
adapted	.0714	.0703	98	87	13%
	.0780	.0652	80		
	.0954	.0671	70		
	.1307	.1190	91		
Dark	.1334	.1090	82		
adapted	.1037	.0901	87	87	13%
	.0751	.0750	100		
	.0926	.0718	78		
	.0812	.0729	90		

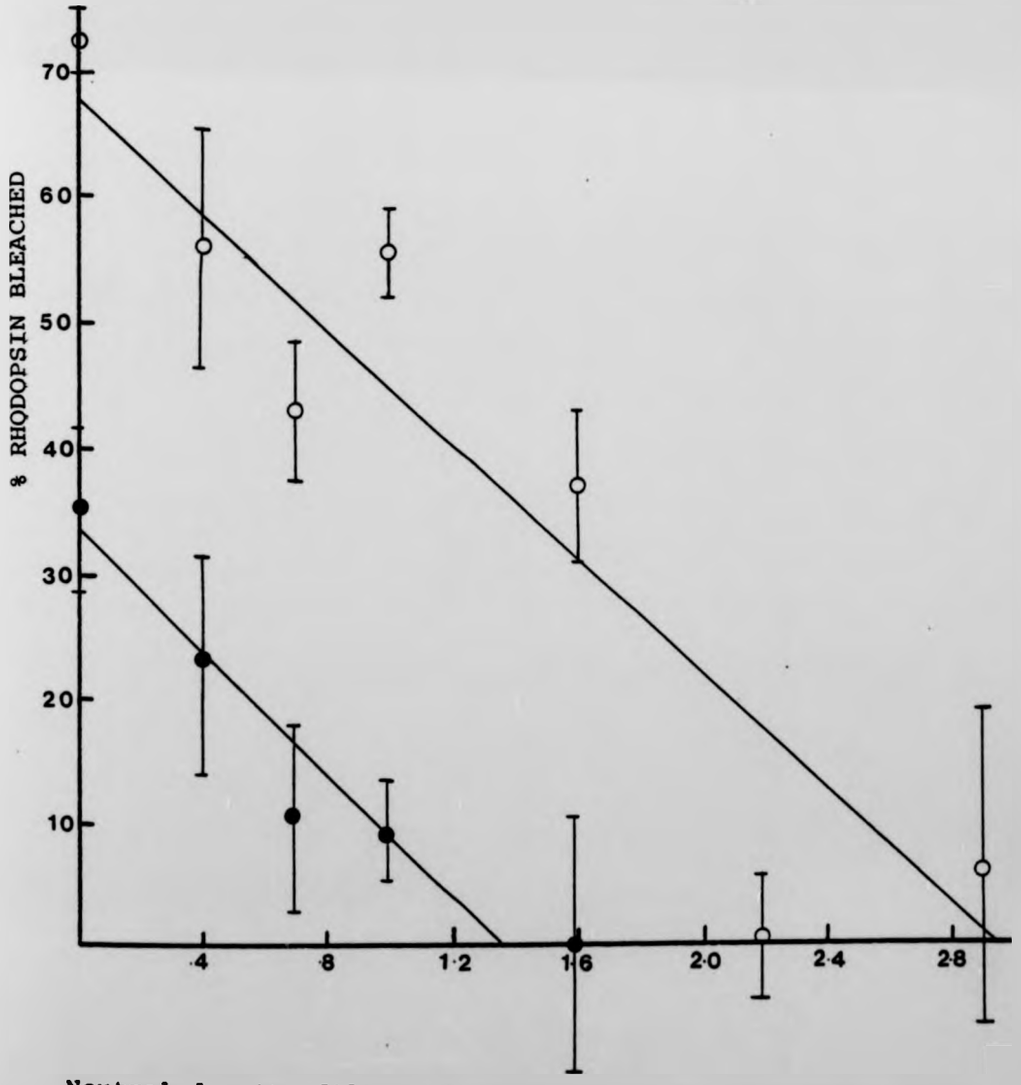
the orbit. This and all subsequent manipulations were carried out using a dim red torch. One eye was then placed directly into McIlvanies buffer, while the other was first exposed to a bleaching spot of light, which completely filled the retina, for forty-five seconds. The visual pigment from both eyes was then extracted as outlined in 3.2. A forty-five second bleach was selected as pilot experiments showed this to be the shortest duration that bleached a reasonable amount of visual pigment in the light adapted retina. By varying the intensity, through the insertion of neutral density filters into the light beam, the amount of visual pigment in both light and dark adapted eyes exposed to different degrees of bleaching was determined. This, expressed as a percentage of the total visual pigment content, gave the relationship between intensity of bleaching light and amount of pigment bleached.

3.5.2 Results. Fig 3.3 shows the percentage of visual pigment bleached by different intensities of light in both light (filled circles) and dark adapted (open circles) eyes. A straight line has been fitted to the points by regression (Sokol & Rohlf, 1969, pp 430-432) with a high level of significance (light adapted $p < 0.001$. dark adapted $p < 0.01$).

From simple examination of fig 3.3 it can readily be seen that a given light intensity bleaches more pigment in dark adapted eyes than in light adapted ones. A measure of the degree of protection given by the photomechanical movements is given by the amount the light adapted line must be transposed along the abscissa in order to differ minimally from the dark adapted curve. As the slopes of the light and dark adapted lines are very similar (-22.81 & -23.22) this is simply a matter of determining the difference between the intercepts on the abscissa. This gives a "protection factor" of 1.56 log units. In other words,

Fig 3.3. Percent of rhodopsin bleached in light (●) and dark (○) adapted hemisected eyes by a forty-five second bleach of varying intensity.

The limits shown are the standard errors of the mean.



the epithelial pigment reduces the incident light reaching the rods by a factor of 36.3.

3.5.3. Discussion. The above evidence again shows that the extractable visual pigment is protected from bleaching in the light adapted condition, photomechanical movements serving to reduce the light intensity affecting the rods by a factor of 36.3. Such a protective function of the epithelial pigment has only been quantitatively demonstrated, in terms of visual pigment content, once before in vertebrates. Bäck, Donner & Reuter (1965) determined the degree of protection in the common frog, Rana temporaria,and found that when the pigment epithelium is in the light adapted position it reduces the effective light intensity at the rods by a factor of three, which is a very much lower value than that observed here for the rainbow trout. Such a large difference in the effectiveness of the pigment epithelium in shielding the rods in two vertebrates requires explanation.

The experimental procedures used in the studies on the frog and trout differ in certain respects and these differences may be part of the cause for this discrepancy. In the first place, Bäck, Donner & Reuter (1965) used excised eyes during the adaptation period that preceded bleaching. Why this was done is not clear, but intact fish were used in the present study to avoid any possible degeneration or abnormal reaction of the retina that may be associated with isolation. Secondly, in the previous study only the small flat portion of the centre of the eye was used, so as to avoid bleaching in peripheral parts of the retina where light would strike at an angle. This was felt to be an unnecessary precaution since the Stiles-Crawford effect is largely restricted to the cones (eg: Flamant & Stiles 1948), and as one is looking at a relative effect. Furthermore,

in the functional eye all light does not strike the receptors normally. The use of such a small area gave rise to several problems in the study on frogs, owing to the small amount of pigment it yielded. The authors had to use eyes that had been dark adapted for fifteen to thirty minutes as their light adapted eyes, presumably because they were unable to get enough pigment from truly light adapted eyes. Although these eyes had their retinal elements in a nearly light adapted position, the use of eyes that have been in the dark for so long is clearly not ideal. Using larger pieces of eye allowed extraction from fully light adapted eyes in the present study. Bäck, Donner & Reuter (1965) also blame the large variation in pigment content of light adapted eyes (25%) on the small amounts of visual pigment. By using larger pieces of retina such problems were also minimised. In the frog study, pigment was only extracted from one dark adapted animal at each intensity of bleach. A greater number was not necessary as the results were confirmed using microdensitometry. In the present study at least three, and on two occasions six, fish had their pigment extracted at each intensity. Similarly, light adapted eyes from the trout were sampled at five intensities of bleaching light, while in the former study light adapted eyes were only sampled at one intensity. Finally, Bäck, Donner & Reuter (1965) used only a twofold range of bleaching intensity (0.3 log units) compared to the range of nearly three log units used in this study. It is felt that a greater range and an increased number of light adapted bleaching intensities allows a more accurate determination of the degree of protection.

Although it is possible that differences in procedure between the two studies may be responsible for part of the observed disparity, it is unlikely to fully account for all of it. The majority of the observed difference in protective

efficiency between Salmo gairdneri and Rana temporaria is probably due to species difference. The photomechanical movements of the trout are thus a good deal more efficent at shielding the rods than those of the frog.

The fitting of a straight line to the data on fig 3.3 is in no way justified on the basis of the underlying photochemistry, but complicating factors such as self screening make it unclear what the exact photochemical basis would be. A straight line was thus only fitted in order to estimate the protective ability of the pigment epithelium and should not be confused with the undetermined photochemical relationship between the amount of pigment bleached and the intensity of the bleaching light.

The three experiments outlined above demonstrate that photomechanical movements serve to protect the rod visual pigment from high light intensity stimuli in nature, and that this shielding effect is a lot more efficient than previously supposed. Further support for the shielding function of the pigment epithelium comes from the work of Blaxter & Jones (1967), Blaxter (1968a), Blaxter & Staines (1970), Ali (1959) and Ali & Wagner (1975), who have shown that almost universally throughout the teleosts, larval stages have pure cone retinas, and that retinomotor movements only develop along with the appearance of rods. This may indicate that in pure cone retinas photomechanical movements would serve no purpose. Additional evidence showing that the epithelial pigment protects the rod visual pigment will be given in the following chapter, by examining the relationship between photomechanical movements and the ERG.

CHAPTER 4 RETINOMOTOR FUNCTION II - RELATIONSHIP BETWEEN PHOTOMECHANICAL CHANGES AND THE ELECTRORETINOGRAM

4.1 INTRODUCTION.

Another way of investigating the functional significance of photomechanical changes, apart from the ones outlined in the previous chapter, is to monitor electroretinographic sensitivity changes during both light and dark adaptation. The amplitude and form of light and dark adapted ERGs differ to a large extent (fig 4.10). By following the change from one form to another, after sudden exposure to light or dark, one can get an indication of sensitivity changes during light and dark adaptation. The intensity of stimulus light necessary to give a criterion b-wave amplitude was therefore determined at various times during adaptation and taken as a measure of sensitivity.

If the rods are shielded from light by the epithelial pigment one would expect that initially, when a fish is transferred from light into darkness, sensitivity would be low as all it's rods, which will be used in darkness, are masked by the pigment epithelium. As the epithelial pigment and cones retract and the rods advance during the course of dark adaptation, an increasing number of rods will be exposed, thus increasing sensitivity, until maximal sensitivity is attained when the retinomotor changes are complete. Conversely, if a fish was suddenly exposed to light, having previously been in the dark, the cones, which are used at high light intensities, would be in an inappropriate position at the back of the eye, masked by the rods. Maximal sensitivity would again only be reached when photomechanical changes are complete and light can reach the cones unimpeded by the layer of rods. If retinomotor movements are involved in mediating sensitivity changes, as described above, one would expect a close temporal relationship

between the time taken to complete retinomotor changes and sensitivity changes during light and dark adaptation. If, on the other hand, the pigment epithelium served solely to isolate cones, such a close relationship would not necessarily be expected.

Such a direct correlation between the ERG and retinomotor responses has never been demonstrated before in vertebrates, although this has been done in several species of arthropods and molluscs (Section 4.4.6.1). The need for such a study, however, has been widely recognised, for instance; "It should be interesting to see what, if any, relationship exists between the retinomotor and electroretinographic responses ..... no direct correlation between the ERG responses and retinomotor responses has been established. This is an aspect which merits further investigation" (Ali 1975).

Form changes of the ERG associated with light and dark adaptation will also be discussed, as they have never before been described in any detail for the rainbow trout. Fonner et al (1973) and Hoffert & Ubels (1979a,b & c) have published rainbow trout ERGs, but their results are not detailed in this respect, as this was not the main concern of their study. Thus observations made during the course of this investigation on such changes, although not exhaustive, are reported and form a useful basis for comparison with published results on other teleosts. Incremental adaptation is also investigated in order to confirm the Weber-Fechner law in trout, and for a comparison of electroretinographically determined Weber fractions to those determined behaviourally (chapter 6).

4.2 MATERIALS AND METHODS.

4.2.1 General. The fish used in this part of the study were generally about seventeen cm long. After collection from the fish farm, and prior to experimentation, they were kept on a twelve hour light/dark cycle for at least one week.

Immediately before the beginning of an experimental period the fish were transferred from the holding tank into 0.45% urethane. This was done in either the light or dark, depending on the experiment (see below). Following anesthesia the fish was placed in a Perspex holder, kept moist by damp tissue paper, a grounding electrode was placed around the body, and urethane kept circulating continuously over its gills (fig 4.1). The holder was then placed in position in the optical system as shown in fig 4.2. Fish treated in this way survived for up to three hours without serious deterioration of the response. Urethane was returned from the collecting tank to the header tank every fifteen minutes by an electric pump.

The temperature was carefully monitored throughout an experimental period as it has been shown to affect both photomechanical movements (chapter 1) and the latency, shape and amplitude of ERGs evoked by both flickering and single flash stimuli (Tamura & Hanyu 1959, Hanyu & Ali 1963 & 1964, Ali & Kobayashi 1967, Thorpe 1973 and Schellart et al 1974). Hoffert & Ubels (1979c) have shown, however, the ERG of *in vitro* rainbow trout retinas to be unaffected by temperature over the fish's normal range (5 - 15^{o}C).

The active and reference electrodes were placed on the cornea of the stimulated and unstimulated eye respectively. In this way all potentials, such as heart beat, which will be picked up by both electrodes, were cancelled out, and only the ERG from the stimulated eye was recorded on the dual beam storage Tektronix oscilloscope. Each separate ERG recorded

Fig 4.1. Rainbow trout in the experimental fish holder. Recording electrodes are shown in the lower portion of the figure.

Fig 4.1. Rainbow trout in the experimental fish holder. Recording electrodes are shown in the lower portion of the figure.

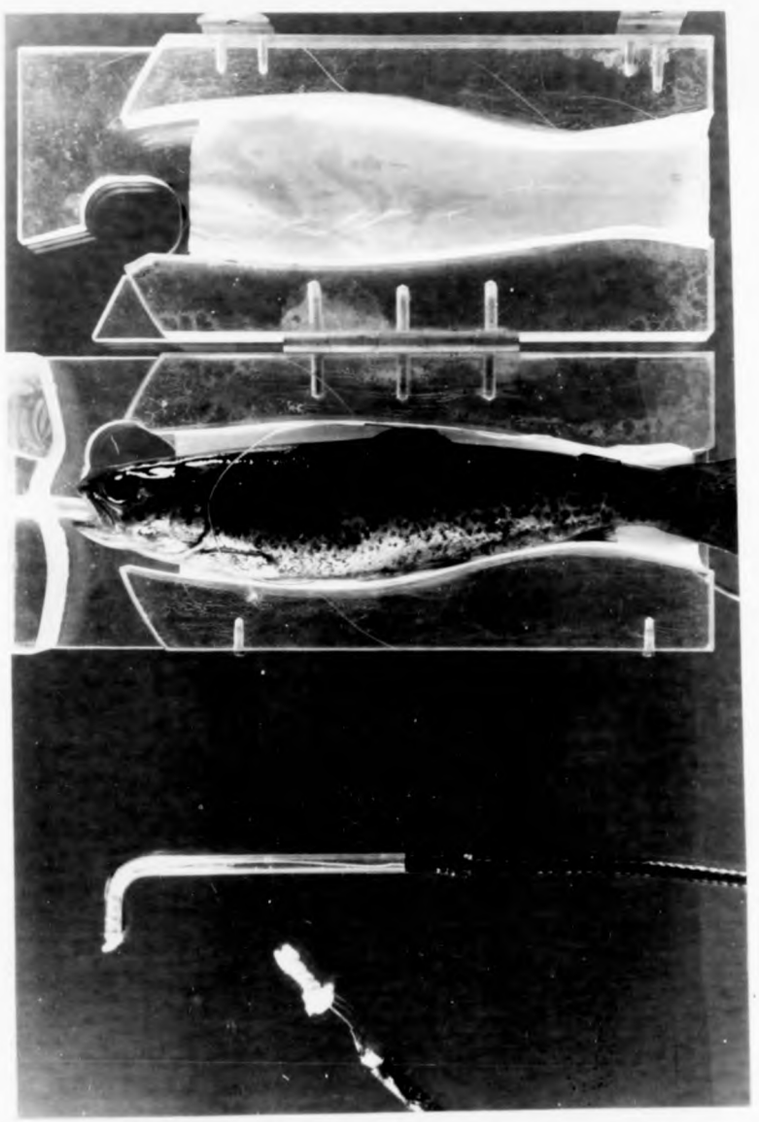
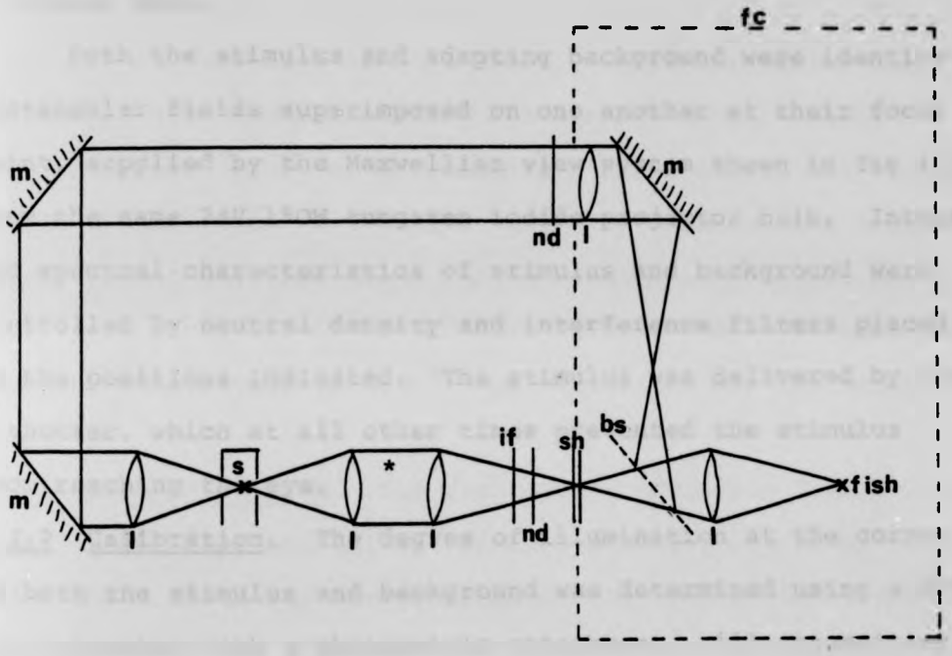


Fig 4.2. Optical system used in determining the time taken to light and dark adapt,scotopic spectral sensitivity and response to flicker.



m-mirror,l-lens,s-light source,if-interference filter,nd-neutral density filter,sh-shutter,bs-beam splitter,fc-Faraday cage, * position in which the sectored disc was positioned so as to obtain a flickering stimulus (chapter 5)

was stored and then either photographed or directly measured. Both electrodes were cotton wicks, soaked in teleost Ringer's, surrounded by chloride coated silver wire and encased in a glass tube to facilitate positioning (fig 4.1). The fish, electrodes, and immediately surrounding optics were enclosed in a Faraday cage.

Both the stimulus and adapting background were identical rectangular fields superimposed on one another at their focal point, supplied by the Maxwellian view system shown in fig 4.2, from the same 24V 150W tungsten iodide projector bulb. Intensity and spectral characteristics of stimulus and background were controlled by neutral density and interference filters placed in the positions indicated. The stimulus was delivered by opening a shutter, which at all other times prevented the stimulus from reaching the eye.

4.2.2 Calibration. The degree of illumination at the cornea of both the stimulus and background was determined using a UDT 40x optometer with a photometric attachment. All intensities, initially determined in terms of neutral density, could now be converted into actual intensities in terms of lux.

In order to define the stimulus completely, the colour temperature of the bulb was determined as follows: The maximum available energy through two interference filters transmitting at opposite ends of the spectrum (433 & 670 nm) was calculated for three colour temperatures (2700,3000 & 3200^{o}K) that straddle the likely value of the experimental bulb. The transmission at all wavelengths for the two interference filters, determined by a Cecil CE505 double beam UV spectrophotometer, was multiplied by the energy at these wavelengths, as determined from the energy vs wavelength curves for black body radiators of the different colour temperatures given in Wysecki & Stiles

(1967). The area under these curves, determined by cutting them out and weighing them, represents the maximum available energy. The ratio of maximum energy at 433nm to the maximum available energy at 670nm can then be plotted against colour temperature.

The maximum available energy at these two wavelengths using the experimental bulb was easily determined using the photometer with the radiometric attachment. These values divided by each other,after correction for the spectral characteristics of the diode (see chapter 5), give a ratio, the corresponding colour temperature for which can be determined from the above determined ratio/colour temperature relationship. In this way, a colour temperature of 3120^{o}K was calculated for the experimental bulb.

4.2.3 Dark adaptation. The fish were transferred from holding tanks to the experimental set-up in a normally lit room. To adapt the fish to its surroundings it was left in the light for ten minutes in the apparatus, after which time all lights were turned off (time 0).

Electroretinographic adaptation. At intervals after being put in the dark the eye was stimulated by a series of 0.2 sec stimuli of differing intensities. Each set of presentations took approximately one minute to complete. In this way a series of response vs intensity curves at different times in the dark was obtained for each fish (fig 4.3). From such a group of curves the intensity of stimulation necessary to elicit a criterion amplitude of b-wave was determined. In three out of the four fish used in this experiment this criterion response was 100 μV. The fourth fish showed such an abnormally large response that a higher value was chosen. When these thresholds are plotted against time a curve representing the time course

Fig 4.3. Relationship between b-wave amplitude and stimulus intensity at various times during dark adaptation.

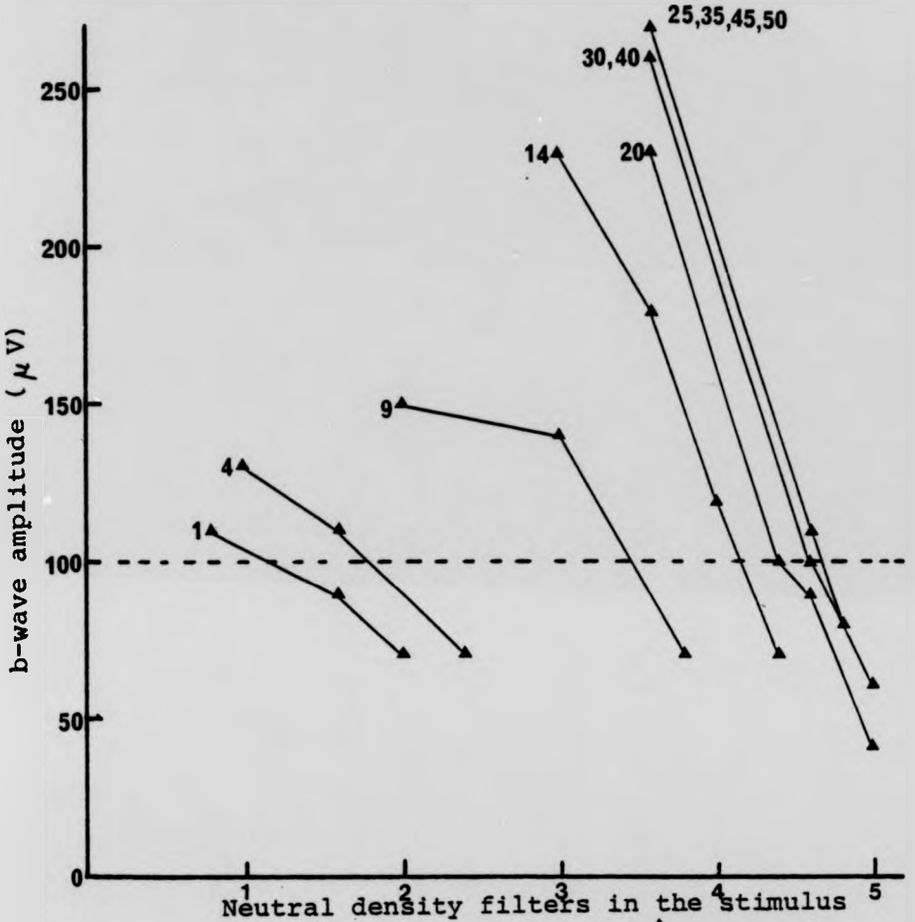
The numbers on the lines indicate the length of time (minutes) that the fish have been adapting.

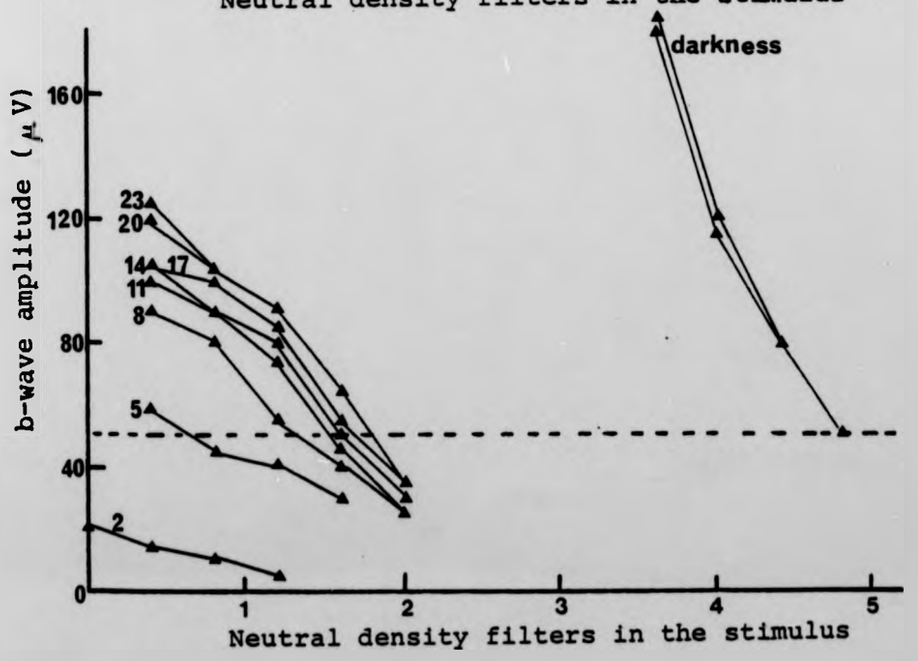
Fig 4.4. Relationship between b-wave amplitude and stimulus intensity at various times during light adaptation.

Fig 4.3. Relationship between b-wave amplitude and stimulus intensity at various times during dark adaptation.

The numbers on the lines indicate the length of time (minutes) that the fish have been adapting.

Fig 4.4. Relationship between b-wave amplitude and stimulus intensity at various times during light adaptation.


25,35,45,50
30,40
20
14
9
4
1
250
200
150
100
50
0
b-wave amplitude (μV)
1
2
3
4
5
Neutral density filters in the stimulus


darkness
23
20
14
17
11
8
5
2
160
120
80
40
0
b-wave amplitude (μV)
1
2
3
4
5
Neutral density filters in the stimulus

of dark adaptation is obtained.

Photomechanical adaptation. A pilot study showed that urethane had no effect on the movement of the retinal elements, confirming the early observation of Arey (1916). It was therefore possible to follow the retinomotor movements of fish exposed to exactly the same conditions as those experienced by fish during the electroretinographic determination of sensitivity changes during dark adaptation. Both groups of fish were treated in an identical fashion, even to the extent of putting electrodes on the eyes of fish later used for histological sectioning. Fish were killed at various stages during dark adaptation, starting immediately after the ten minutes preliminary adaptation to the apparatus. Three fish were killed at each time up to, and including, thirty minutes in darkness, after which only one fish was used. Only individuals alive at the end of their adaptation period were used.

4.2.4 Light adaptation. Fish were dark adapted for at least three hours before being transferred to the holder using a dim red torch. The fish were then left in the apparatus in complete darkness until the ERG reached a stable form and amplitude. At this point it was assumed they were totally dark adapted. The eye was then exposed to a background illumination of 12 lux (1.0 neutral density). At backgrounds of higher intensity a measurable b-wave could not be recorded.

Electroretinographic adaptation. At various times after the adapting light came on (time 0), response vs amplitude curves were constructed as before. Fig 4.4 shows a typical family of curves. Such a set of curves was obtained for each of the eight fish used, and the intensity needed to elicit a b-wave amplitude of 50μV determined at various times during light adaptation. Threshold plotted against time represented

the time course of light adaptation.

Photomechanical adaptation. The measurement of the extent of movement of the retinal elements differed slightly from the method used in other parts of this study. Following adaptation for the desired length of time the whole eye was fixed in Bouin's solution. After at least twenty-four hours the eye was hemisected and its periphery removed. The remainder of the retina was divided into four pieces and each piece embedded separately. Sections were subsequently taken from the middle of each "quarter". In this way the differential affect of the adapting light on parts of the retina, caused by the light being smaller than the area of the eye and the partial covering of the eye by the electrode, was controlled for.

Although most of the eye was adapted to the background, evidence was found in four cases that some parts of the retina were more light adapted than others. In these cases the most light adapted values of pigment and cone indices were used. In all other eyes the indices from all four sections were very similar. These values were averaged to give the index for that eye.

As during dark adaptation, electrodes were placed on the eye to simulate recording conditions, but this time ERGs were actually recorded. Recording the ERGs of eyes that were later used to determine the time course of retinomotor adaptation served two purposes. Firstly, it ensured that eyes used for sectioning and eyes used for determination of the ERG time course underwent exactly the same treatment. Secondly, it ensured that the fish was initially totally dark adapted and that its condition did not subsequently deteriorate. A fish in poor physiological condition begins to show an abnormally large a-wave and the b-wave amplitude decreases. As soon as this was observed the experiment was terminated and the eye fixed. As the time

between recordings was only three minutes, it was felt that the eyes could still be used for histological sectioning, as such a short time would not have a significant effect on the retino-motor movements.

At each point sampled during light adaptation a different number of fish were killed and the results of all fish at one time averaged (0, 5 & 20 minutes - 3 fish, 10 & 25 minutes - 4 fish, 15 minutes - 6 fish, 30 minutes -2 fish).

4.2.5 Increment thresholds. Dark adapted fish were transferred to the experimental apparatus from holding tanks as previously described, and their thresholds determined in complete darkness. Once a steady threshold level was reached, after between twenty and forty minutes, the eye was exposed to a very weak background light and thresholds were once more determined until the response stabilised after about ten minutes. The eye was then exposed to a more intense adapting background and the procedure repeated. This was continued until the fish had been exposed to five different intensities of background illumination. At least two response vs stimulus intensity curves, after the response had reached a steady level, were obtained for each level of adapting background (fig 4.5). The threshold for each intensity of adaptation was taken as the average intensity of stimulus light needed to elicit a b-wave of 50 μV, once the eye was fully adapted.

If the response decreased at any time, this was taken as an indication that the fish was in poor physiolgical condition and the experiment was immediately terminated.

4.3 RESULTS

4.3.1 Form changes. Since the principal objective of the present study was to determine the time course of adaptation, form changes were not examined in great detail. Therefore the description

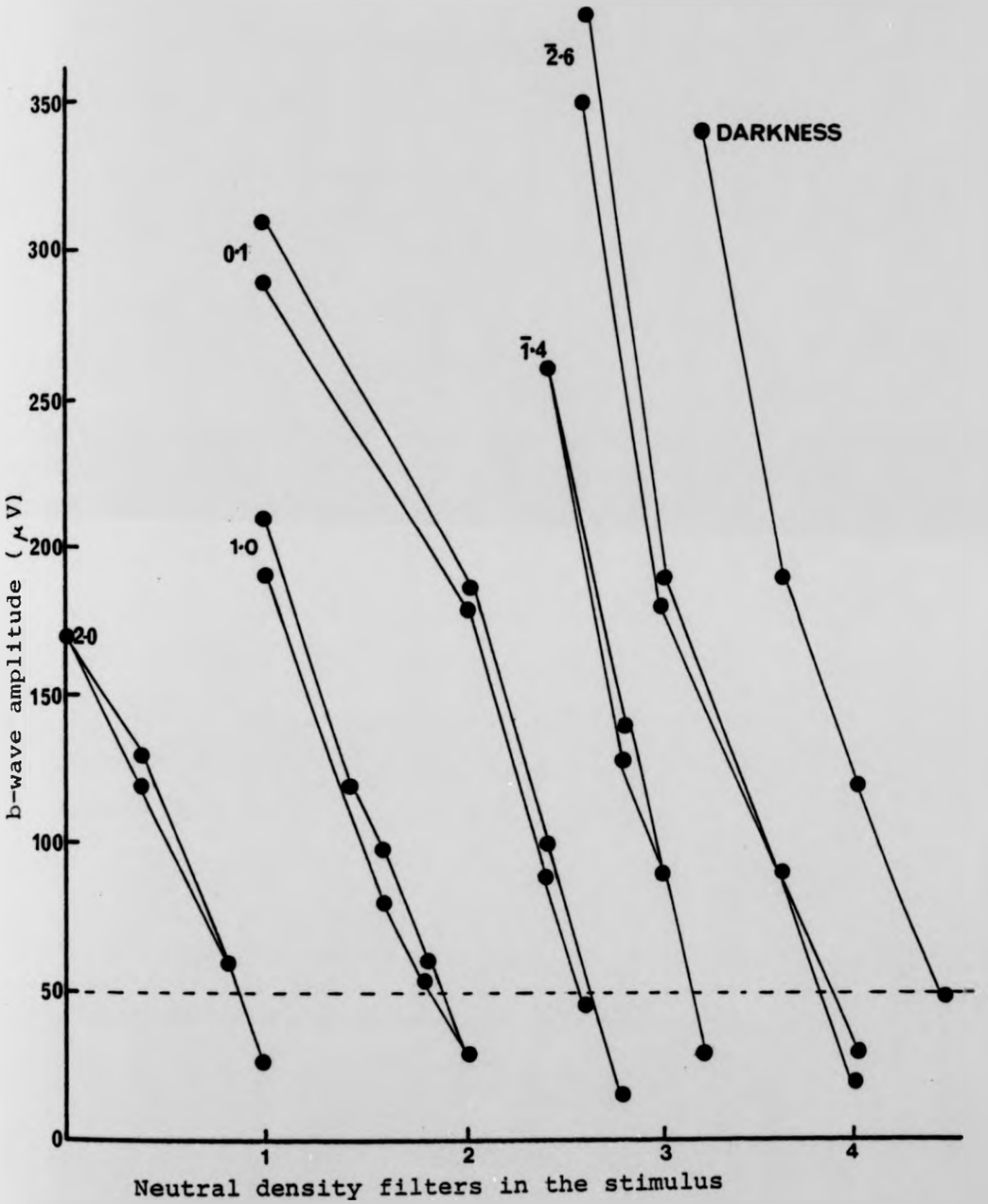


Fig 4.5. Amplitude of the b-wave of an individual in response to different intensities of stimulation with various intensities of background illumination.

The numbers on the lines are the intensity of the background illumination (log lux).

given below for both light and dark adaptation is not exhaustive, and is sometimes based on data from only three or four fish.

Fig 4.6 is a schematic representation of a typical response, indicating the parameters measured.

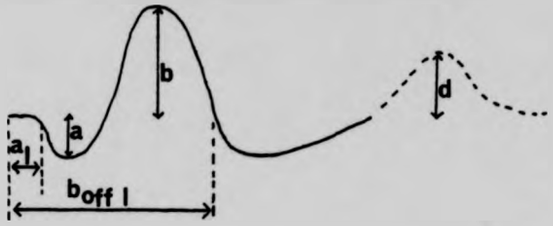


Fig 4.6 a,b,d - wave amplitudes, a_1 - a wave latency, $b_{off\ 1}$ - duration of response.

4.3.1.1 Light adaptation. Fig 4.7 a & b show typical series of changes in the form of the ERG associated with light adaptation recorded from two individuals in response to a constant stimulus. The most obvious changes are that during adaptation both the a- and b-wave increase in amplitude until they reach a constant size. If any latency changes do occur they are too small to be measured by the present methods. A d-wave is apparent almost immediately after exposure to light.

In fig 4.7a, a secondary positive going wave occurs after the d-wave, which diminishes as light adaptation proceeds. There is also an indication of two positive responses after the stimulus has ceased in fig 4.7b, with the second wave taking-off from the first. An intermediate state between these two extremes, observed in a third individual, is shown in fig 4.7c, eight minutes after the begin of light adaptation. Such secondary positive responses after the stimulus had ceased were observed very frequently during the course of the investigation.

4.3.1.2 Dark Adaptation. After turning off all adapting lights and during subsequent dark adaptation, several changes occur in response to a stimulus of constant intensity (fig 4.8). The

Fig 4.7. ERGs of three individuals at various times after the onset of illumination in response to a 631 lux stimulus.

The numbers to the left of each response are the times since the begining of light adaptation. A stimulus marker is shown below each set of traces.

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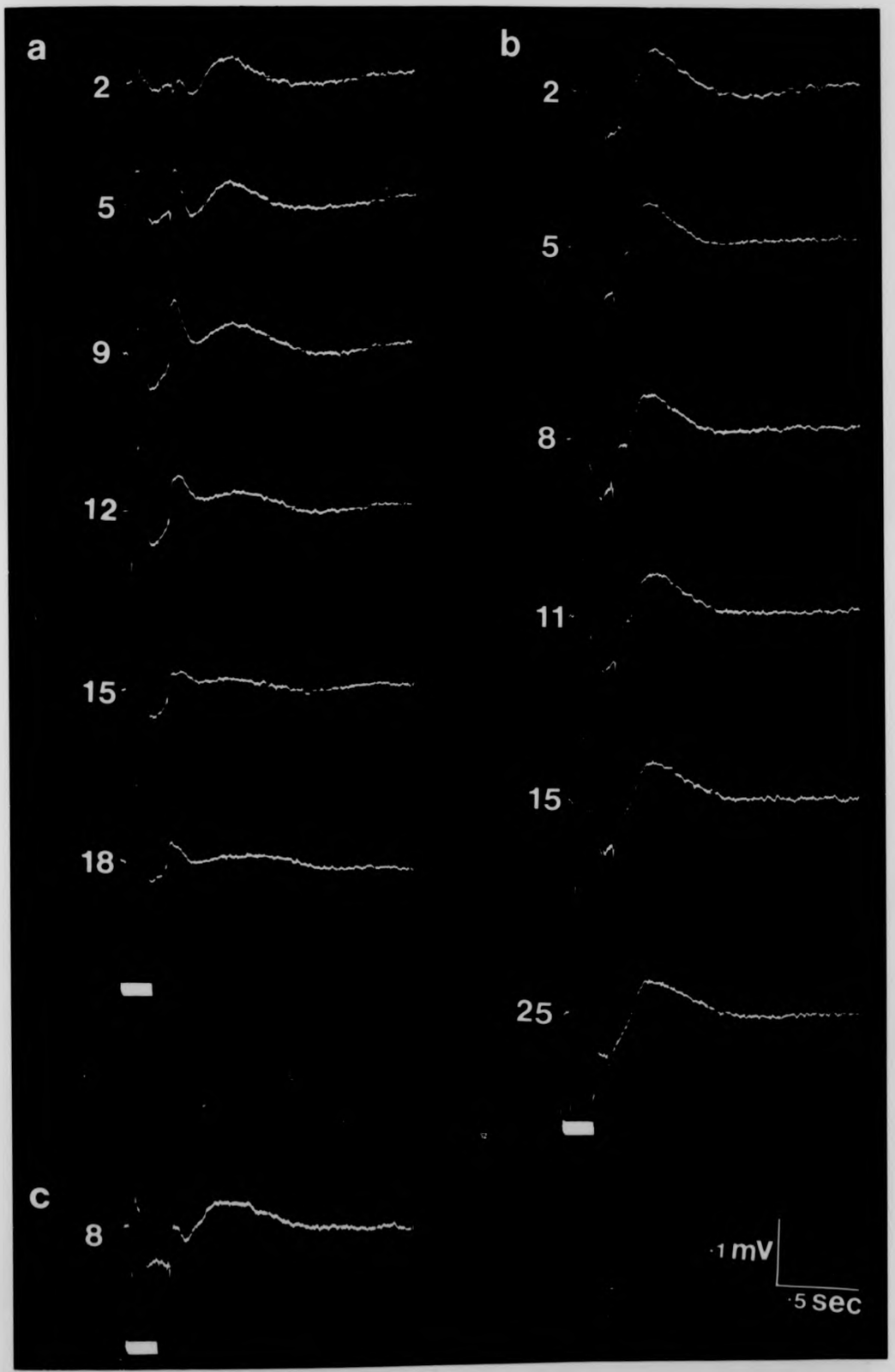

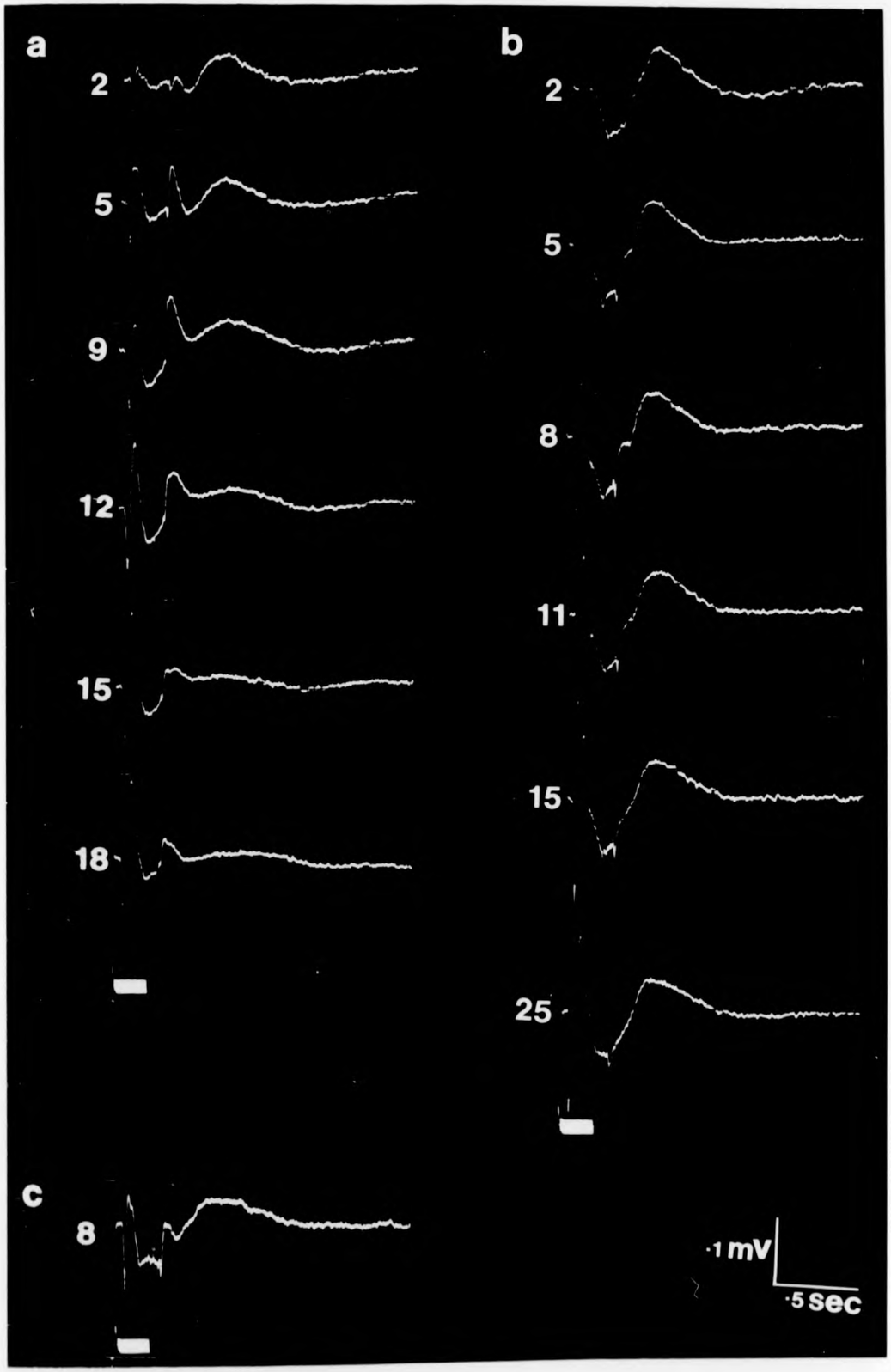

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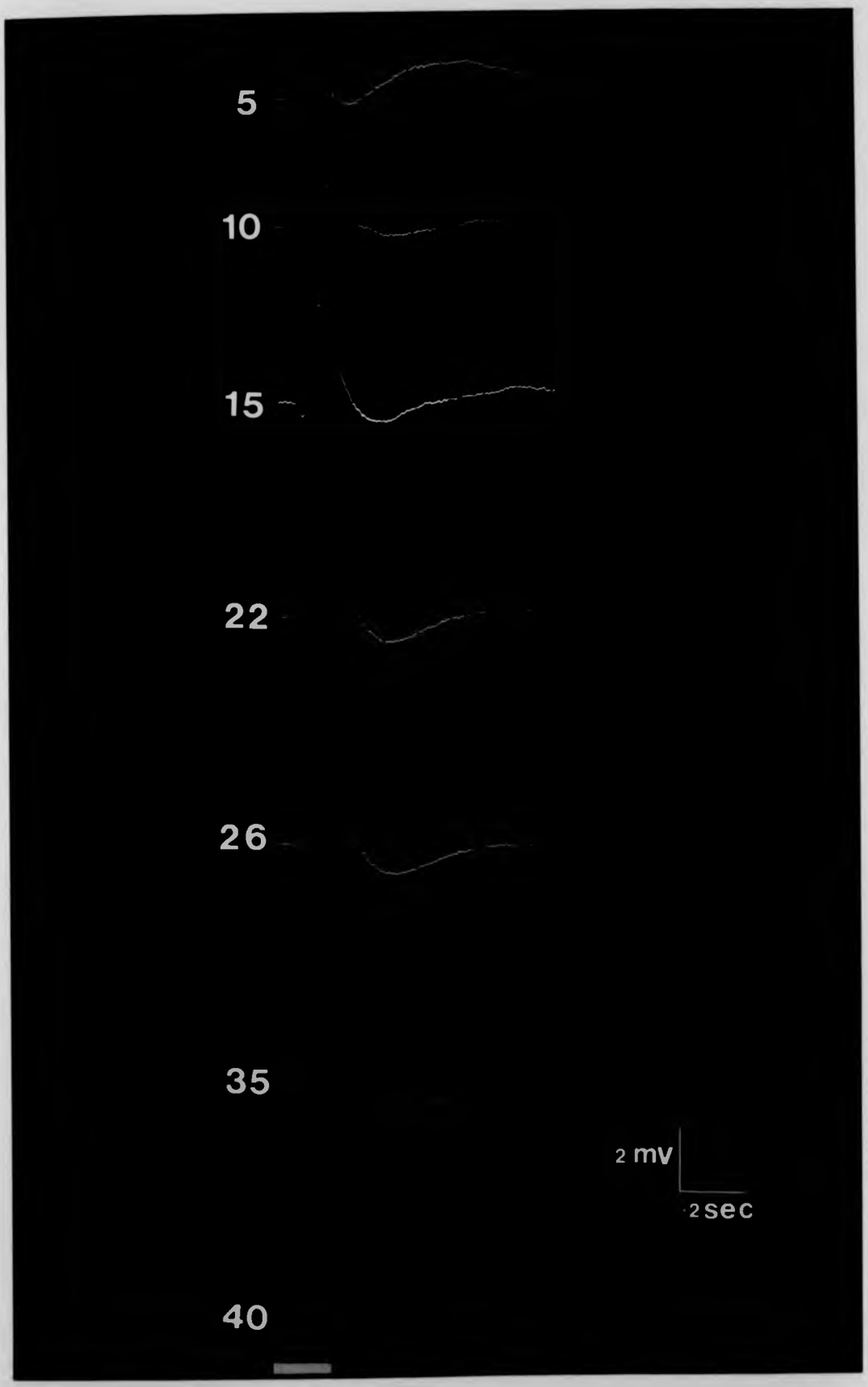
Fig 4.8. ERGs of an individual at various times after the onset of darkness in response to a 5 lux stimulus.

This intensity of stimulus is the strongest possible without depressing the b-wave amplitude (see 4.3.1.3). The numbers to the left of each ERG are the times after the begining of darkness.



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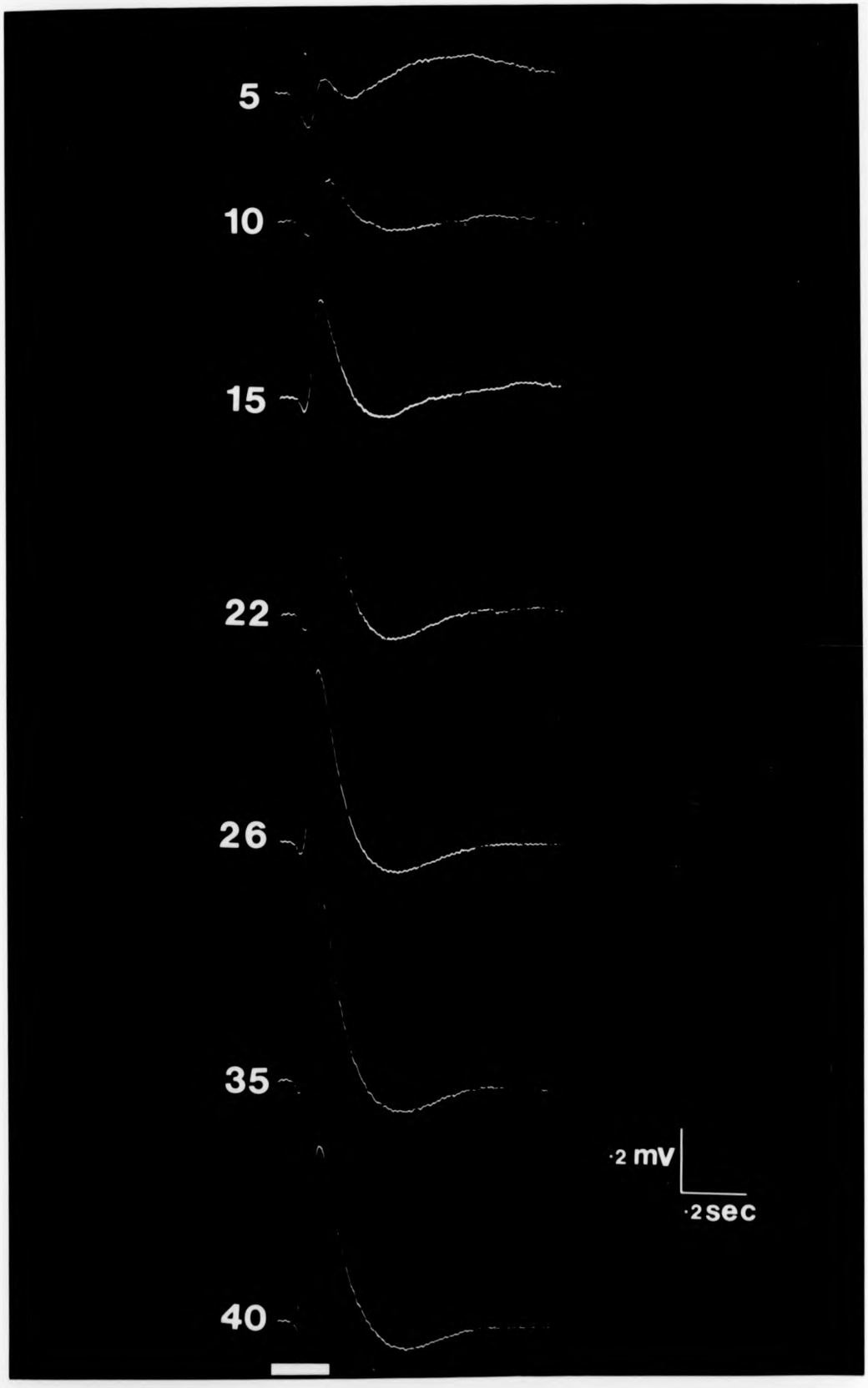

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most rapid change is that the d-wave, shown very clearly in the light, decreased in amplitude immediately the fish was put in darkness and after two minutes is no longer recognisable as a definite wave, although, as shown in fig 4.8, five minutes after the onset of darkness there is a slow positive going response after the cessation of the stimulus. After ten minutes this wave has also disappeared and the response remains on the baseline for the remainder of the period in the dark. The dip below the baseline after the b-wave is an artifact due to AC coupling (0.1 KHz & 1 Hz low frequency cutoff employed).

Changes in a- and b-wave amplitude and latency during dark adaptation for one fish are graphically displayed in fig 4.9. During adaptation the b-wave increases in amplitude until it reaches a constant height and the whole response becomes slower, as is indicated by the rise in $b_{off\ 1}$. The a-wave latency seems to stay constant, but this cannot be stated with certainty as small changes may be occurring which cannot be measured using the slow time base of the present study.

Fig 4.9 shows the a-wave amplitude as changing little throughout adaptation, but this may be because of the low level of stimulation used. The b-wave response of a dark adapted eye reaches its maximum amplitude at lower intensities than the a-wave. Therefore, a high level stimulus for the b-wave is not necessarily so for the a-wave. Thus the behaviour of the a-wave throughout dark adaptation was not thoroughly investigated, as stimulation at the high light levels necessary to obtain a reliable a-wave may alter the time course of adaptation by partially light adapting the eye. However, on several occasions the response of the eye both while light adapted, prior to dark adaptation, and when fully dark adapted to maximal (1600 lux) white light stimulation was investigated. In all such cases,

Fig 4.9. ERG amplitudes (▲) and latencies (△) of an individual during dark adaptation in response to a constant stimulus (5 lux,white light).

b-b-wave amplitude,a-a-wave amplitude,$b_{off\ 1}$-end of b-wave,a_1-a-wave latency.

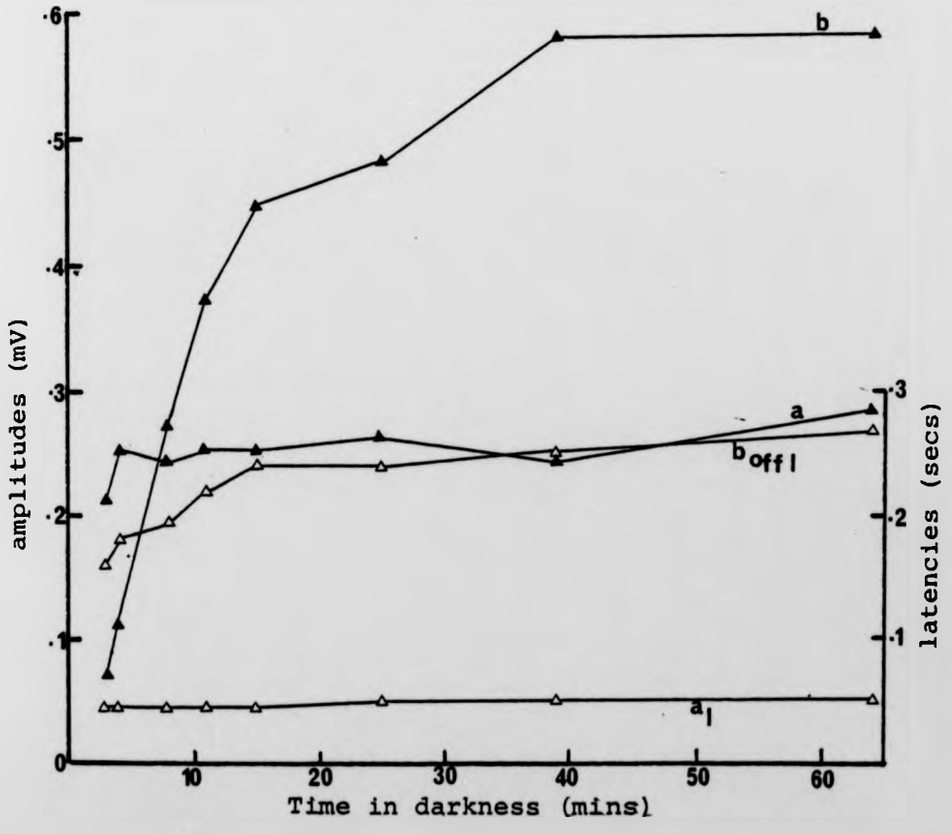
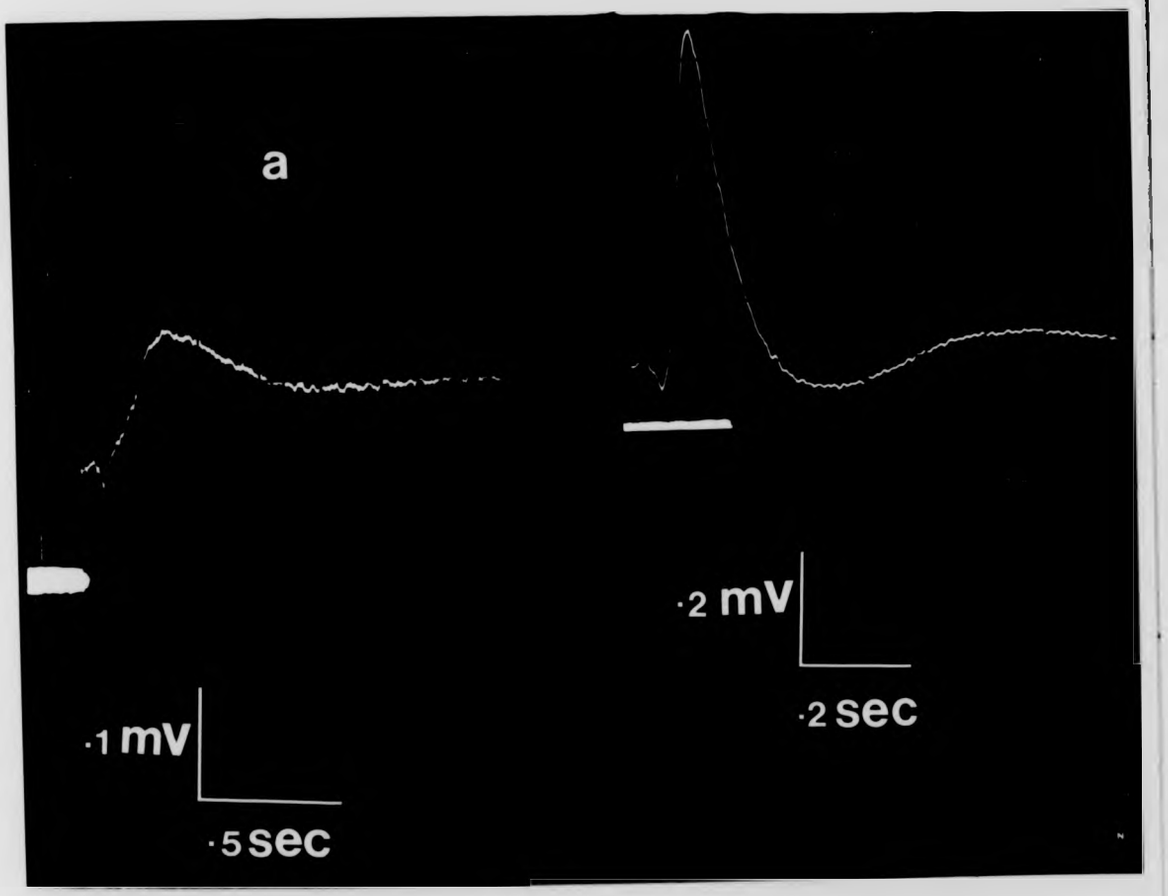


Fig 4.10. ERGs of an individual (a) after 28 minutes light adaptation in response to a stimulus of 600 lux and (b) after sixty minutes in darkness in response to a stimulus of 6 lux. Note the dark adapted b-wave is larger than that of the light adapted eye although the stimulus is 100 times as weak.



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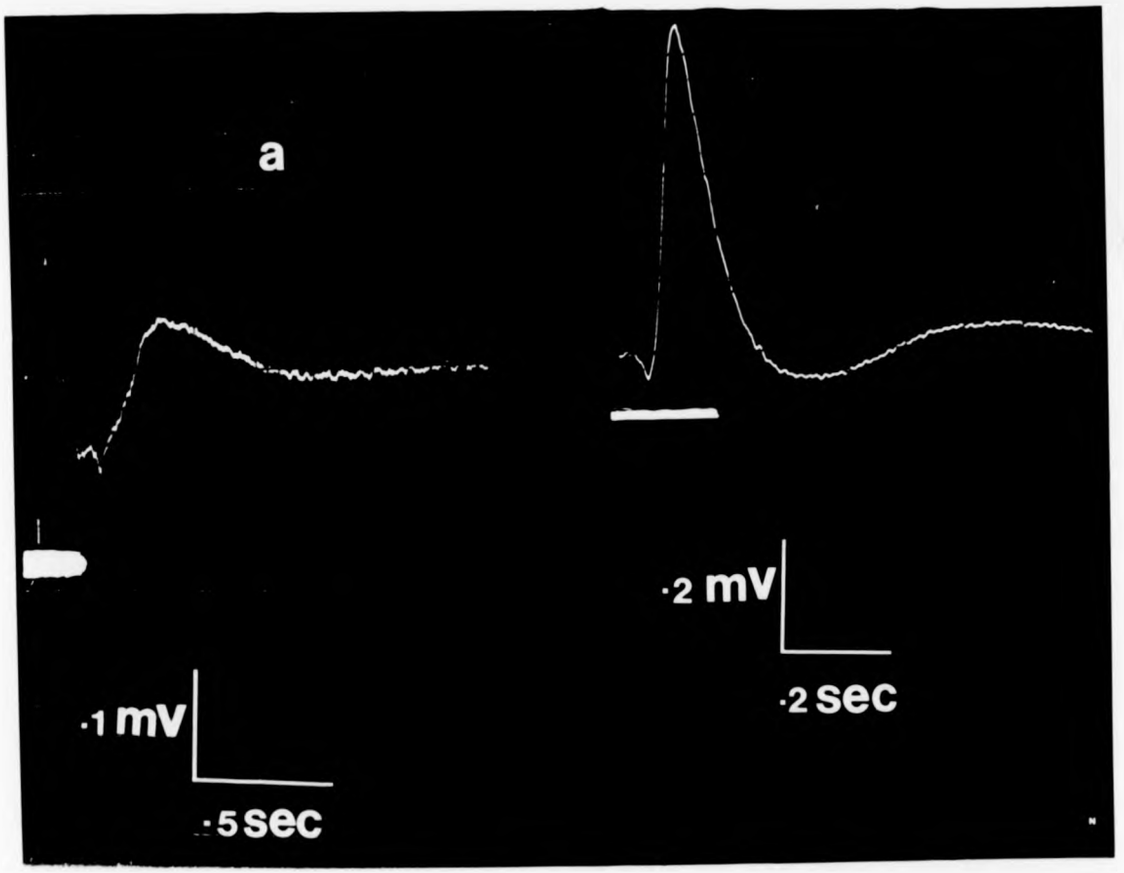
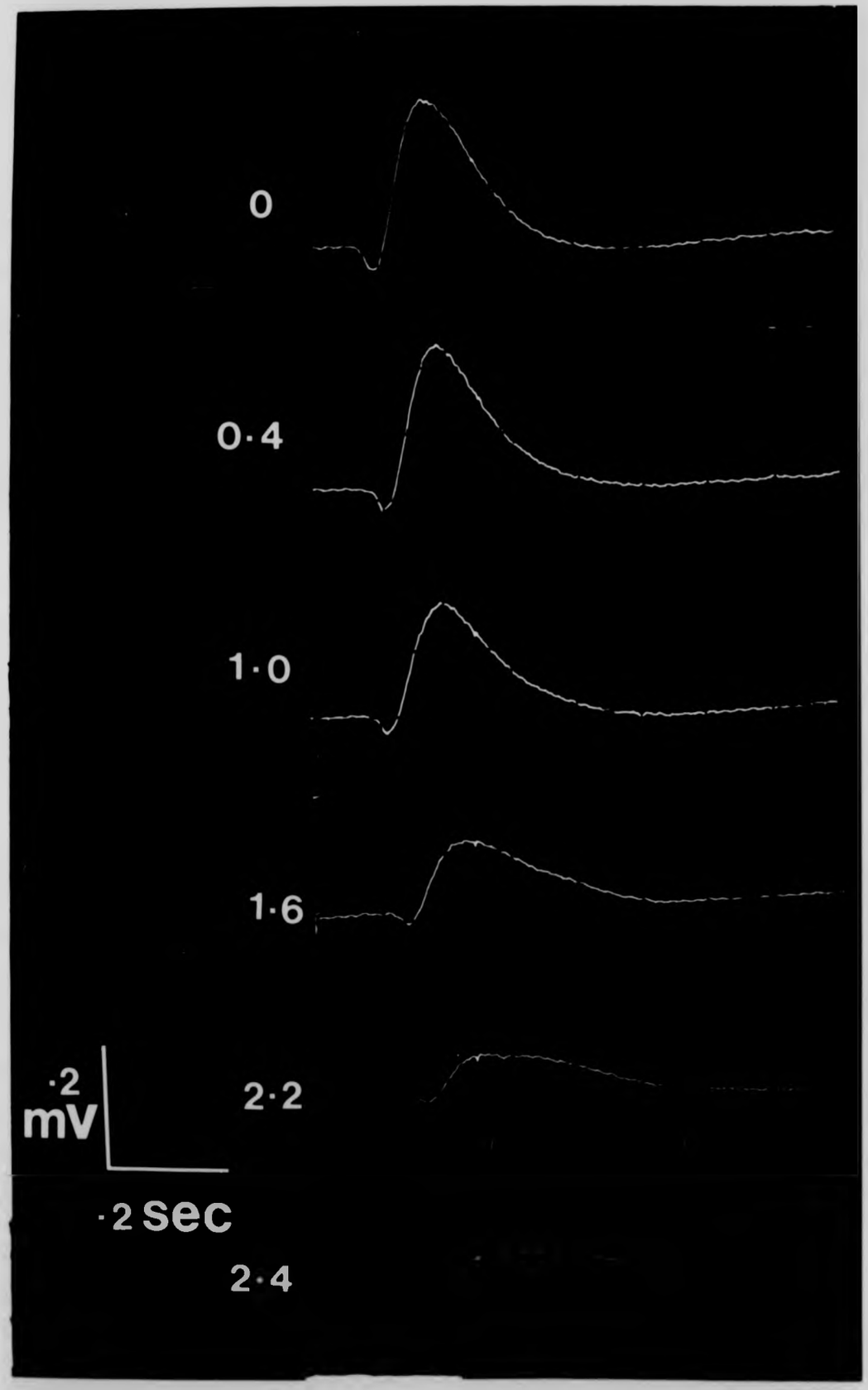


Fig 4.11. Scotopic ERGs of an individual in response to differing strengths of 513 nm stimuli. The values refer to the neutral density filters in the stimulus beam.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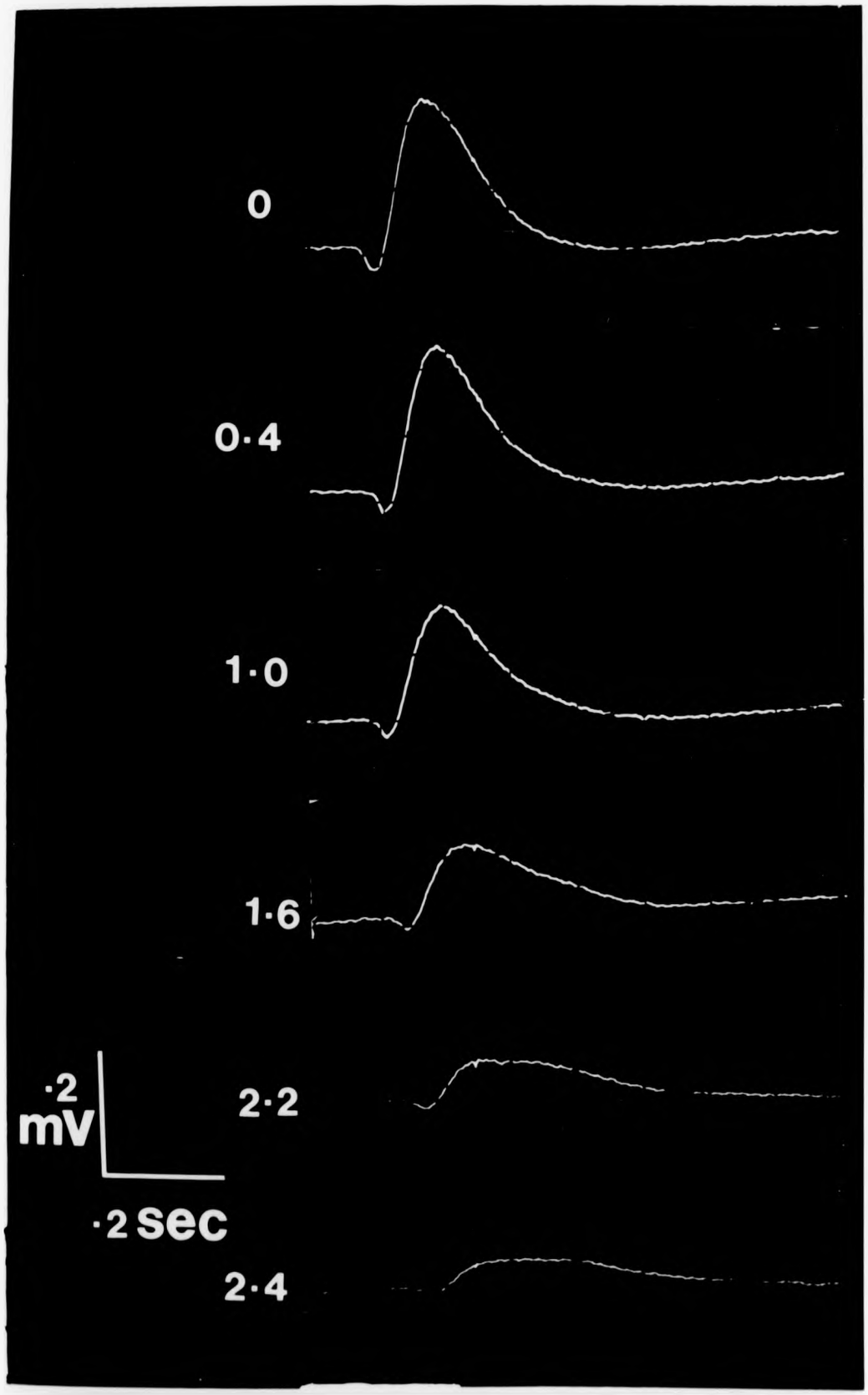


when an a-wave was observed before and after dark adaptation, the amplitude was found to be two to four times greater in the dark adapted state.

The a-wave was often observed to have two peaks, but no systematic study of this was carried out. That these peaks were not just noise is indicated by the fact that they were perfectly repeatable if the eye was stimulated several times in succession.

4.3.1.3 Changes in the form of the dark adapted ERG with intensity of stimulation. Such changes have been examined in detail at several wavelengths. Fig 4.11 shows these changes for a 513nm stimulus, which are graphically represented in fig 4.12. In general, as the intensity of stimulation decreases the amplitude of both a-and b-waves decreases, with the a-wave disappearing at a higher intensity than the b-wave. The latency for the onset of the response (a_1) also increases and the b-wave gets broader ($b_{off\ 1}$) with decreasing intensity, resulting in an overall slower response.

Typical responses of a fully dark adapted eye to different intensities of white light are shown in fig 4.13. At lower intensities of stimulation the amplitude of the b-wave decreased regularly with a lowering of the stimulus intensity, but at high intensities of white light the b-wave response was depressed. This reaction was observed in six out of seven fish in which dark adapted responses to white light were investigated. Such a depression of b-wave amplitude not only occured in fully dark adapted eyes but also at all times after the first few minutes of dark adaptation. The a-wave showed no such reaction to high intensity stimulation. Within the intensities used the a-wave amplitude increased with increasing stimulation.

Fig 4.12. Amplitudes (●) and latencies (○) of a dark adapted individual's ERG at various intensities of 513 nm stimulation.

a - a-wave amplitude, b - b-wave amplitude, $b_{off\ l}$ - end of b-wave, a_l - a wave latency.

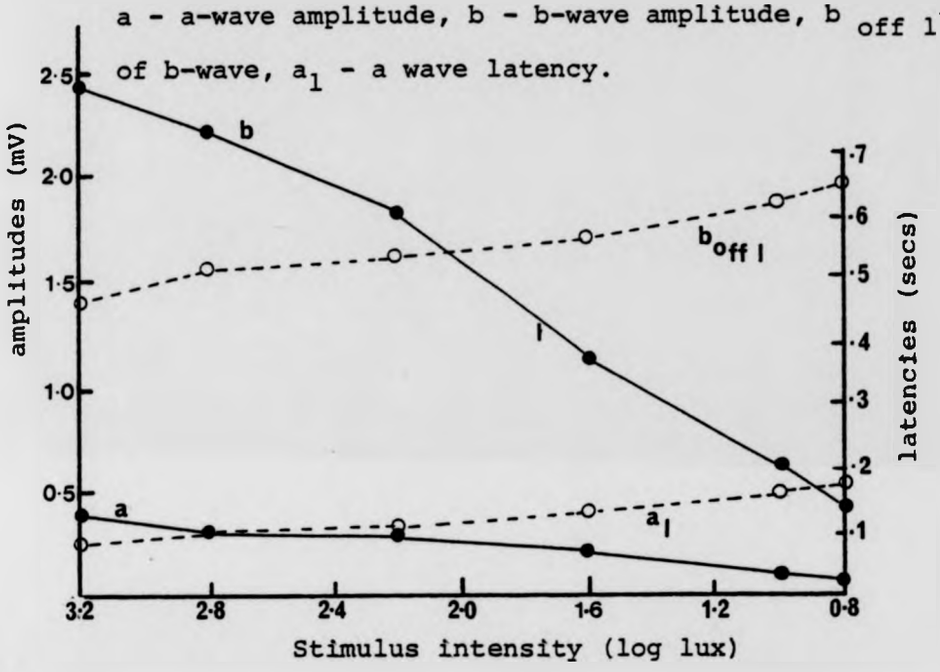
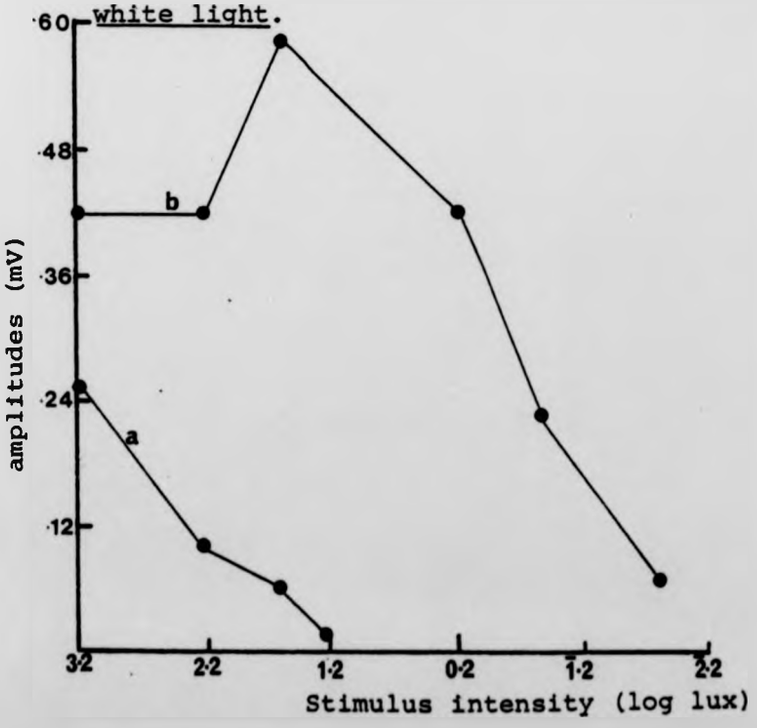


Fig 4.13. Amplitudes of a dark adapted individual's ERG in response to stimulation by various intensities of white light.



4.3.2 Time course of adaptation

4.3.2.1 Light adaptation - photomechanical. Both the pigment and cone indices show full light adaptation after fifteen minutes (fig 4.14). The rise in the pigment index at thirty minutes is not a true indication of a change in the pigment position as it is based on only two fish and may be due to individual variation.

Light adaptation - electroretinographic. Immediately on exposure to light the sensitivity dropped to a very low level, so that during the early stages of adaptation the stimulus light was not bright enough to elicit a b-wave. During subsequent light adaptation the sensitivity slowly increased, as shown for all eight fish used in the part of the study in fig 4.15, until a steady light adapted threshold was reached. Individual fish varied in their absolute sensitivity, although their time courses for adaptation were very similar. Table 4.I shows the difference between the dark adapted threshold, determined immediately prior to exposure to the adapting background, and the final light adapted threshold. The average decrease in sensitivity with light adaptation is 3.36 log units.

To give an indication of the average time course of adaptation, the final response of each fish was arbitrarily assigned as 100%, and all other thresholds for that fish plotted relative to this (fig 4.16). The line joining the average of these responses gives a rough indication of the adaptation time course. Some errors will occur due to adaptation perhaps being incomplete in fish 70 and 71, but they will be minimal. Adaptation is completed after about fifteen minutes. This agrees well with the time course for photomechanical adaptation, although this may be completed a little earlier.

The slope of the response vs intensity curves varies with

Fig 4.14. Retinal pigment and cone indices at various times during light adaptation.

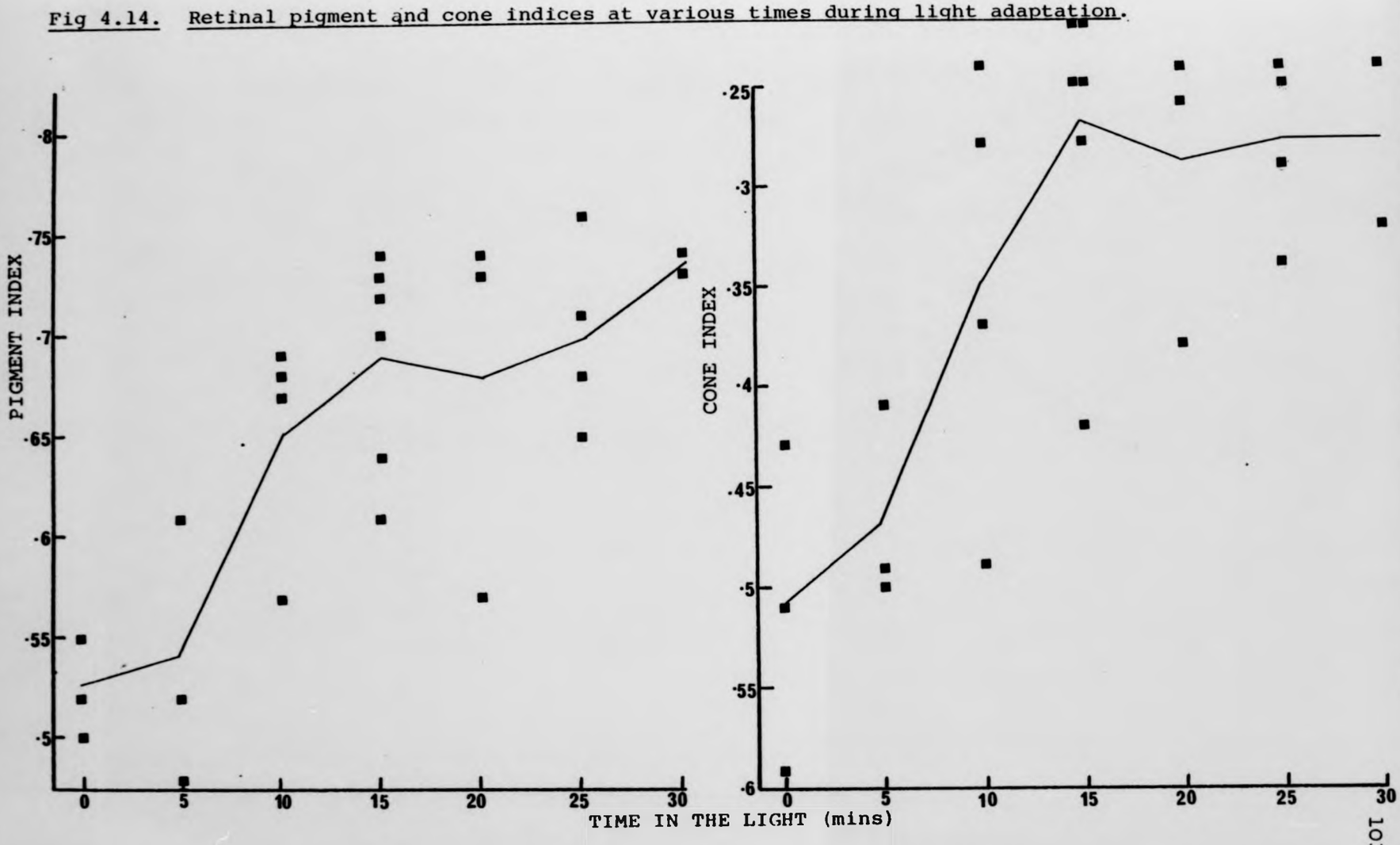
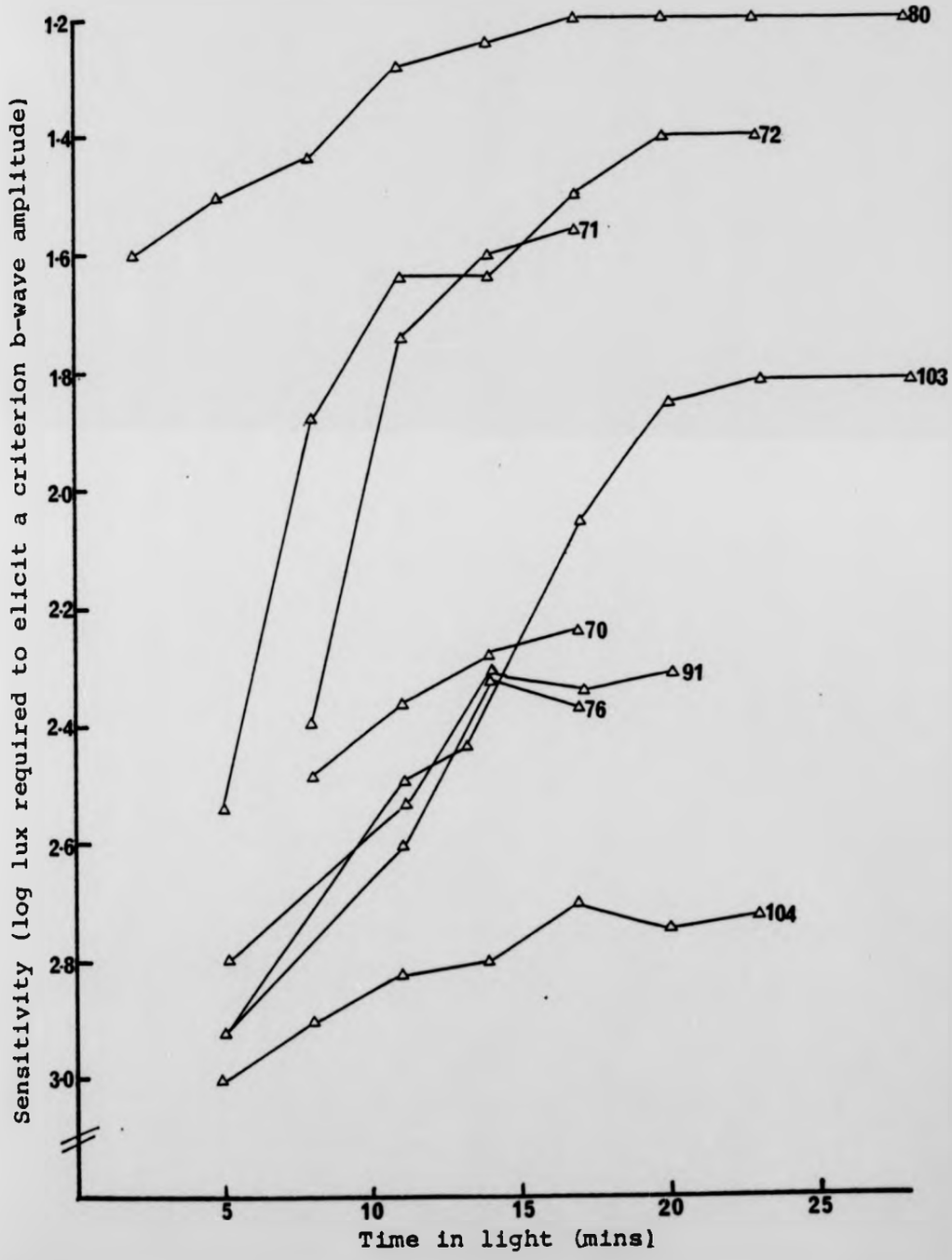


Fig 4.15. Sensitivity changes in eight individuals during light adaptation measured by the ERG b-wave.



The numbers on the lines are the fish identification.

Fig 4.16. Average sensitivity of eight fish during light adaptation expressed as a percentage of the final response.

The retinal pigment (■) and cone (○) indices are also plotted.

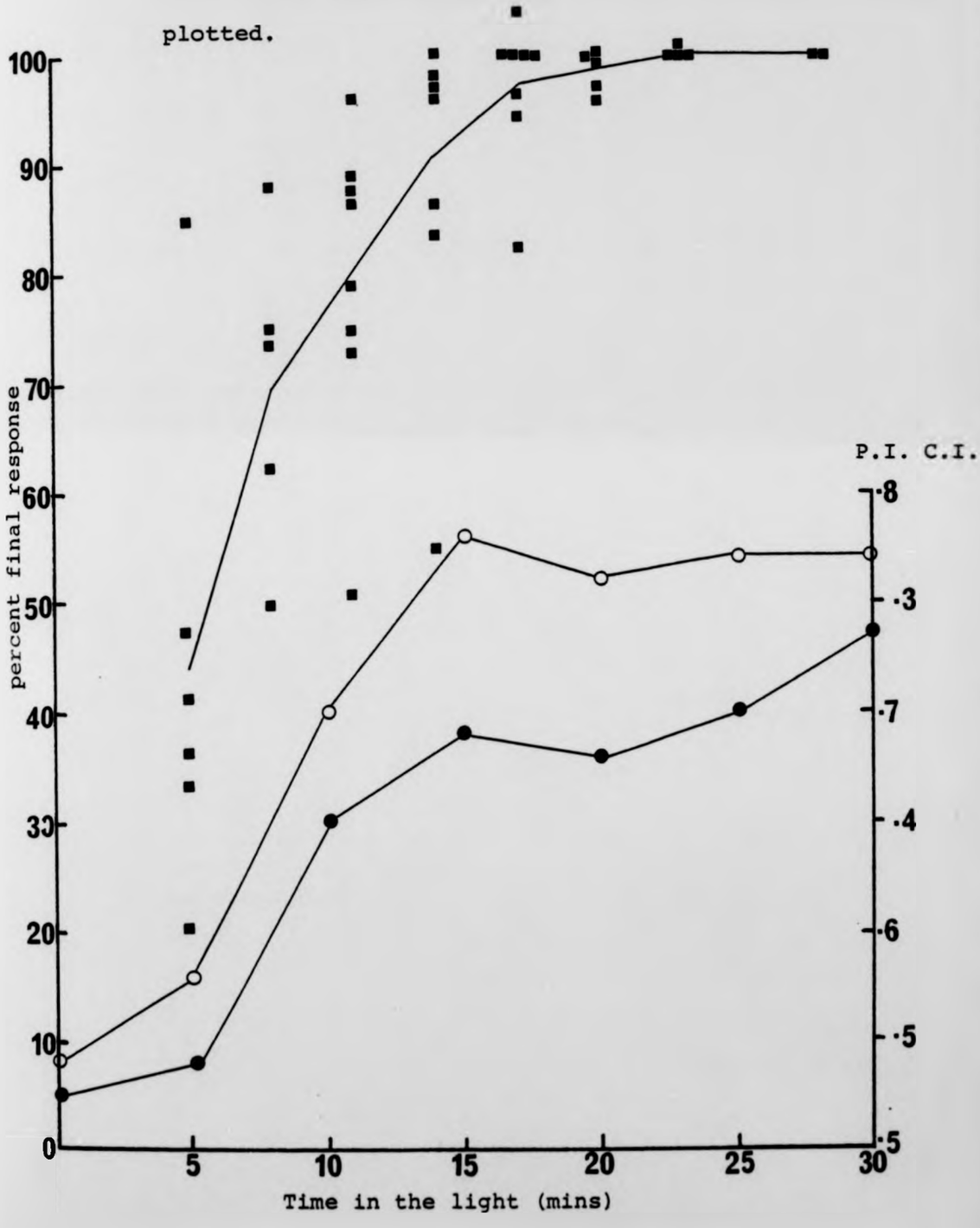


TABLE 4.I Light and dark adapted thresholds to white light in the eight fish used in the determination of the time course of sensitivity change during light adaptation.

Fish	50 μV dark adapted threshold (Log lux)	50 μV light adapted threshold (Log lux)	threshold change (Log lux)
70	$\bar{2}$.38	2.2	3.82
71	$\bar{2}$.52	1.5	2.98
72	$\bar{2}$.2	1.32	3.12
76	$\bar{2}$.34	2.32	3.98
80	$\bar{2}$.62	1.1	2.48
91	$\bar{2}$.57	2.32	3.75
103	$\bar{2}$.56	1.76	3.20
104	$\bar{2}$.08	2.7	4.62

time, tending to become steeper with light adaptation (eg: fig 4.4). This was the case in all eight fish.

4.3.2.2 Dark adaptation - photomechanical. Fig 4.17 shows the position of the retinal elements in relation to the time in darkness. Adaptation was complete twenty-five to thirty minutes after the onset of darkness. Reasons for the large variation will be discussed in a later section.

Dark adaptation - electroretinographic. The sensitivity changes associated with dark adaptation are shown in fig 4.18, along with the associated retinomotor changes. In two out of four fish no b-wave that reached threshold level could be recorded during the early stages of dark adaptation. The fish were fully dark adapted between approximately twenty-five and thirty minutes after the beginning of darkness.

It is interesting to note that, as during light adaptation, the slope of the response vs intensity curves increases during adaptation, as shown in fig 4.3.

4.3.3 Increment thresholds. The relationship between the threshold and the intensity of the adapting background was determined for four fish (table 4.II). As it was only possible to elicit a measurable b-wave at the highest intensity of adapting illumination in two fish, the thresholds from all four fish could not be combined directly to give an average increment threshold function (ΔI vs I) covering all intensities. To accomplish this, the difference between the dark adapted threshold and the threshold at each adapting illumination was determined for each fish. The average of these values, for all four fish, could then be plotted together, as all values were expressed relative to the dark adapted state, to give a classic increment threshold function (fig 4.19). At the higher levels of adapting illumination the function was a straight line of slope 47.7^{o}, thus closely

Fig 4.17. Retinal pigment and cone indices at various times during dark adaptation.

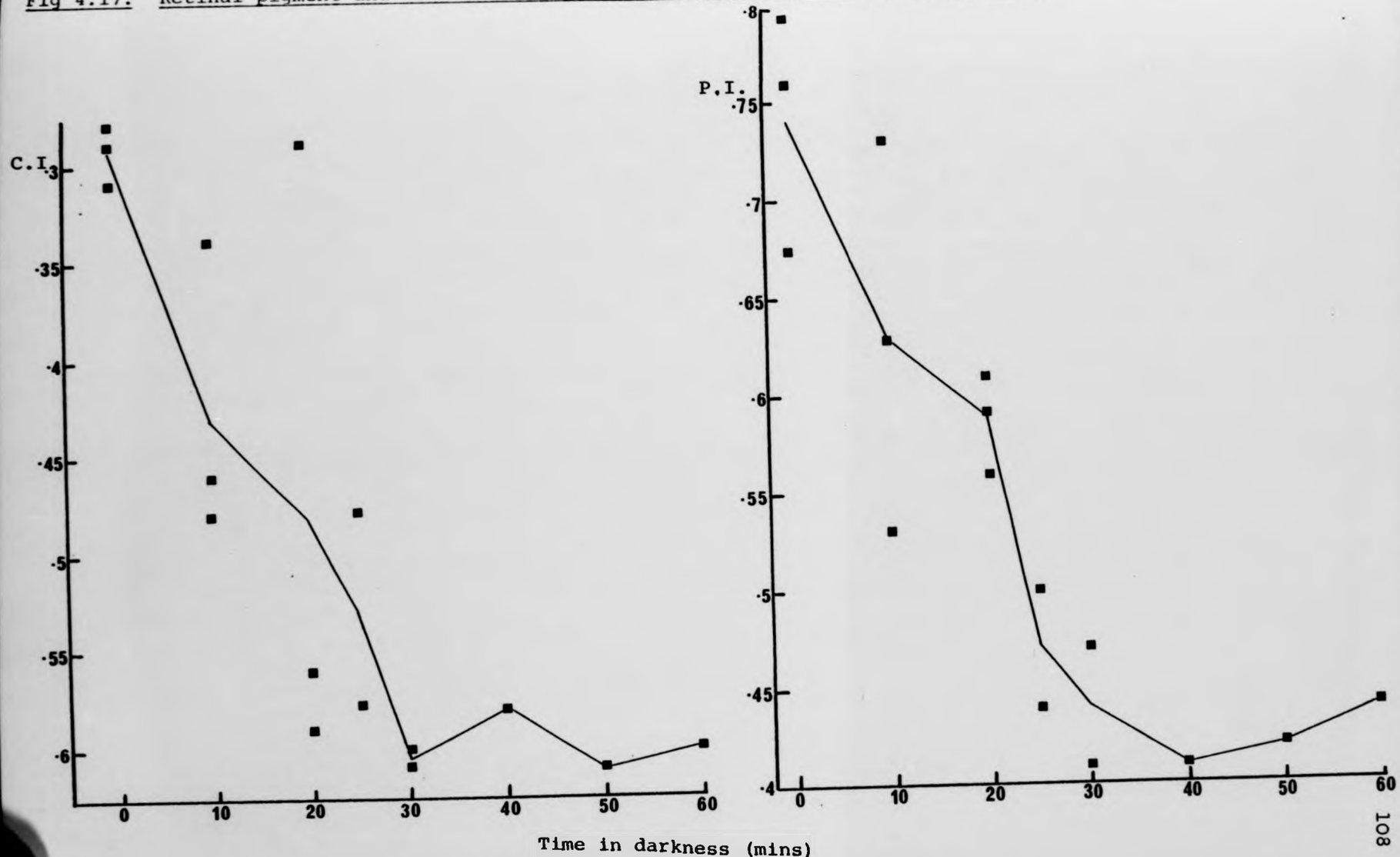


Fig 4.18. Sensitivity changes of four individuals during dark adaptation as measured by the ERG b-wave.
Retinal pigment (▲) and cone (△) indices are also plotted

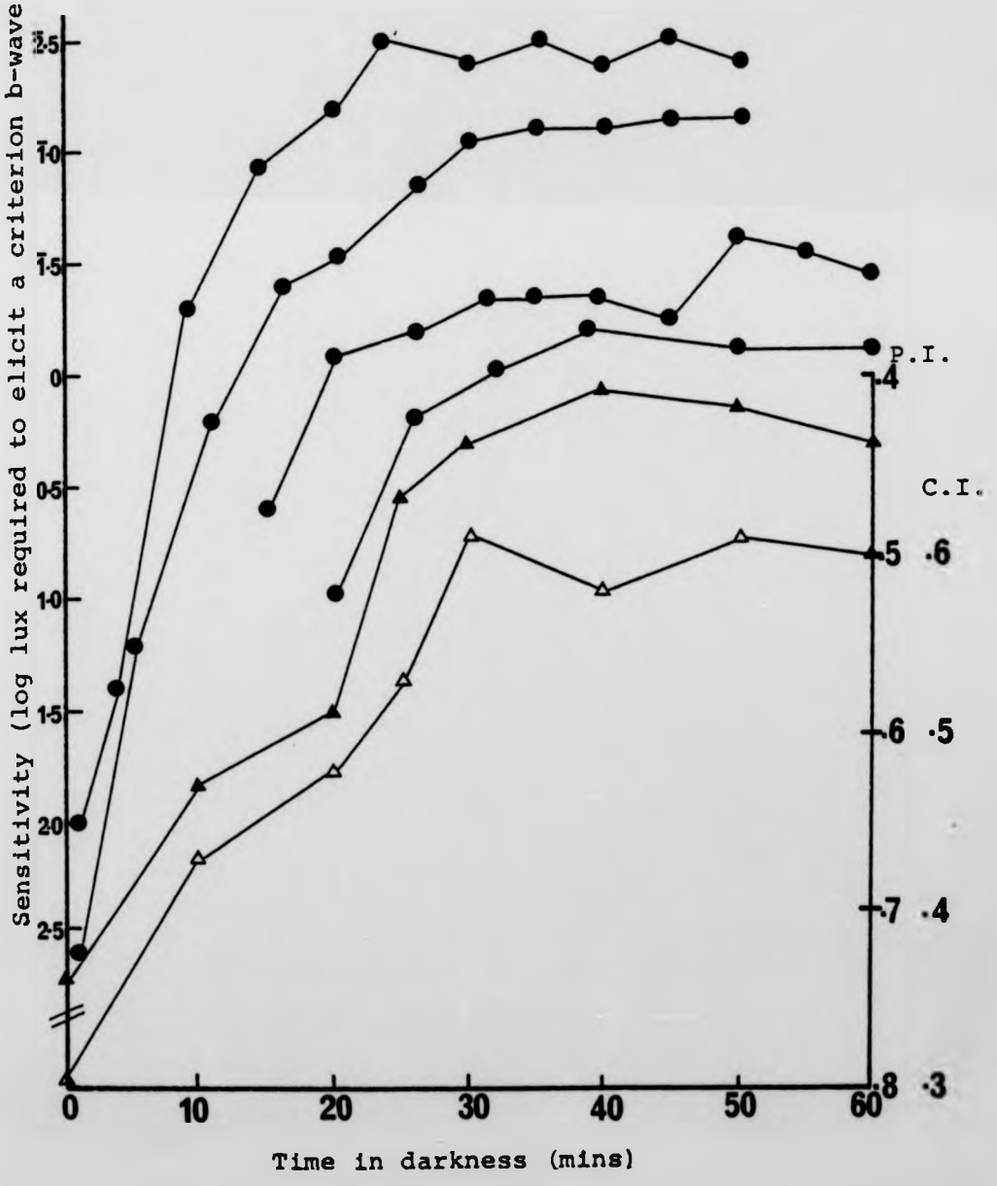


Fig 4.19. Increment threshold function - average difference between between the dark adapted threshold and the threshold at different intensities of background.

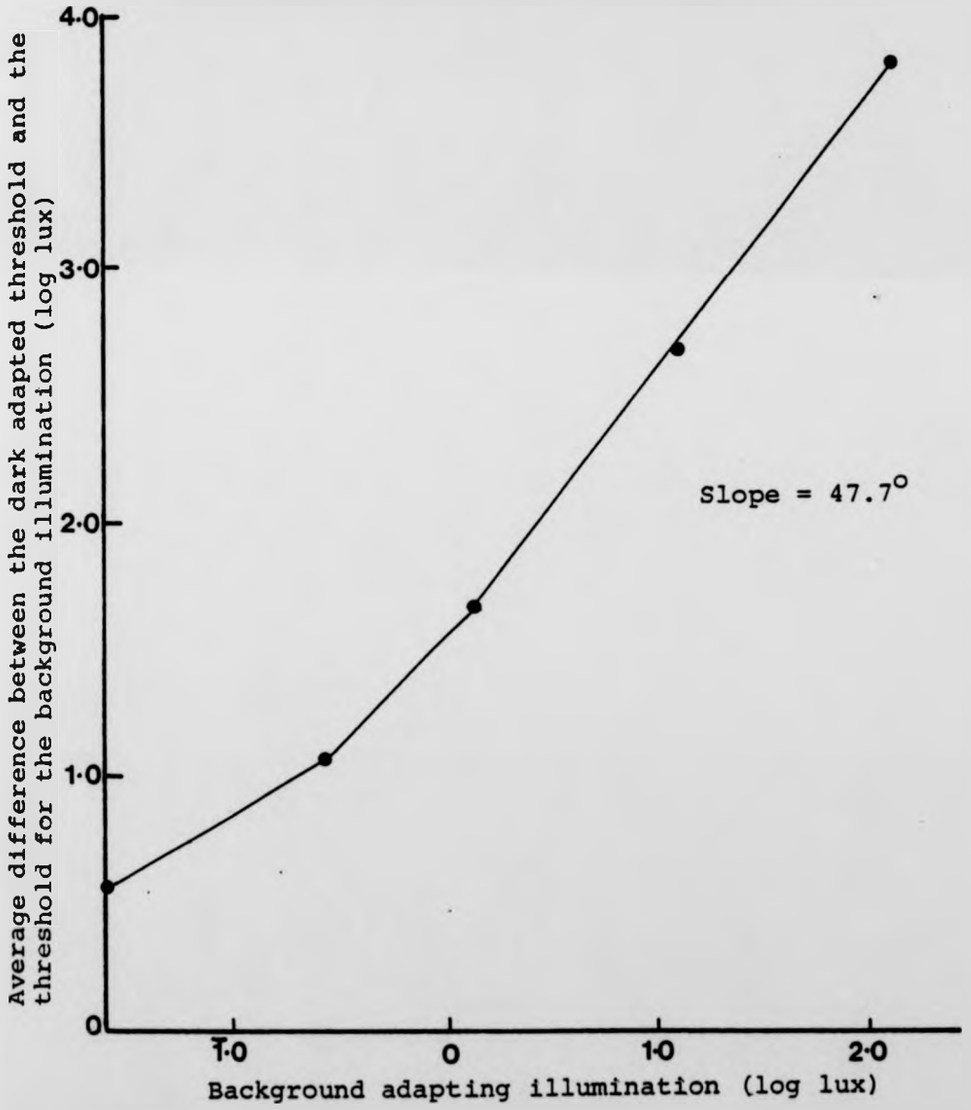


TABLE 4.II Intensity of stimulation required to elicit a b-wave of 50 μV at different intensities of background illumination.

Fish No	39	40	45	48
Background intensity (Lux)	Stimulus intensity necessary to elicit a 50 μV b-wave (Lux)			
0	1.32×10^{-1}	3.98×10^{-2}	4.07×10^{-2}	8.32×10^{-2}
2.51×10^{-2}	3.47×10^{-1}	1.51×10^{-1}	1.58×10^{-1}	2.19×10^{-1}
2.63×10^{-1}	6.92×10^{-1}	3.47×10^{-1}	8.32×10^{-1}	7.94×10^{-1}
1.38	2.29	3.16	2.95	2.29
1.20×10^{1}	7.59×10^{1}	2.29×10^{1}	1.82×10^{1}	1.91×10^{1}
1.2×10^{2}	-	-	2.09×10^{2}	6.31×10^{2}
Weber factions ($\Delta I/I$)	3.99	2.10	1.80	2.83

approximating the Weber-Fechner law (slope = 45°). As the illumination reached the absolute threshold at low levels of background this law was no longer obeyed (slope < 45°).

The Weber fraction for each fish, determined by dividing the response threshold by the background illuminations at backgrounds 1.38, 12 and 120 lux and averaging them together for each fish, are shown in table 4.II. The average Weber fraction of the four fish is 2.68.

4.4 DISCUSSION

The literature pertaining to both ERG sensitivity and form changes during light and dark adaptation is so vast that a comprehensive review is beyond the scope of this study. Therefore, in the following discussion the general findings concerning adaptational changes in the rainbow trout are only briefly compared to previous work on some teleosts. The review of the literature relating photomechanical movements to the ERG, on the other hand, is more comprehensive, as all observations concerning the relationship between retinomotor movements and both electroretinographically measured sensitivity and ERG form have never been considered together in one review.

4.4.1 Form changes of the ERG associated with adaptation. In general, the ERG of the rainbow trout reported here is typical of most teleosts. During dark adaptation it gradually changes from a fast response with an a- and d-wave and a low amplitude b-wave, to a much slower response with a higher amplitude of b-wave and no d-wave. The a-wave latency seems to stay constant throughout dark adaptation, but as Gramoni & Ali (1970a) demonstrated in a very detailed study, an increase of the a-wave latency by as little as 2 msecs, as occurs in Salmo salar ouaniche,

may be significant. Such small changes could not be measured by the methods employed in this study.

The increase in a-wave amplitude observed during dark adaptation is similar to that found by Hoffert & Ubels (1979c) in Salmo gairdneri and Kobayashi & Ali (1968) in Lepomis gibbosus. The latter authors explain this observation, which is contrary to that usually observed in vertebrates, in terms of retinomotor movements. During dark adaptation all of the rod and some of the cone outer segments are exposed, due to the retraction of the epithelial pigment, while in the light adapted state only the cones are exposed as the rods are shielded by the pigment epithelium. If, as is believed, the a-wave at least partially originates from the receptors (Armington 1974 for review), it is logical that more visual pigment will be bleached in the dark adapted state when more receptors are exposed, than when light adapted.

The component of the ERG over which there is most disagreement is the c-wave. Generally it is associated with the dark adapted retina and thought to be absent under light adapted conditions (Gramoni & Ali 1970a, Salmo salar ouaniche, Mühlmann 1967, Perca fluviatilis and Fonner et al 1973, Hoffert & Ubels 1979a, Salmo gairdneri). However, Hanyu & Ali (1964, Salmo salar) and Ali & Kobayashi (1968a, albino Salvelinus fontinalis) have recorded a c-wave from light adapted fish, while it was observed in neither the light nor dark by Ali & Kobayashi (1968a, pigment Salvelinus fontinalis) and Kobayashi & Ali (1968, Lepomis gibbosus). As Gramoni & Ali (1970a) point out, Millodot (1967) claims to have shown a c-wave for light adapted Salvelinus fontinalis, but as can be seen from his diagrams, the c-wave is probably due solely to slow fluctuations of the baseline. The results of Mühlmann (1967) are equally unconvincing, as what he considers a c-wave is just a slight rise at the end of the trace that is hard to identify positively. Thus the c-wave shows no consistency in its

presence or absence from one study to another. Part of the reason for this inconsistency may be that the c-wave, due to its slow time course, would be lost by AC coupling, depending on the time constants used.

Gramoni & Ali (1970a) have shown that these differences between studies are not due to the type of electrode used, as c-waves have been recorded with both intracellular and surface wick electrodes. Hamasaki & Bridges (1965), Hamasaki et al (1967) and Kobayashi (1962), on the other hand, have shown that the choice of anesthetic can influence the ERG. Hoffert & Ubels (1979a) therefore blamed the inconsistency of the c-wave behaviour in the studies of Ali and his co-workers on the use of MS -222, an anesthetic which Fonner et al (1973) had shown to have a deleterious effect on the ERG. The concentration of the anesthetic may also be important, as Ookawa (1971) was able to suppress the c-wave, normally present in chicks, by using deep anesthesia. Fonner et al (1973) and Hoffert & Ubels (1979a & b), without the use of anesthetic, showed that the amplitude, and thus the appearance of the c-wave, is also influenced by temperature and ventilatory flow rate, which in turn influence the arterial and intraocular $_{p}O_{2}$. Another factor that may influence its presence is the length of the stimulus. Gramoni & Ali (1970a) were unable to elicit a c-wave after sixty minutes dark adaptation with a stimulus of 128 msec, but were able to do so with one of one second duration. Length of adaptation is also important as after several hours dark adaptation these authors were able to elicit a c-wave with a stimulus of only 128 msecs.

Although positive potentials arising after the b-wave, other than the d-wave, were observed in the present study, it is unlikely that they are true c-waves as they are normally

associated with intermediate states of adaptation and, unlike the c-wave, they are fast and transitory. A c-wave has however been reported in dark adapted Salmo gairdneri by Fonner et al (1973) and Hoffert & Ubels (1979a,b & c). Any of the above mentioned differences in technique might explain the failure to find a c-wave in this study, but most likely the reason is that only one second of the response was usually recorded, while the above authors recorded up to twenty seconds after stimulation. As the c-wave is a relatively slow response it may be present, but has just not been observed due to the short duration of recording. Some evidence for a dark adapted c-wave will be given in the following chapter (Fig 5.12).

The secondary positive potentials observed after the cessation of the stimulus in the present study may be explained in another way. They could be related to the "delayed off" responses or "e-waves" seen in the ganglion cell response and ERGs of frogs (Pickering & Varjú 1967, 1969 & 1971, Varjú & Pickering 1969, Pickering 1968, Sickel & Crescitelli 1967, Crescitelli & Sickel 1968, Crescitelli 1970, Sickel 1972, Chino & Sturr 1975a & b, Newmann & Lettvin 1978, Tomita et al 1978a & b and Tomita 1978). Some form of dark adaptation is required to elicit these waves, which can occur up to sixty-five seconds after the stimulus, the latency increasing the longer the frog has been in darkness. Several authors have shown these responses to be related to rod activity, so that Sickel (1972) refers to them as "scotopic off effects", while Tomita et al (1978a) call them "rod specific d-waves." The dependence of this waves latency on the state of adaptation as well as the intensity of stimulation means that it has a great mobility in time. Such mobility often results in it not being distinguishable from the d-wave, when one is present. Crescitelli & Sickel (1968),

for instance, obtained records of the two waves completely separate and in various stages of fusion. As in the present study, fig 4.7 b & c, the second wave was often seen taking-off from the first. These same authors note that although such delayed off components have never previously been described they "should be a fundamental entity of the vertebrate ERG."

Although it is not possible to say with certainty whether or not the potentials observed in rainbow trout are the same as the delayed off responses in the frog, some factors do indicate that they may be related. Firstly, the disappearance of the response in fig 4.8 during dark adaptation may be explained by the increase in latency during such adaptation. The response may thus occur at a time after that recorded on the oscilloscope in the later stages of dark adaptation. Secondly, the variable position of the response in relation to the d-wave recorded in fig 4.7, is very similar to the situation noted by Crescitelli & Sickel (1968) for the frog e-wave. Lastly, the e-wave is a rod specific scotopic response, which could explain why the observed response was lost during light adaptation.

4.4.2 Effect of stimulus intensity on the ERG. The slowing of the response, accompanied by a decrease in amplitude, with decreasing stimulus intensity, is common to all vertebrates. A phenomenon also often noted, as in this study, is the decrease in b-wave amplitude at high levels of stimulation. It has been reported among teleosts in Lepomis gibbosus (Kobayashi & Ali 1968), Salvelinus fontinalis (Ali & Kobayashi 1968), Cyprinus carpio, Saurida undosquamis and Scorpaenodes guamensis (Kobayashi 1962), Plecoglossus altivelis (Hanyu & Tamura 1978), Katsuwonus pelamis (Hanyu et al 1973) as well as in the elasmobranchs Mustelus mamazo (Kobayashi 1962), Negaprion brevirostris, Ginglymostoma cirratum, Dasyatis sayi (Hamasaki et al 1967), and other vertebrates,

such as the rat (Dodt & Echte 1961 and Cone 1964), the frog (Riggs 1937 and Sickel 1960), and humans (Karpe & Tansley 1948). Similar depression has never been observed in the a-wave, which, due to its higher threshold, continues to increase in amplitude at all examined levels of stimulation. This higher threshold of the a-wave is also the reason why, when the level of stimulation is decreased, the a-wave always disappears at a higher intensity than the b-wave.

Kobayashi (1962) found b-wave depression in only three of the twenty-five marine and freshwater teleosts he examined. He correlated this to the low light levels (deep or turbid water) in which these fish normally live. Such a correlation clearly does not hold in the case of the rainbow trout which can inhabit clear shallow water.

4.4.3 Measurement of sensitivity changes during light and dark adaptation. The time course of adaptation can be ascertained in either of two ways; (1) By measuring the response to a constant stimulus or (2) by finding the stimulus intensity necessary to elicit a constant criterion response. These two measures, used within one study, can give widely differing results. Kobayashi (1962), for instance, found that, "The recovery of the amplitude of the ERG during dark adaptation requires a long time in general, while the recovery of the threshold intensity is complete more rapidly ..... The retina of nocturnal fish ..... showed a wide range of the change in threshold intensity, in spite of indistinguishable increase in the amplitude of the ERG".

There are several problems associated with the use of the constant stimulus method. Firstly, as shown above, soon after the beginning of dark adaptation the b-wave amplitude no

longer rises proportionately to the intensity of stimulation. Thus the high level stimulus necessary to elicit a response at the beginning of dark adaptation may give a false indication of sensitivity in the later stages of adaptation. The danger of this was pointed out as early as 1948 by Karpe & Tansley working on humans. "In recording a dark adaptation curve in terms of the size of the b potential it is important to use a stimulus which is neither too strong or too weak ...if...the stimulus is too strong, the b-wave will reach its maximum before the adaptation process is complete." Secondly, the intensity of stimulation needed to elicit a response at the beginning of dark adaptation is so high, in comparison to that necessary when the animal is fully dark adapted, that stimulation at such high levels throughout may retard final adaptation. Thirdly, by measuring the intensity necessary to elicit a constant response one is measuring a threshold and is thus able to ascertain sensitivity changes during adaptation. The response to a constant stimulus, on the other hand, is not a true measure of sensitivity.

Thus, the constant response method is usually preferred as an indication of sensitivity, although it too has a drawback. The constant responses are only constant with respect to the particular parameter measured, in this case the b-wave amplitude. Therefore, responses that are equal in amplitude at different times during adaptation may, for instance, have different latencies and slopes. No intensity of stimulation will elicit an ERG in a fully dark adapted eye that is similar in all respects to one obtained at the beginning of adaptation (Johnson 1949).

The criterion amplitude chosen can also have an effect on the form of the sensitivity curve if the response vs intensity curves are not parallel, as was the case in this study. During

both light and dark adaptation the slope of these curves increased (figs 4.3 & 4.4). Similar increases in slope during adaptation have been noted in the teleost Katsuwonus pelamis (Hanyu et al 1973), three species of elasmobrach Negraprion brevirostris, Dasyatis sayi & Ginglystoma cirratum (Hamasaki & Bridges 1965), the rat (Dodt & Echte 1961) and in humans (Elenius & Ahlas 1961). The criterion chosen will thus determine the slope of the sensitivity versus time function. For the present purposes this does not matter as the important point is when adaptation is complete. This will be the same regardless of the b-wave amplitude chosen as a criterion. Such non-parallel response vs intensity curves will only be of consequence when examining the exact shape of the adaptation curve or when determining spectral sensitivity (Chapter 5).

4.4.4 Sensitivity change during light and dark adaptation. The absolute sensitivity difference between the dark and light adapted state observed for the rainbow trout (2.4 - 4.4 log units) compares quite favourably to absolute values observed in other fishes (table 4.III). This difference obviously depends on the intensity of the adapting background (Kobayashi 1962), which will account for much of the observed differences between species.

As in all vertebrates, adaptation is initially very fast, gradually becoming slower, before finally levelling off onto a plateau. In no case during dark adaptation was there a distinct break in the adaptation curve, as is found in some vertebrates, indicating a change from cone to rod function. Despite the fact that the majority of fish have duplex retinas, such a biphasic curve has never been observed electrophysiologically in any fish, although Crozier & Wolf (1940, for review) have found such curves using the optomotor reaction. The reason for this is not clear. Hamasaki et al (1967) suggest that, "The absence

TABLE 4.III Absolute sensitivity differences between light and dark adapted fish.

SPECIES	Difference (log units)	Background illumination (lux)	Reference
Negaprion brevirostris Dasyatis sayi Ginglymostoma cirratum	4 - 6	Not known	Hamasaki & Bridges (1965)
Narke japonica	1.5 - 3	0.1 - 15	Kobayashi (1962)
Holorhinus tobijei	1 - 2	0.6 - 12	Kobayashi (1962)
Urolophus fuscus	1 - 4	0.01 - 12	Kobayashi (1962)
Misgurnus anguillicaudatus	0.5 - 3	3 - 200	Kobayashi (1962)
Anguilla japonica	1 - 2	0.03 - 35	Kobayashi (1962)
Chrysophrys major	0.5 - 1.5	0.02 - 15	Kobayashi (1962)
Kareius bicoloratus	1 - 3	3 - 15	Kobayashi (1962)
Katsuwonus pelamis	3	10	Hanyu et al (1973)
Cyprinus carpio	3.4 - 4.0	90,000 photopic trolands	Witovsky (1968)

of a cone : rod break in dark adaptation curves would perhaps indicate that the cones are not the limiting factor in the rate of dark adaptation during the early stages as they are in other mixed retinas, as in man for example." It is tempting to speculate that this limiting factor in fish is the retinomotor movements. Unfortunately this cannot be the case as certain elasmobranchs, which have a duplex retina (Gruber 1975), but do not show photomechanical changes, have a monophasic adaptation curve, while frogs, which do exhibit retinomotor changes, have biphasic dark adaptation curves (Riggs 1937, Dodt, Echte & Jessen 1960 and Dodt & Jessen 1961a).

One possible reason for the absence of such a cone : rod break is that in fish the rods, and at least some cones, are relatively close in sensitivity. Powers & Easter (1978a &b) have shown both rods and red cones to be functioning near the threshold in goldfish.

4.4.5 Increment thresholds. Weber's law states that, the increment of intensity just distinguishable (ΔI), expressed as a fraction of the ambient intensity (I), should be constant ($\Delta I/I = k$). That is, the sensitivity of the eye should decrease by the same amount as the adapting illumination increases. This relationship has been confirmed in many species over a wide range of stimulus conditions, and is again confirmed by the work presented here, as is evidenced by a slope of approximately unity in fig 4.19. As is the case in most species so far examined, the constancy of $\Delta I/I$ breaks down at low levels of adapting illumination as the absolute threshold is approached. At these low levels factors such as background noise start to obscure the relationship.

Dodt, Echte & Jessen (1960) noted that the loss in sensitivity was less than the increase in the intensity of the

adapting light in the frog. The authors erroneously attributed this to photomechanical movements, suggesting that the intensity of the adapting light is decreased relative to the receptors by the epithelial pigment during light adaptation. In actual fact, the increment thresholds should be totally independent of retinomotor changes, as the pigment epithelium will not only reduce the intensity of the adapting illumination reaching the receptors, but will also reduce the stimulus intensity to the same extent.

The Weber fraction ($\Delta I/I$) is a measure of the ability to distinguish brightness differences. Therefore, a low Weber fraction indicates that two lights differing only slightly in brightness can be distinguished by the animal. Weber fractions, measured using the ERG, for several species of fish were determined by both Kobayashi (1962) and Protasov (1964). Their results are concisely tabulated by Blaxter (1970, pp 219 - 222). Kobayashi obtained results ranging from 540% - 2% in teleosts and observed a value as high as 1400% in the dogfish, Mustelus manzo. He related this large variation between species to ecological differences. Thus, fish relying little on vision had the highest Weber fractions ($>$180%). Protasov (1964) obtained very much lower values ranging from less than 0.01% to 12%. According to Kobayashi's classification the present results would indicate that rainbow trout should not rely heavily on vision for their behaviour, which is obviously untrue as the trout is known to be highly dependent on visual stimuli. An obvious reason for such a high Weber fraction for the trout is that the ERG b-wave is not a good measure of the "true" sensitivity of the animal. Such a measure can only be obtained using behavioural training techniques. Animals in a behavioural situation respond at intensities which no longer elicit an ERG (chapter 6). $\Delta I/I$ will also depend heavily on the criterion amplitude chosen as

the threshold. The larger the amplitude, the higher the threshold and thus the larger the Weber fraction. Differences between the criterion amplitudes chosen, limited by factors such as degree of background noise, thus cause differences in the values of $\Delta I/I$ observed. Electrophysiologically determined Weber fractions are therefore only useful as a rough indication of a fish's visual ability and are most useful as a comparative tool within a single study (eg: Kobayashi 1962).

4.4.6 Relationship between ERG sensitivity changes and photomechanical movements. That retinomotor movements may mediate the sensitivity changes is indicated by the parallel time course of the photomechanical movements and the ERG sensitivity changes during both light and dark adaptation. When fish are transferred from one light condition to another, sensitivity is initially low as the relevant retinal elements are not positioned so as to function optimally. Subsequently, sensitivity rises as the photomechanical changes occur and is maximal when retinomotor movements are complete, as the receptors are now in an optimal position to receive the impinging illumination. If the movements served solely to shield cones from one another, such a close relationship between sensitivity changes and retinomotor movements would not necessarily be expected.

The data for the adaptation of the photomechanical movements is very variable for intermediate states of adaptation (light adapted 10 minutes, dark adapted 10 & 20 minutes). This greater variability in semi-adapted states is probably due to the tendency of retinas in this condition to separate from the epithelial pigment, consequently causing errors in measurement (section 1.2.3). When the eye is fully light or dark adapted the pigment epithelium interdigitates between the cones thus binding it to the retina. In intermediate states of adaptation such interdigitation is less complete. Consequently, the pigment

epithelium and receptors are more easily separated, resulting in tearing (eg; fig 1.2). Individual differences may account for some of the variation, but this is likely to be fairly minor as the time courses of sensitivity change during adaptation are very similar for the different fish.

4.4.6.1 Invertebrate review. Such a direct link between sensitivity changes and pigment migration, as has been shown here for the rainbow trout, has never before beeen demonstrated in vertebrates, although a similar parallel has been drawn for invertebrates.

Various classes of invertebrates have been shown to possess some form of photomechanical response. Most studied have been those in the eyes of Arthropoda. Kuiper (1962) gives detailed descriptions of the two types of arthropod compound eyes. The superposition eye, usually found in nocturnal insects and other arthropods associated with dim habitats, have their sense cells a considerable distance from the crystalline cone. They also possess a migrating retinal pigment in secondary iris cells, which in the light adapted condition moved down between the crystalline cones, and while dark adapted is aggregated distally. The apposition eye, usally associated with diurnal animals, on the other hand, has its sense cells in close contact with the crystalline cones and generally lacks this form of migrating pigment. Kuiper (1962) suggested that the function of this pigment in the superposition eye is to protect the sense cells from overstimulation in bright light, a proposal analogous to that of Exner & Januschke (1906) and Herzog (1905) for vertebrates, although the mechanism of such protection is completely different. The crystalline cones have a high refractive index and therefore act as waveguides. When light adapted the pigment moves around the cones. As the refractive index of this pigment is rather

high, where it makes contact with the crystalline cones it reduces internal reflexion. Consequently, the amount of light transmitted along the crystalline tract and reaching the sense cells is reduced.

That this mechanism may be in operation has subsequently been confirmed by many workers. Bernhard & Ottoson (1960a & b, 1964) and Bernhard et al (1963) demonstrated, by intracellular recording, a close temporal relationship between the position of the screening pigment and the second phase of sensitivity change during dark adaptation in nocturnal Lepidoptera. If pigment migration failed to occur this second phase of adaptation was absent, showing that this part of the sensitivity change during dark adaptation was mediated by the migratory pigment.

A similar close temporal correlation between sensitivity changes during light adaptation and pigment position might therefore also be expected in these nocturnal moths. That this is not so was demonstrated by Höglund (1963, 1966) and by Post & Goldsmith (1965), who showed sensitivity changes to reach their final value within seconds, while pigment migration required up to sixty minutes. These authors showed, however, that such observations are not inconsistent with pigment migration reducing the intensity of incident light, as such migration has two opposing effects during light adaptation. The stimulus reaching the receptors is decreased in intensity, leading to a fall in sensitivity which is counterbalanced by the adapting light intensity also being decreased by the pigment, causing the receptors to gain in sensitivity. These two antagonistic effects tend to cancel one another, leaving the sensitivity unchanged by pigment migration.

Further support for Kuiper's (1962) hypothesis came from Höglund & Struve (1970) who showed that at all wavelengths tested

the sensitivity of dark adapted eyes with the pigment in the proximal position was lower than when the pigment was distal or completely removed. The difference between the proximal and distal curves (2 - 3 log units) served as a measure of the increase in screening effect caused by pigment migration.

The Insecta are not the only class of Arthropoda whose pigment movements have been studied. For instance, Arechiga & Fuentes (1970) demonstrated that the slow increase in ERG amplitude during dark adaptation in the crayfish, Procambarus bouvieri, is paralleled by changes in the position of the retinal pigment. In fully dark adapted animals the injection of sinus gland extract, which causes the pigment to move to a light adapted position, reduced the size of the ERG, thus showing that the retinal pigment modulates the amount of light reaching the photoreceptors. Some influence of pigment migration on the amount of light reaching the rhabdomeres in three species of prawn and a mysid was also suggested by de Bruin & Crisp (1957), but not backed up by any experimental evidence.

Hagins & Liebman (1962) suggest that the retinal pigment movements occuring in Mollusca may also be associated with shielding the photoreceptors during light adaptation. Accordingly, Byzov et al (1962) demonstrated that in squid and octopus sensitivity changes during dark adaptation were closely paralleled by changes in pigment position. Daw & Pearlman (1974) investigated the effectiveness of this screening pigment in the squid, Loligo peali, and found it to act as a filter of 0.6 log units.

Thus, by demonstrating a close temporal relationship between retinal sensitivity changes and pigment migration in certain species of arthropod and mollusc , the retinal pigment has been implicated as shielding the receptors in the light adapted state, a conclusion directly parallel to the one reached

for rainbow trout, by a similar method, in this study.

4.4.6.2 Vertebrate review. Although a direct link between photomechanical movements and the ERG has never before been demonstrated in vertebrates, Gramoni & Ali (1970a) did note two similarities between the fish ERG and retinomotor movements. Both responses depend on the intensity of pre-adapting illumination, and light adaptation is more rapid than dark adaptation. There have also been several indirect correlations made, indicating that the epithelial pigment may shield the rods and mediate sensitivity changes.

Dodt & Jessen (1961a) found that in the frog the threshold changed by a factor of thirty-two during light adaptation. Injection of dark adapted frogs with adrenalin, which causes the pigment epithelium to move to a light adapted position, reduced the sensitivity change to a factor of two to four over subsequent light adaptation. The authors thus conclude that pigment migration changes sensitivity by a factor of eight to sixteen.

Ali & Kobayashi (1968a) compared the ERGs of albino and pigmented Salvelinus fontinalis, and found the components of the albino's ERG to have higher amplitudes and shorter latencies compared to pigmented fish. These differences indicate a higher sensitivity of the albino trout, presumably caused by the lack of rod shielding by the absent pigment epithelium. Similarly, at low levels of illumination the ERG flicker fusion frequency of the albino was higher than that of the normal trout, and the maximum fusion frequency was obtained at a much lower intensity in the albino(150 lux) compared to the normal trout (2500 lux) (Ali & Kobayashi 1968b). These differences can also be explained in terms of the pigment shielding the receptors in the normal fish, thus reducing the effective amount of light and consequently the response.

A relationship has also been shown to exist between retinomotor activity and the ERG during hypoxia in rainbow trout, by Hoffert & Ubels (1979b). Initially Fonner et al (1973) and Hoffert & Ubels (1979a) showed that the dark adapted ERG of Salmo gairdneri includes a low amplitude c-wave, the amplitude of which is greatly increased during hypoxia. Such a c-wave is absent in the light adapted condition and hypoxia abolishes the complete electrical response of the eye. Subsequently, hypoxia during dark adaptation was found to cause the retinal elements to move from their normal dark adapted positions (Hoffert & Ubels 1979b), so that the cones contract and take up a position near the e.l.m. as in the light adapted condition, similarly, the rods expand toward the lamina basalis. The epithelial pigment expands to some degree but not to its full light adapted extent. Hypoxia had no effect on the light adapted retinal positions. These photomechanical changes can be used to explain the increased c-wave amplitude in the hypoxic retina. The c-wave is generally accepted as originating from the pigment epithelium, and is thought to be generated by the decrease in extracellular K^+ concentration in response to photic stimulation of the rods (Hoffert & Ubels 1979 a & b for review). The stimulating effect this K^+ concentration has on the epithelial cells decreases with distance (Oakley & Green 1976). Therefore, the greater the distance between the pigment and rods, the smaller the c-wave generated. Thus, in the dark adapted condition the c-wave is small, as the distance between the rods and epithelial cells is relatively large, but as hypoxia decreases this distance, by causing the rods and pigment epithelium to expand, the c-wave amplitude increases. No c-wave is generated when light adapted, as the rods are not stimulated due to being totally surrounded by the pigment. The suggestion that the c-wave amplitude increase is related to aberrant photomechanical changes during

hypoxia, finds support in the observation that in mammals, which do not show these changes, hypoxia causes a decline in the c-wave amplitude.

Over a wide range of both vertebrate and invertebrate species there is thus ample evidence that photomechanical movements determine the time course of sensitivity changes during adaptation, and that the epithelial pigment serves to shield the rods from stimulation when light adapted.

Since the initial suggestion of a pigment protection function for the epithelial pigment there has been very little evidence cited that would serve to disprove it. Granit et al (1939) did, however, challenge the theory, as they found the rise in retinal sensitivity in the frog, measured by the amplitude of the b-wave in response to a constant stimulus, to lag behind the rise in visual pigment concentration during dark adaptation. The authors initially speculated this was due to the covering of the rods by the epithelial pigment in the light adapted state, thus stopping the rods being stimulated and consequently leaving the sensitivity unchanged at a low level. Since the screening pigment does not move immediately on being stimulated, there is a lag in the rise of sensitivity, a point of view supported by the present work. To test this hypothesis Granit et al (1939) observed the rise in sensitivity in response to different intensities of stimulation. The authors conclude that for weaker stimulation the lag would be longer as, "the shielding by the pigment would be relatively more significant for the weaker test lights." For all intensities of stimulation the lag was found to be the same. The authors thus concluded that the pigment does not shield the rods. Such a conclusion is in no way valid as the pigment cuts out a certain proportion of the incident light regardless of its intensity. Thus, the

percentage change in sensitivity caused by the migration of pigment will be the same at all stimulus intensities and no difference in the lag periods before the rise in sensitivity would be expected. Granit et al (1939) found further support for their hypothesis by making sections of eyes during dark adaptation and observing if the retina was easily separated from the eye cup. They observed that this, "did not lead to the expected close correlation between dark adaptation and withdrawl of pigment." Such a statement again is unwarranted as they carried out no controlled experiments, and this conclusion is largelly based on incidental observations.

The only real contradiction to the shielding function of the pigment epithelium came from Therman (1939), who measured the b-wave in response to red and blue stimuli in both the light and dark adapted frog retina. When dark adapted, the blue response was large relative to the response to red light (Blue response /Red response = 0.91). In the light adapted eye the situation was reversed, with the red response being relatively larger (Blue/Red = 0.38). This is a demonstration of the Purkinje shift. When dark adapted frogs were injected with adrenalin, which caused the retinal elements to become light adapted, the response was still that of a dark adapted retina (Blue/Red = 0.94). No Purkinje shifted occurred, despite the movement of the retinal elements. Therman (1939) therefore concludes, "To judge by these results there is hardly any reason to believe that the dispersion of the pigment epithelium should have any significant protective purpose." This is the only real evidence against the pigment protection theory and at present cannot be explained.

4.4.7 Mechanism of rod shielding effect. Bäck, Donner & Reuter (1965) considered two possible ways in which the pigment might shield the rods. (1) In the light adapted condition the

epithelial pigment surrounds the ellipsoid and may reduce the amount of light entering in a way analogous to a pupil. For the frog, in which light adaptation caused the intensity relative to the rods to decrease by a factor of three, this would mean a reduction in receptive area from 1 (arbitrary diameter of rod outer segment) to 0.6 (diameter of exposed area at myoid/ellipsoid junction). In trout the reduction of light by the pigment epithelium was much higher, decreasing it by a factor of thirty-six, requiring a reduction in the surface area from 1 to 0.17. That is, the exposed area of the receptor at the myoid/ellipsoid junction would have to be approximately six times as small as the rod outer segment diameter. Such a reduction in area is quite feasible if one examines the electronmicrographs of isolated brook trout rods, as shown by Anctil et al (1973, p110 fig 2a - d), especially as the myoids are elongated and thus have a decreased diameter when light adapted. If such a reduction in area occurs, on the other hand, is open to question. The exact distribution of the epithelial pigment over the surface is unclear and requires further study to show if the exposed area is actually reduced by the required amount during light adaptation.

(2) The epithelial pigment acts as a filter, reducing the intensity of the illumination by absorbing the light scattered in the retina.

(3) Another possible way that the light reaching the rods may be reduced in the light adapted condition has been suggested by Miller & Snyder (1972) and concerns not only the pigment epithelium but retinomotor movements as a whole. It has long been known that the outer segments of the retinal receptors can act as waveguides in humans, as well as lower vertebrates, due to the higher refractive index of receptors relative to their surrounding medium (eg: O'Brien 1951, Toraldo et al 1952, Tansley & Johnson

1956, Sidman 1957, Barer 1957, Stiles 1962, Enoch 1963 & 1967 a & b, Denton & Nicol 1964, Röhler & Fischer 1971, Kirschfeld & Snyder 1976, Wijngaard 1971 and Zyznar & Ali 1975). Miller & Snyder (1972) propose the same is true for the myoids of frogs, Rana catesbeiana. In the light adapted condition the myoids elongate, thus decreasing their diameter. As the fraction of light within the myoid decreases with diameter, less light will be transmitted, thus reducing the amount reaching the rods. Light intensity is then further reduced by the pigment epithelium surrounding the receptors. As the epithelial pigment has a high refractive index it will tend to increase light loss by refraction, a suggestion analogous to that for invertebrates made by Kuiper (1962). Further, the pigment will absorb the energy transmitted on the outside of the receptor; "Thus the light loss resulting from a decrease in diameter of the myoid would be accentuated, and the rod myoids could function to protect the rod visual pigment from bleaching in bright light. ... pigment migration around the photoreceptor organells and myoids of receptors may function as a neutral density filter to reduce indiscriminately the intensity of light in the receptor organelles." (Miller & Snyder 1972).

4.5 SUMMARY

The results presented above (chapters 3 & 4) indicate that the epithelial pigment serves to protect the rod visual pigment in the light adapted retina. By measuring the visual pigment levels in both the light and dark adapted retinas, the eye was found to possess a nearly full complement of visual pigment in bright light when the rods are covered by the pigment epithelium. This masking pigment reduces the incident light by a factor of

approximately thirty-six, proving to be a much more effective barrier to light in the trout than it is in the frog.

The close correlation between sensitivity changes during light and dark adaptation and the position of the retinal elements shows that retinomotor changes mediate sensitivity and that it cannot be maximal until these movements are complete, indicating that the pigment shields the rods when light adapted. This conclusion is directly similar to that reached for the function of the migrating pigment in invertebrates by similar means. The involvement of the epithelial pigment in rod protection is further supported by various incidental observations of other authors, concerning the properties of the ERG related to the position of the retinal elements.

CHAPTER 5 RESPONSE OF THE LIGHT AND DARK ADAPTED EYE TO MONOCHROMATIC STIMULI AND FLICKER, AS MEASURED BY THE ERG.

5.1 INTRODUCTION.

The state of adaptation can affect visual function in several ways. It can alter, among other things, (1) sensitivity (2) spectral sensitivity and (3) the ability to temporally resolve stimuli. Changes in sensitivity have been described in detail in the preceeding section. The present chapter reports the effect of light and dark adaptation on the last two of these functions.

In an animal with a duplex retina scotopic vision is known to be mediated by a homogeneous class of receptors, the rods, containing, in the case of the rainbow trout, a mixture of vitamin A_1 and A_2 based visual pigments (Munz & Beatty 1965). Consequently, scotopic action spectra, determined either electrophysiologically or behaviourally, are usually monophasic and often agree well with the absorption spectrum of the extractable scotopic pigment, although on some occasions the long wavelength arm of the action spectrum may be too broad to fit the visual pigment absorption spectrum. Photopic vision, on the other hand, depends on the relative contributions of different cone types, each population containing a pigment absorbing maximally at a different wavelength. Photopic action spectra are thus often more complex, showing more than one peak. Attempts have been made to explain these curves in terms of the underlying cone pigments, with varying degrees of success.

As neither light nor dark adapted ERG action spectra have ever been described for the rainbow trout, spectral sensitivity curves were determined using the criterion response technique. The results are discussed in terms of the under-

lying visual pigments, and some of the assumptions made concerning the relation between scotopic visual pigments and the action spectrum questioned. Further, the photopic action spectrum serves as a comparison to the spectral sensitivity obtained using a behavioural technique (chapter 6).

The ability to temporally resolve stimuli is indicated by an animal's response to flickering stimuli. At a certain critical flicker fusion frequency it ceases to respond to individual stimuli. This relationship between flicker rate and response amplitude can be investigated either behaviourally, usually using the optomotor response (Brown 1965 for review), or electrophysiologically. In the present section the response of light and dark adapted eyes to flickering light, as measured by the ERG, is investigated and discussed in relation to the retinal morphology and life style of the trout.

5.2 MATERIALS AND METHODS

All fish used in this part of the study had been adapted to a twelve hour light/dark cycle for at least one week prior to experimentation. The temperature, as in chapter 4, ranged from 14°C to 17°C.

5.2.1 Scotopic spectral sensitivity. The action spectrum of six fish was determined in May 1979. Dark adaptation was carried out in the experimental set-up as described in chapter 4, using the same apparatus. Fish were assumed to be dark adapted when the threshold of the b-wave response to white light remained constant.

Wavelengths were presented in a random order, and an intensity versus b-wave amplitude curve constructed for each wavelength. Between presentations of different wavelengths the fish was exposed to a standard white light stimulus, which

was of the same intensity throughout an experiment. The average b-wave response to this stimulus before and after each wavelength trial was taken as 100%, and all responses to that wavelength expressed relative to this. This controlled for variations in the sensitivity of the eye, due to deterioration of the preparation during the course of an experiment. In this way, a family of curves such as that shown in fig 5.1 was obtained for each experiment. The intensity of stimulation necessary to elicit a response of 50% the size of the standard white light response was arbitrarily taken as threshold.

5.2.2 Photopic spectral sensitivity. As demonstrated in chapter 4 (fig 4.10) using a white light stimulus, the photopic ERG is of a very much smaller amplitude than the scotopic response. This already small response is reduced even further when the intesity of the stimulus is decreased by the addition of interference filters. Using the previous apparatus (fig 4.2) the response was so small that it could not be distinguished reliably from the general background noise. To get around this problem one either has to use some form of averaging technique, or the intensity of the stimulus has to be increased. The latter solution was effected in the present study by increasing both the voltage and wattage of the light source (colour temperature, 4560^{o}K), using the bulb and condenser of a Rank Aldis tutor 2 projector to give the stimulus and modifying the optical system accordingly (fig 5.2). A rotating sectored disc, giving equal periods of light and darkness, was inserted to present a flickering stimulus. The bulb used to give the background was the same as that used to give stimulus and background illumination in all other experiments.

The procedure for presentation and determination of the threshold was as described for determination of the scotopic spectral sensitivity described above, except that the stimulus

Fig 5.1. Relationship between the b-wave amplitude and the intensity of stimulation at various wavelengths in a dark adapted individual.

The b-wave amplitude is expressed as a percentage of the response to a standard white light stimulus.

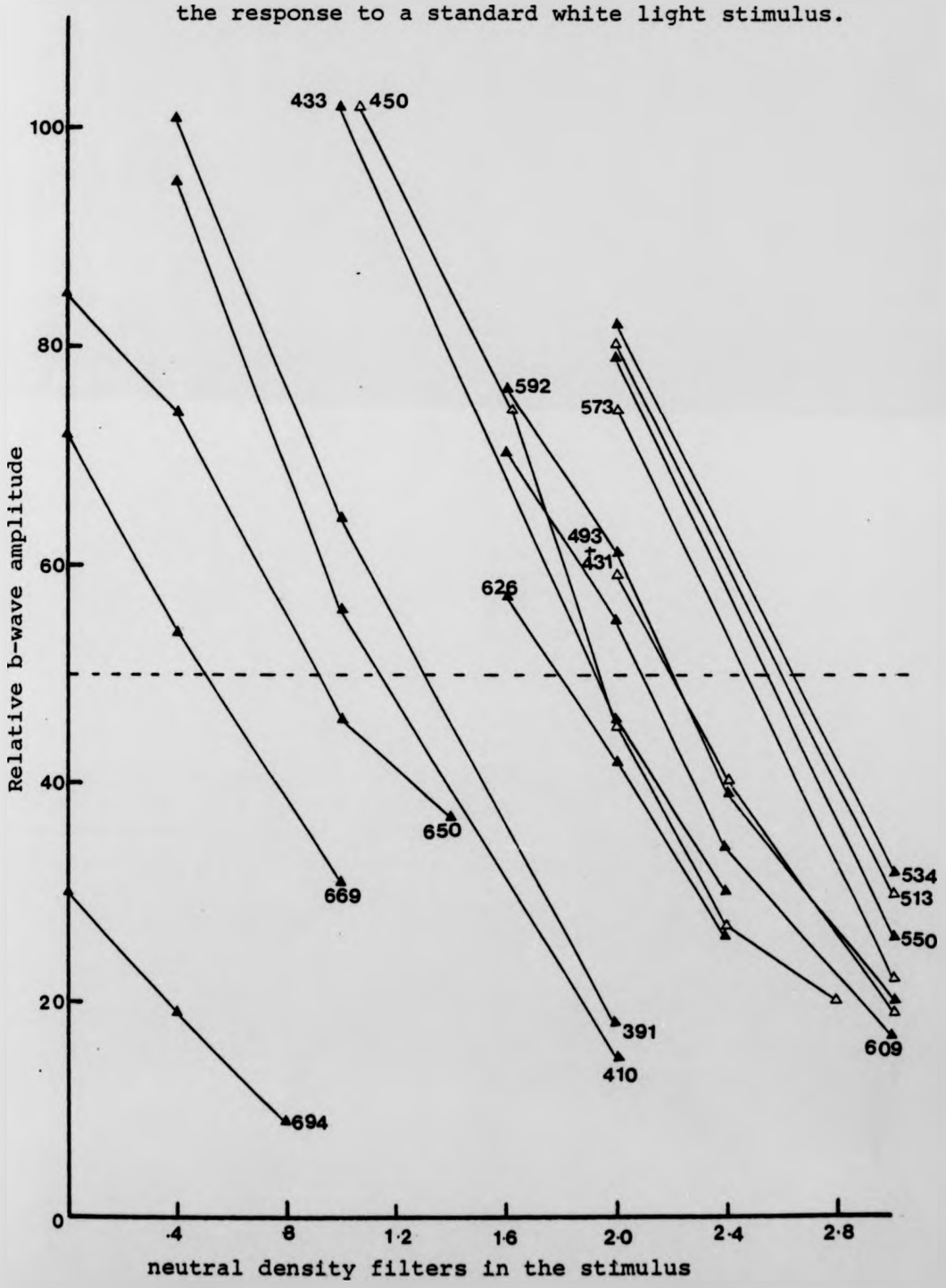
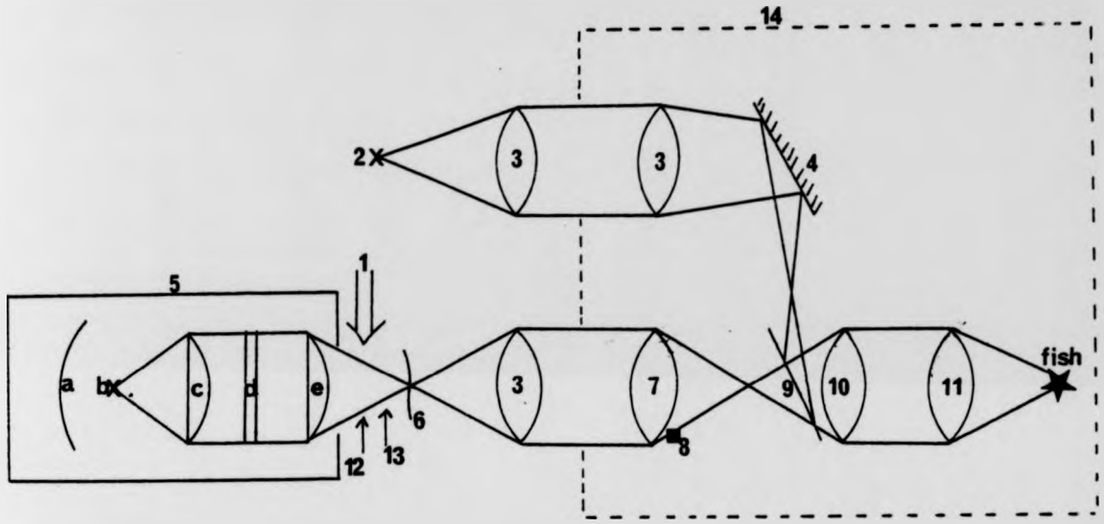


Fig 5.2. Optical system used in determining photopic spectral sensitivity.



1. Cooling fan.
2. light source (12V 36W).
3. Achromatic doublet (10 cm focus).
4. Mirror.
5. Rank Aldis tutor 2 projector.
   a. reflector.
   b. 24V 250W bulb.
   c. aspheric condenser.
   d. heat glass.
   e. front condenser.
6. Rotating sectored disc.
7. Achromatic doublet (12.5cm focus).
8. Photodiode.
9. Beam splitter.
10. Bi/convex lens (15cm focus).
11. Bi/convex lens (10 cm focus).
12. Interference filter
13. Neutral density filter.
14. Faraday cage.

was presented at a frequency of four hertz. Each one second stimulus presentation therefore gave four responses, which were averaged for greater accuracy.

Fig 5.3 shows the type of response versus intensity relationship observed in three out of the five fish used. A criterion response of 50% of the white light response was again taken as the threshold, as in this area the lines of the amplitude/intensity relationship for each wavelength were most parallel. At higher intensities the "saturation response", described for white light in chapter 4 caused unrepresentative results. In the remaining two fish, response versus intensity curves such as that shown in fig 5.4 were obtained, where the response to 626nm stimulation was relatively flatter. In this case a 50% criterion was still used, although it is clear that by taking a higher criterion response the sensitivity maxima at 626nm would be diminished and finally disappear.

5.2.3 Calibration for light incident on the cornea. Thresholds obtained in this way for both photopic and scotopic conditions are, in the first instance, in terms of "apparent neutral density". In order to obtain the true degree of light incident on the cornea, differential spectral absorption by the elements of the optical system must be corrected for. The degree of illumination at the focal point, where the fishes eye would normally be, was therefore determined using a U.D.T. 40x optometer. In this way, a relationship between the apparent neutral density and actual illumination for all interference filters was obtained. Using these curves, apparent density thresholds could be converted to thresholds in terms of $\mu W/cm^2$. A further correction was also made as the optometer has an uneven spectral response. Thus a value for the actual illumination impinging on the cornea was obtained, accounting for any uneven spectral absorption of the

Fig 5.3. Relationship between the b-wave amplitude and the intensity of stimulation at various wavelengths in a light adapted individual.

The b-wave amplitude is expressed as a percentage of the response to a standard white light stimulus.

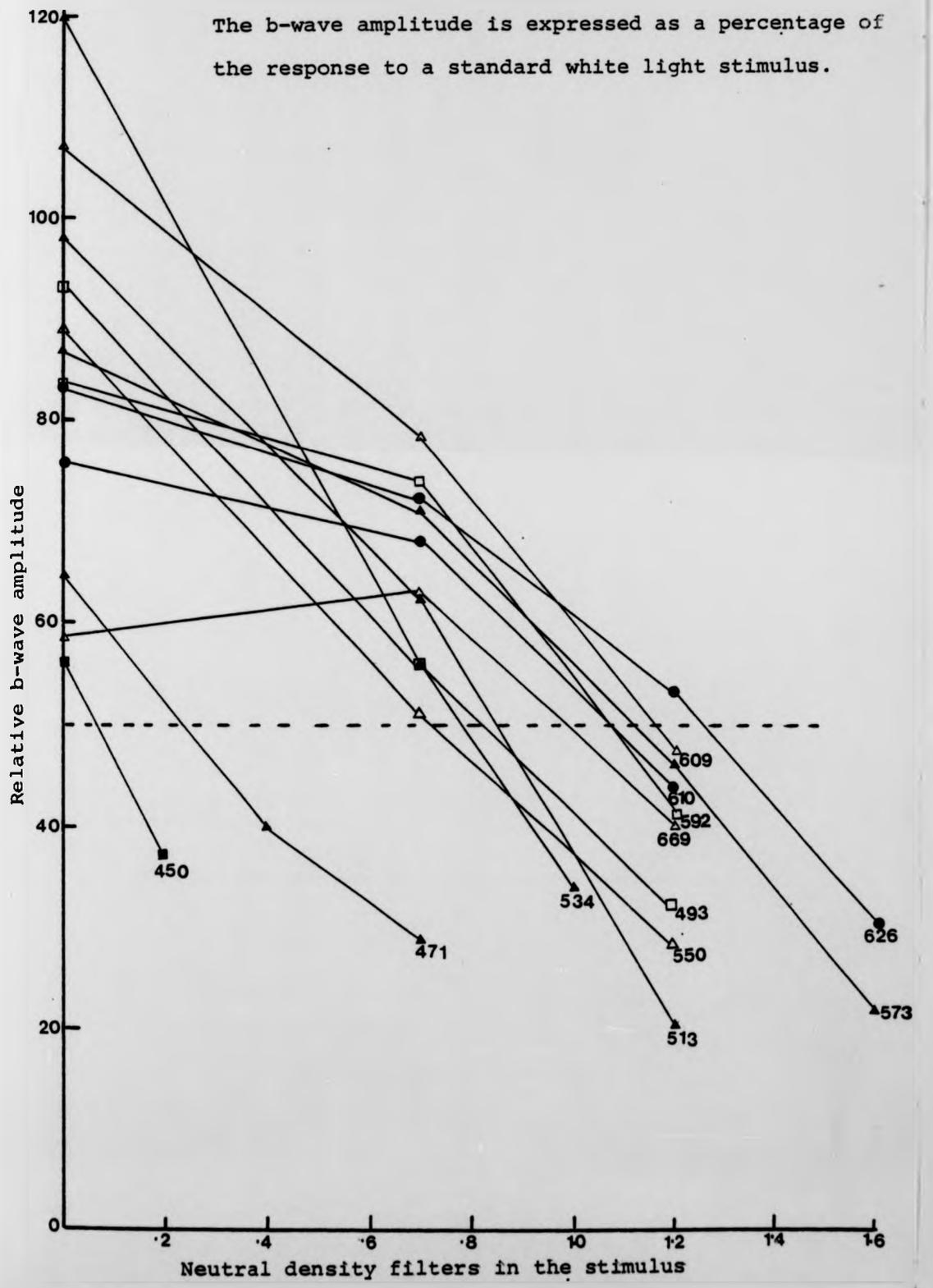
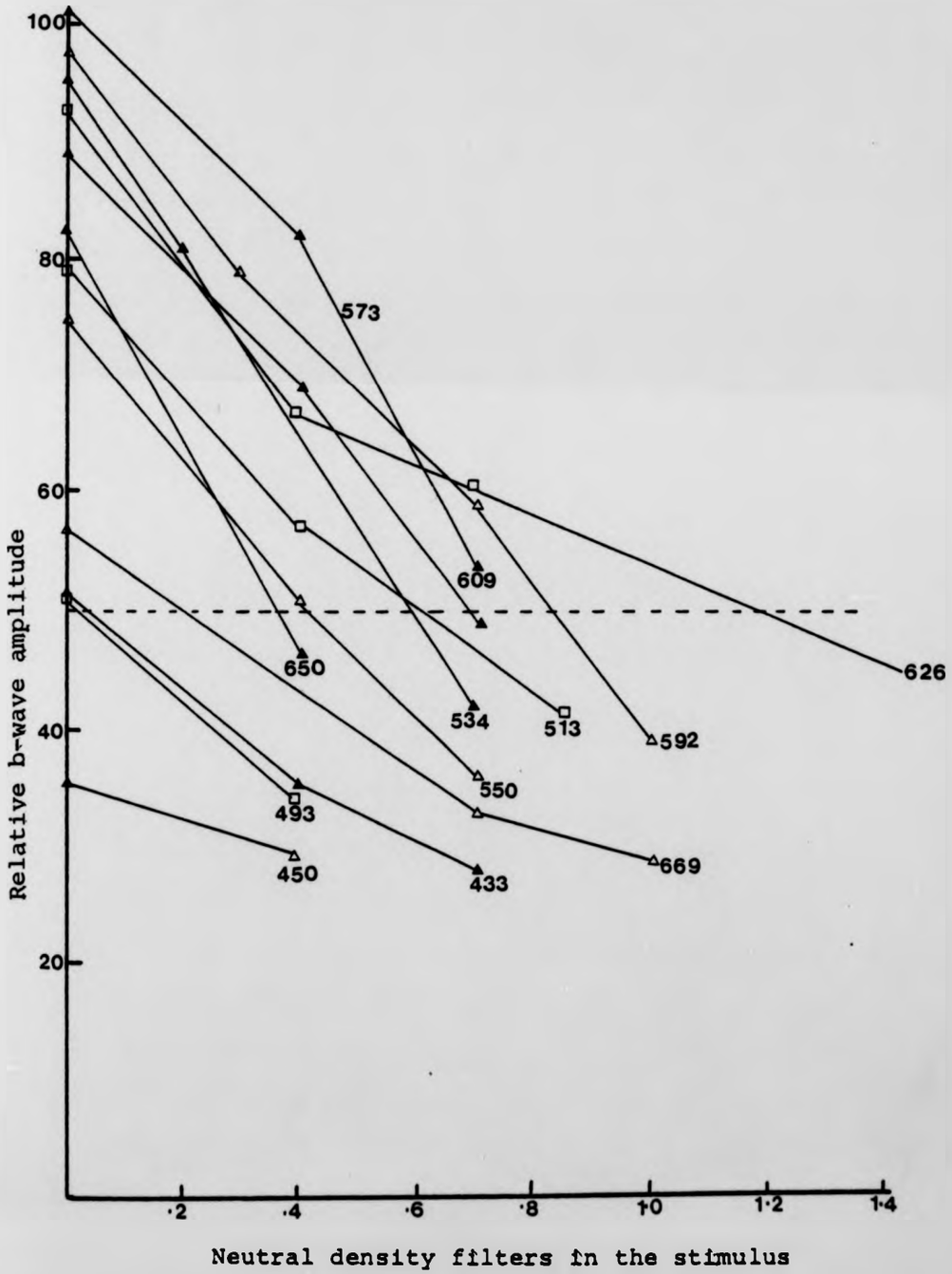


Fig 5.4. Relationship between the b-wave amplitude and the intensity of stimulation at various wavelengths in a light adapted individual.

The b-wave amplitude is expressed as a percentage of the response to a standard white light stimulus.



neutral density filters, lenses, beam splitter and interference filters.

5.2.4 Calibration for light incident on the receptors. In order to relate ERG spectral sensitivity to the spectral absorption of the visual pigments, corrections must also be made for the selective spectral absorption by the ocular media, so that the degree of illumination actually impinging on the receptors can be determined (Dartnall 1953). Many teleosts, for example, have coloured lenses and corneas, which absorb shorter wavelengths to a greater extent than the long wavelengths (Muntz 1972 for review). The failure to account for this in determining the threshold would result in a sensitivity curve apparently lower in the blue end of the spectrum than is actually the case. McCandless et al (1969) spectrophotometrically measured the absorbance of rainbow trout lenses, corneas, and aqueous humour. The aqueous humour was found to absorb selectively between 250 - 300 nm, but at all wavelengths after this approximately 90% of incident light was transmitted. The authors give a 50% transmission at 356 nm for the lens, but furnish no further details. As fish lens cutoffs are usually very sharp (eg: Muntz 1972), it is probable that all wavelengths after 390 nm are transmitted to the same extent. Thus neither the lens or aqueous humour need be corrected for. The results obtained by the same authors for the cornea, however, must be questioned. At 390 nm the cornea was reported to transmit only 40% of the incident light and at 700 nm only about 60% (fig 5.5). It is very unlikely that a cornea will have evolved that is so inefficient, allowing only 50% of incident light to reach the eye between 400 - 700 nm, effectively acting as a 0.3 neutral density filter. Although the relative spectral transmission obtained may be valid, this unlikely net transmission casts doubt on these results, and

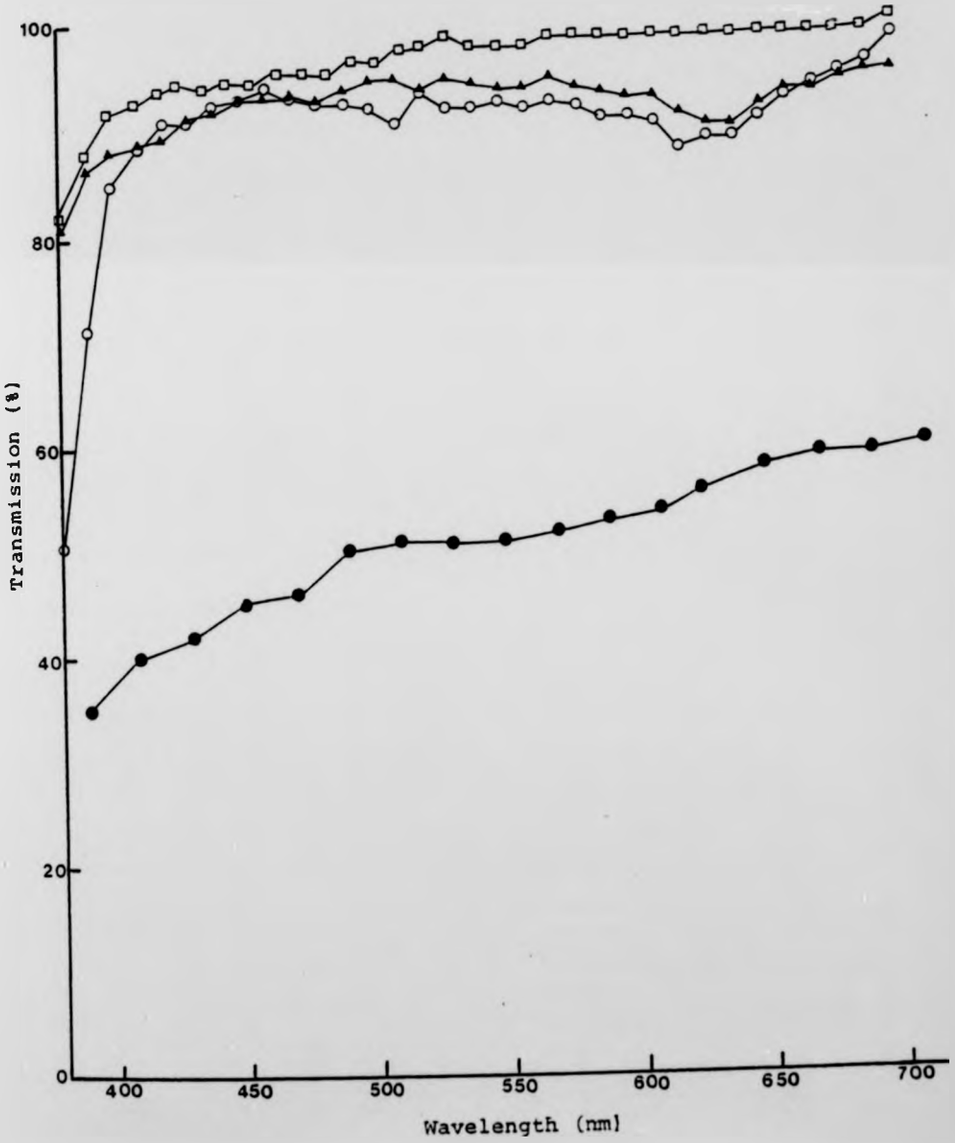
they were thus repeated.

Three rainbow trout corneas, mounted in teleost Ringer's, were examined for their spectral characteristics using a special purpose spectrophotometer (Muntz 1973 & 1976a for details). Fig 5.5 clearly shows that the results obtained differ from those of McCandless et al (1969). On average the cornea was found to transmit more than 90% of all incident light above 420 nm, which seems a more realistic value than that of McCandless et al (1969). The unlikely results of McCandless et al (1969) might be explained by corneal deterioration due to osmotic shock. The cornea is made of collagen fibres, the spacing of which is critical (Maurice 1957), and if the osmotic medium is changed these fibres may shift, causing a certain degree of opaqueness. Therefore, an average curve derived from the three corneas of the present study was used to make appropriate corrections. The corneas are seen to be spectrally neutral at wavelengths above 420 nm, below this the short wavelengths are preferentially absorbed. A correction at all wavelengths was effected by multiplying the threshold by the reciprocal of the corneal transmission.

In this way, the relative amount of light impinging on the photoreceptors was determined. One further correction must be made in order to compare successfully the spectral sensitivity to the absorption of the visual pigments. As Warburg (1920) indicated, the effectiveness of a visual stimulus depends on the number of absorbed quanta and not on the energy. As the energy of such photons is proportional to the reciprocal of its wavelength, the sensitivity (1/threshold) at all wavelengths must be divided by the wavelength, in order to obtain an equal quantum spectrum. That such quantal curves are more suitable for comparison to the visual pigment, than ones based on energy, has been shown in detail by Scheibner & Schmidt (1969).

Fig 5.5. Spectral transmission of three rainbow trout corneas.

(●) - results re-plotted from McCandless et al (1968).



These relative quantal sensitivities plotted against wavelength form an action spectrum which can be compared to the absorption spectrum of the visual pigments.

5.2.5 Visual pigments. For comparative purposes the scotopic visual pigments were extracted, using digitonin, from five dark adapted fish, kept under the same conditions as fish whose action spectra were determined using the electroretinogram.

Dartnall (1953) noted that the rhodopsins of several species had absorption spectra of similar shape when plotted against wavenumber instead of wavelength. This led him to propose a nomogram from which the spectral characteristics of any rhodopsin could be determined if its λ_{max} was known. A similar nomogram was subsequently proposed by Munz & Schwanzana (1967) for porphyropsins, whose absorption spectra are somewhat broader.

As the rainbow trout is a paired pigment species, with scotopic pigments $VP503_1$ and $VP527_2$ (Munz & Beatty 1965), the extracted visual pigments cannot be directly analysed using either nomogram individually. The difference spectra obtained from these extracts were therefore analysed using the computer program outlined by Muntz & Northmore (1971b). The difference spectra of the trout visual pigment can be thought of as the weighted sum of the component spectra of the two visual pigments in its retina. The program calculates these weighting factors, giving a least squares fit to the data. The basic form of the $VP503_1$ and $VP527_2$ spectra were obtained using the nomograms of Dartnall (1953) and Munz & Schwanzana (1967) respectively.

The above procedure was used for scotopic pigments, but as there is as yet no way of extracting individual cone visual pigments, the only reliable way to identify single photopic visual pigments is by microspectrophotometry. Since this

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technique was not available, hypothetical cone pigment absorption spectra were constructed using visual pigment nomograms. Loew & Dartnall(1976) have shown convincingly in the rudd, that the cones, as well as the rods, contain visual pigments based on vitamin A_1 and A_2. Furthermore, the ratio of these two pigments in the cones is the same as that in nearby rods. The authors thus conclude that both rods and cones draw their visual pigment prosthetic group from a common pool. If, as is now generally accepted, the cones contain similar visual pigments to the rods, nomograms such as those described above might be used to describe the absoption spectra of their visual pigments. Yet these nomograms are not universally applicable, as they are based on the assumption that the spectral characteristics will be the same for all pigments, independent of their λ_{max}. Recently, using techniques such as internal recording from single cones (Tomita et al 1967) and microspectrophotometry (eg: Harosi 1976), this has been shown not to be the case. Long wavelength pigments have narrower, and short wavelength pigments wider, absoption curves than predicted by the nomograms of either Dartnall (1953) or Munz & Schwanzara (1967). The absorption bandwidth of pigments seems to vary continuously with wavelength, and no single nomogram can therefore cover all areas of the spectrum. This led Ebrey & Honig(1977) to propose three nomograms, one for each the short (400 - 470 nm), middle (470 - 530 nm) and long (530 - 610 nm) wavelength regions of the spectrum. The nomograms for the middle area of the spectrum are still the original ones of Dartnall (1953) and Munz & Schwanzara (1967), thus leaving the computer program described above unaffected, as the λ_{max} of the rainbow trout will always lie between 503 and 527 nm.

5.2.6 Response to flicker. Fully light adapted fish,

anesthetized and placed in the experimental apparatus described in chapter four, were maintained in a state of light adaptation by a 120 lux background on the eye being stimulated. After ten minutes adaptation to these conditions the fish were presented with differing frequencies of flickering white light (1600 lux) for 1.15 seconds. The eye's response to the first second of each flicker rate was recorded on an oscilloscope and photographed for analysis. Flicker was presented in an order of increasing frequencies until single responses could no longer be distinguished. When this point was reached several of the lower frequencies were re-presented to ensure that the condition of the fish, or state of adaptation, had not changed. The fish were then dark adapted and the above procedure repeated for dark adapted eyes.

At all frequencies the peak to trough amplitude of the response was measured and all responses at each frequency averaged. Abnormally large responses immediately following stimulation, expecially in the dark adapted state, were ignored, as they differ greatly from the norm. Fig 5.6 shows the amplitudes measured at different frequencies in both the light and dark adapted condition. Amplitude measurement in the dark adapted eye was complicated by a large positive component following the initial b-wave (fig 5.6), causing amplitudes for dark adapted eyes to be abnormally large, especially at lower flicker frequencies. This positive component could be either a c-wave from the initial response or be due to d.c. drift, although the latter is less likely as the observed drift was always in the upward direction.

The critical flicker fusion frequency is usually taken as that frequency at which individual responses can no longer be distinguished from the background noise. It is therefore, to a certain extent, a subjective determination. In the present

Fig 5.6. Showing the amplitudes measured during the determination of the relationship between frequency and amplitude in both the light and dark adapted retina.

The lower trace at each frequency is the stimulus marker recorded from a photodiode placed in the stimulus beam. The upper part of the square wave represents the time when the stimulus is on. Each trace represents one second of response.

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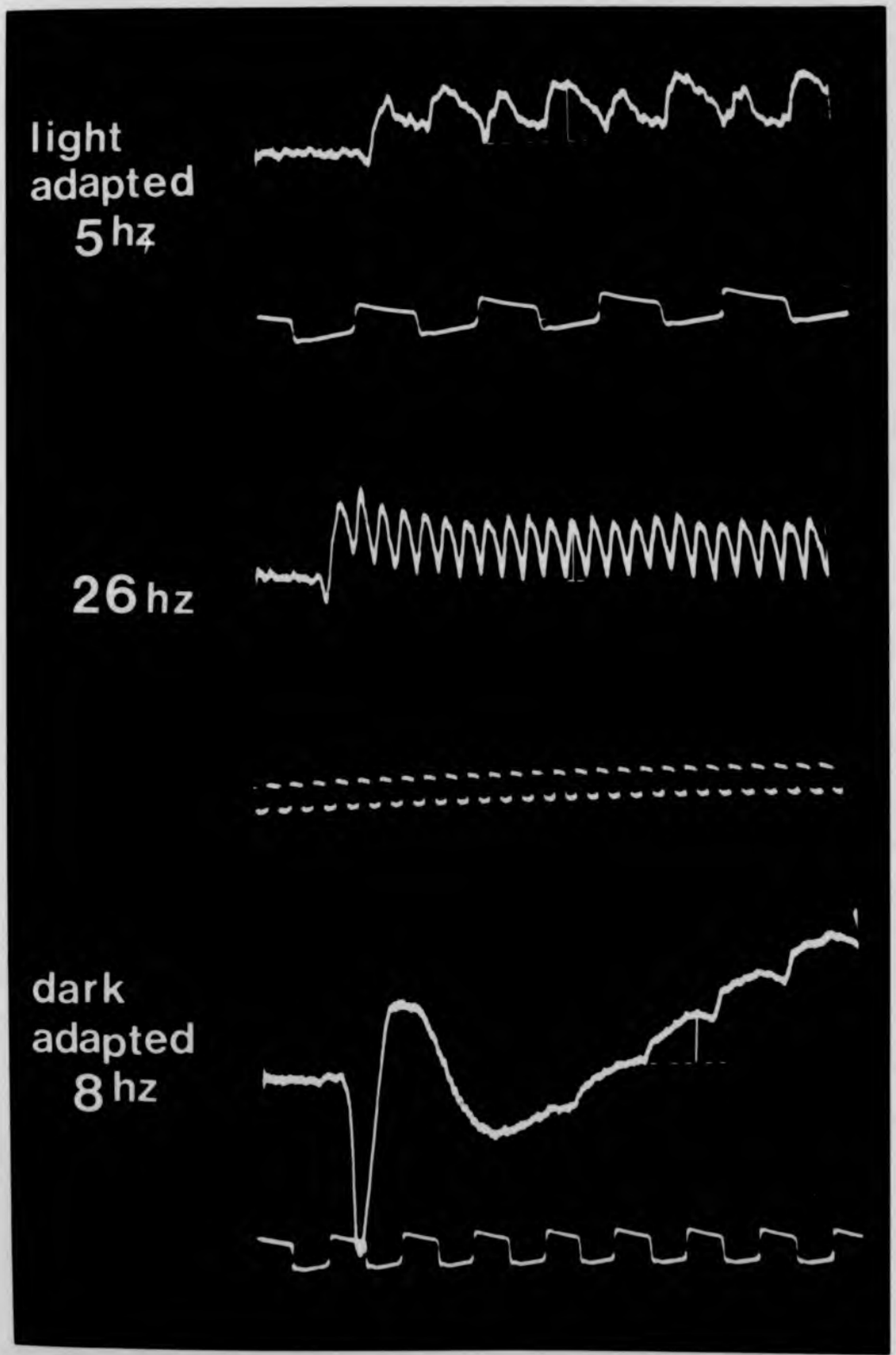

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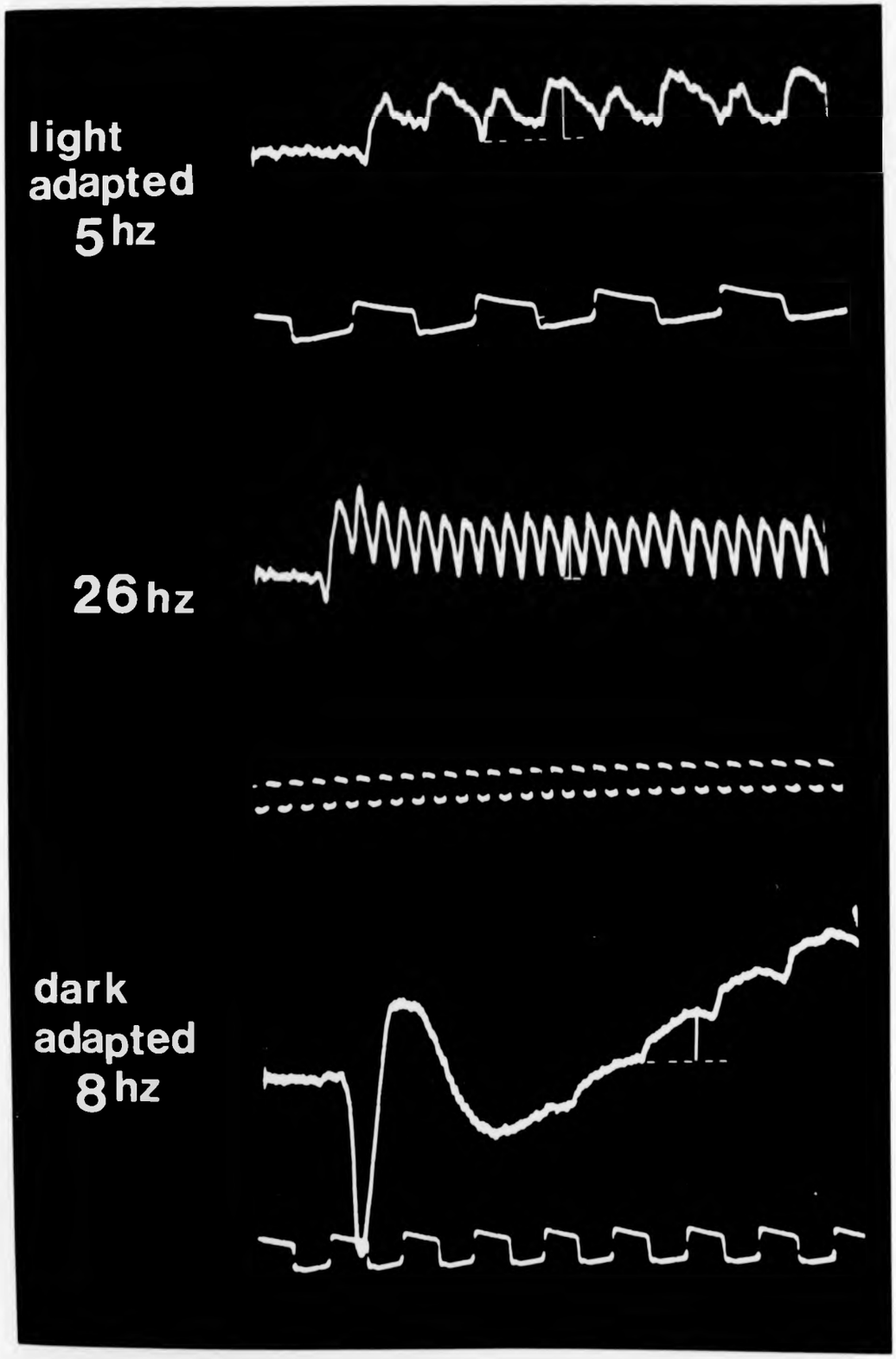


series of experiments this point was taken as that frequency which gave a response amplitude of 0.025 mV, which was just above the noise level in most cases. Individual responses could be observed at slightly higher frequencies but were hard to measure due to their small amplitude. Owing to decreased accuracy of amplitude determination at the higher frequencies, measurement often stopped at higher amplitudes than the criterion, and the results were extrapolated (fig 5.11) to 0.025 mV.

5.3 RESULTS

5.3.1. Scotopic spectral sensitivity. The relationship between sensitivity and wavelength for all six fish is shown fig 5.7. The curves have been arbitrarily shifted along the ordinate for clearer separation between fish. An average response for all fish (dotted line fig 5.7) was constructed by minimising the differences between individual curves, as outlined by Muntz & Northmore (1970). The average threshold for all wavelengths for each fish, over all points they have in common (410-650 nm), was determined. This value, subtracted from that of a fish chosen as the standard (S - fig 5.7), gives a "correction factor" for each fish, which can be added to its thresholds at all wavelengths to superimpose that fish's curve onto the standard, with a minimum of difference. An average for all fish of these corrected thresholds can now be calculated.

The λ_{max} of the extracted visual pigment from the five fish ranged from 512.2 nm to 515.5 nm, with an average of 514.4 nm (55% A_1 based pigment). The absorption spectrum determined using the program described above for a pigment mixture with this λ_{max} (solid line) is shown with the average ERG action spectrum (dotted line) in fig 5.8. The fit between the two curves is very

Fig 5.7. Scotopic action spectra of six individuals determined using the ERG b-wave.

(·····) is an average action spectrum determined as described in the text.

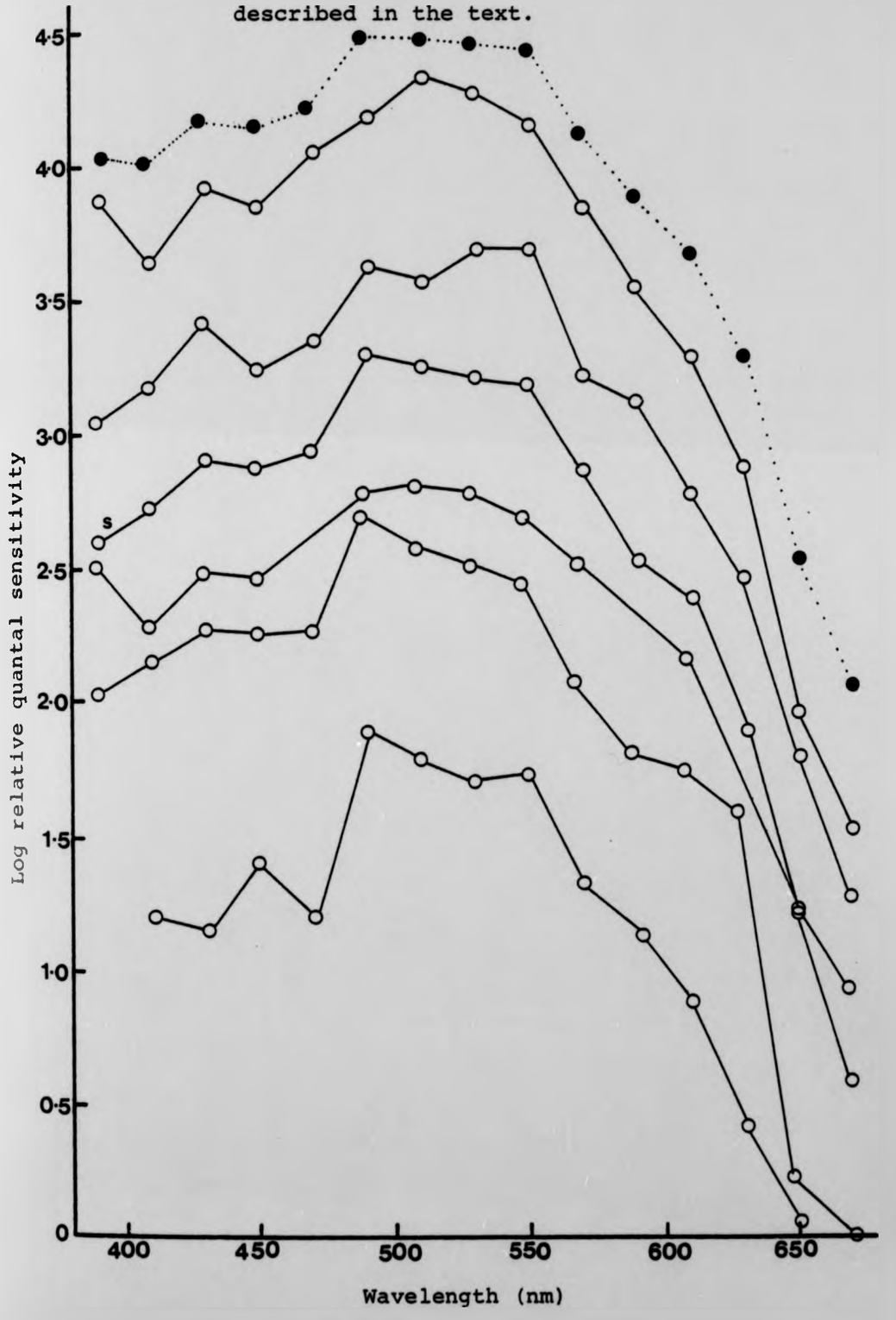
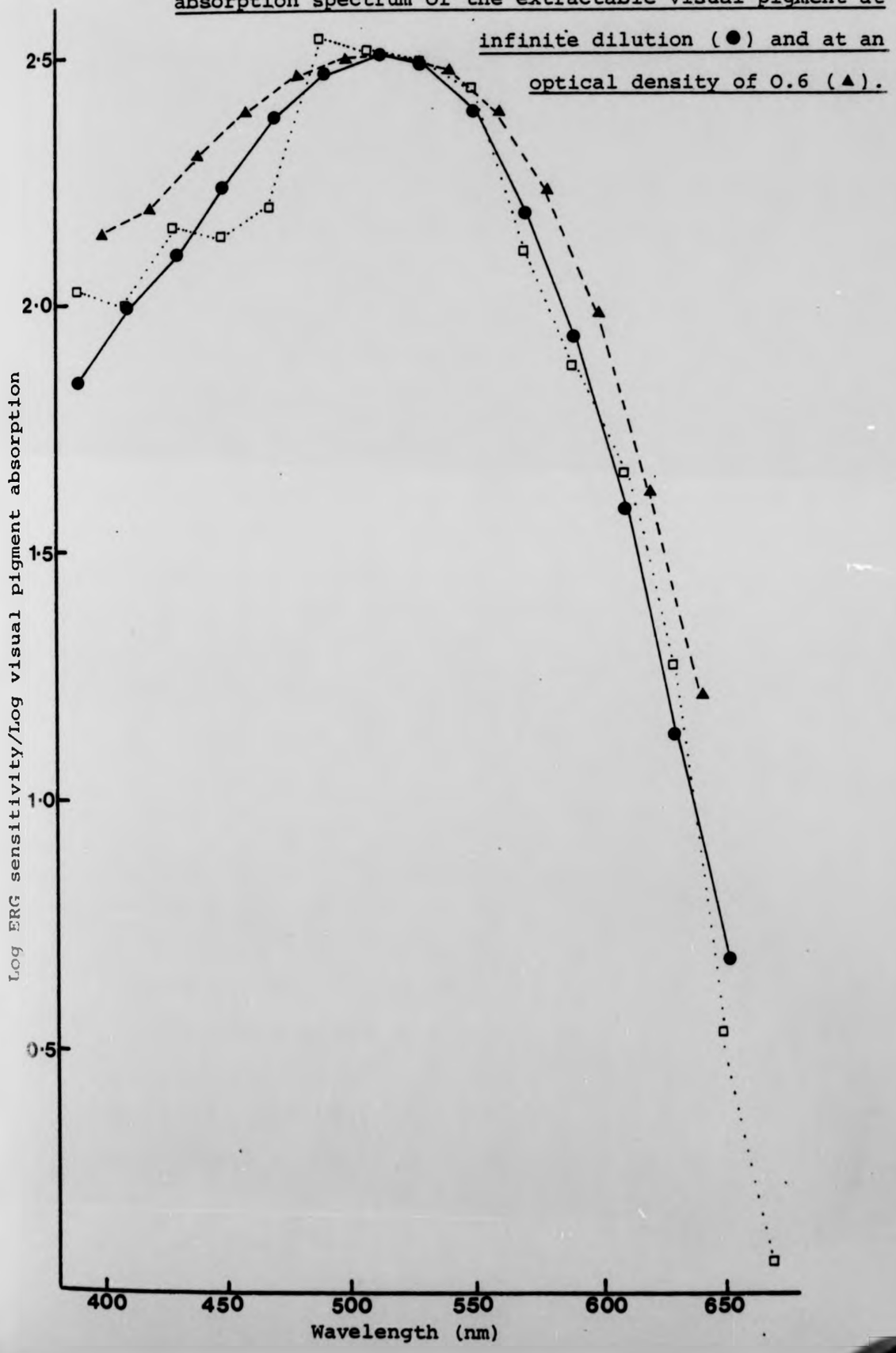


Fig 5.8. Average scotopic ERG action spectrum (□) and absorption spectrum of the extractable visual pigment at infinite dilution (●) and at an optical density of 0.6 (▲).



good.

5.3.2 Photopic spectal sensitivity. Three maxima can be seen in the photopic action spectra of all five fish, although their exact positions differ between fish (fig 5.9). All curves have again been shifted along the ordinate to facilitate separation, and an average curve was calculated as outline above for all fish except 121. Records for this fish are incomplete as one film was lost during processing. The average curve (dotted line fig 5.9) shows three maxima at 493 - 513 nm, 573 nm and 626 nm.

Fig 5.10 shows a series of ERGs in response to different intensities of 471 nm stimulation. Any systematic change of ERG form, related to wavelength or intensity of stimulation, was not investigated in detail.

5.3.3 Response to flicker. The amplitude/flicker frequency relationship in both the light and dark adapted state was determined for six fish (fig 5.11 a-f). Fig 5.12 a & b shows a typical set of records. In all six dark adapted eyes there was a large slow positive component, often larger than that shown in fig 5.6, which was never seen in the light adapted condition. An indication that this drift is only transient is given by fig 5.12 c, where the time base of the trace has been slowed down. This, added to the fact that it was only observed in the scotopic condition, might indicate that it is a c-wave following the initial response.

At low frequencies the light adapted response shows all the characteristics of a single flash light adapted ERG, as outlined in chapter four, exhibiting distinct a-, b- and d-waves (fig 5.12, 4Hz). As the frequency of flicker increases the b- and d-waves become closer together until they eventually fuse. The frequency of this fusion is indicated in fig 5.11 b,c,e,f & g by a distinct amplitude peak, usually around

Fig 5.9. Electroretinographic photopic spectral sensitivity of five individuals.

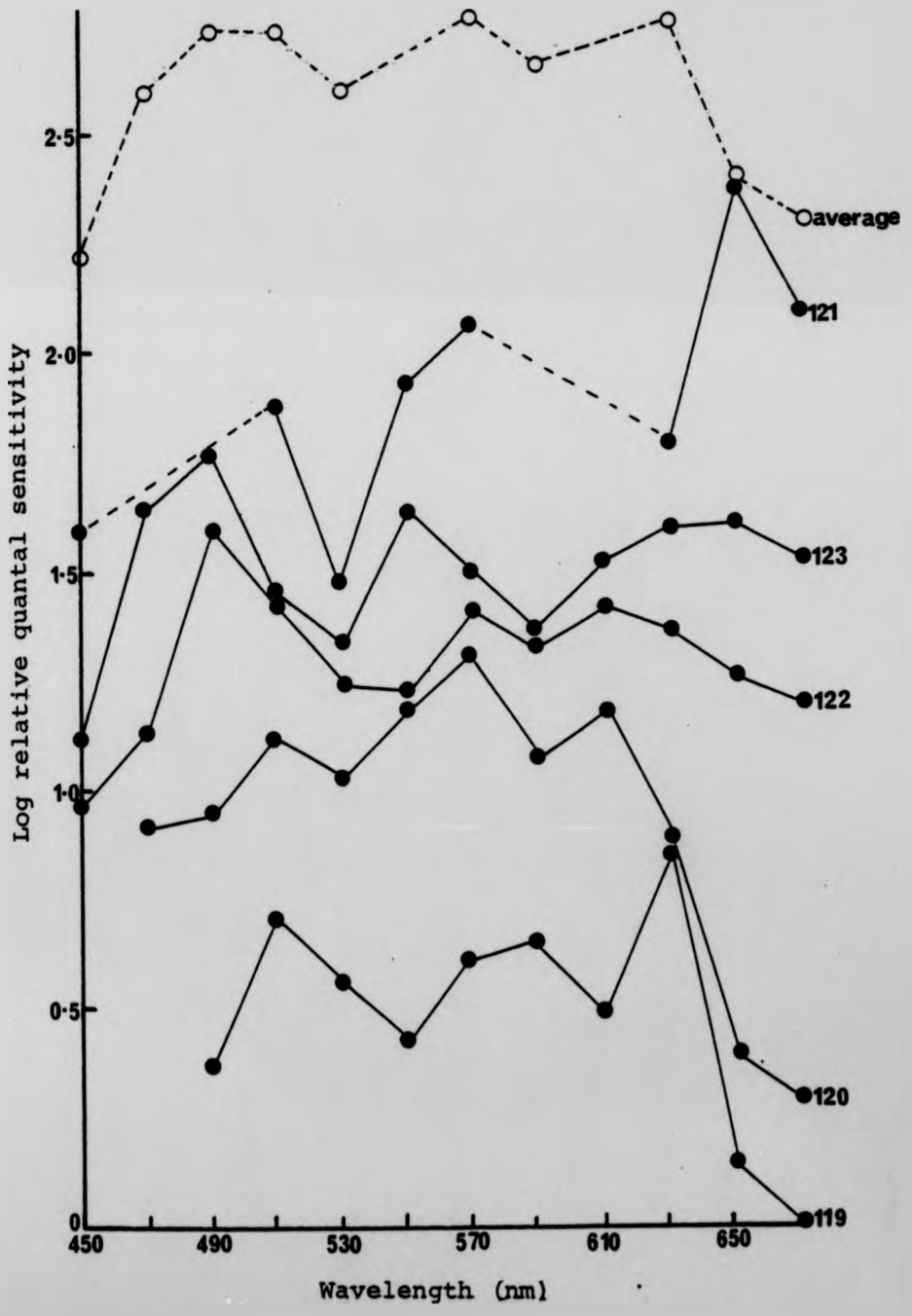
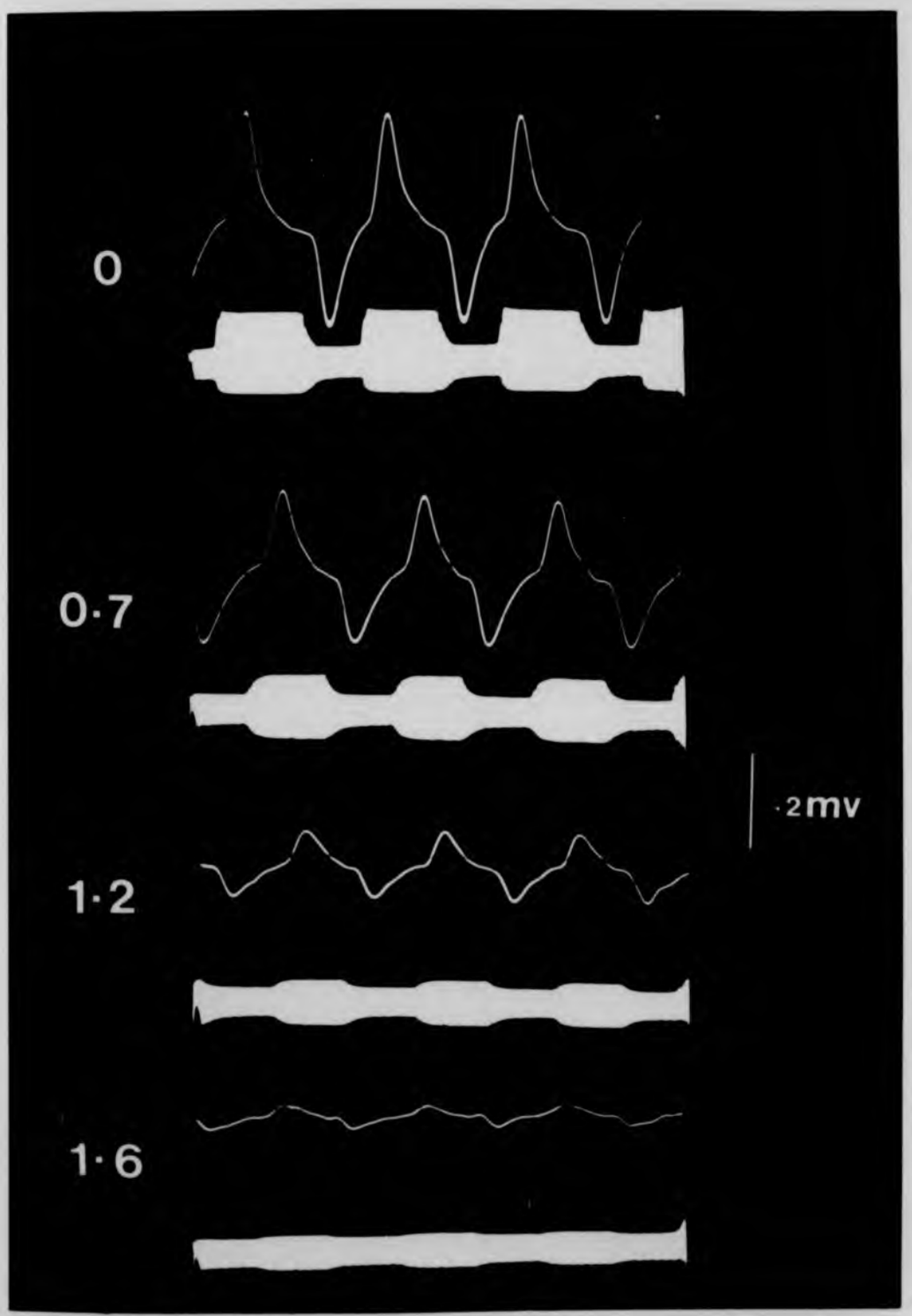


Fig 5.10. Photopic ERGs in response to different intensities (neutral density in stimulus) of a flickering (4Hz) 471 nm stimulus.

different

in stimulus)

stimulus.



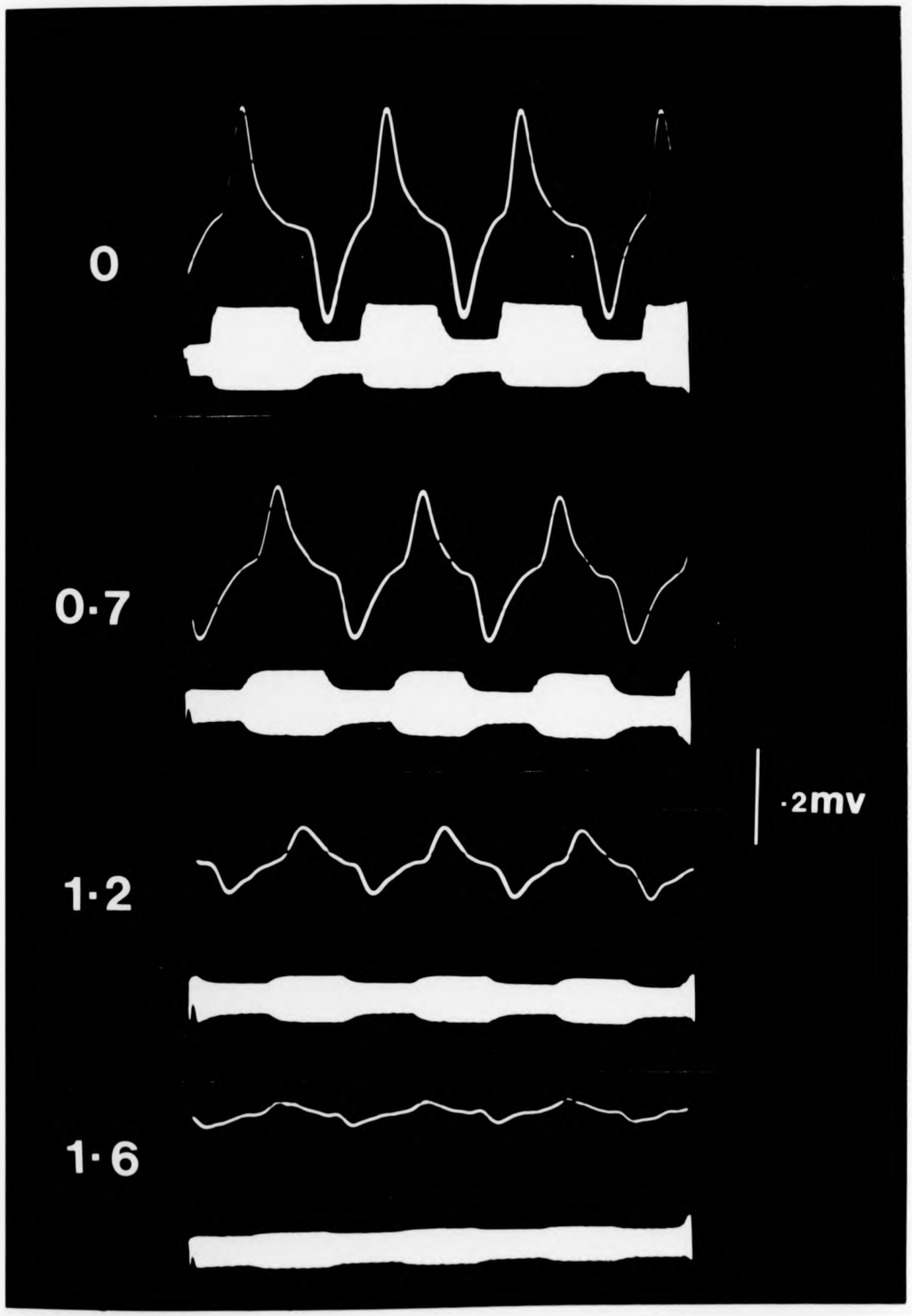
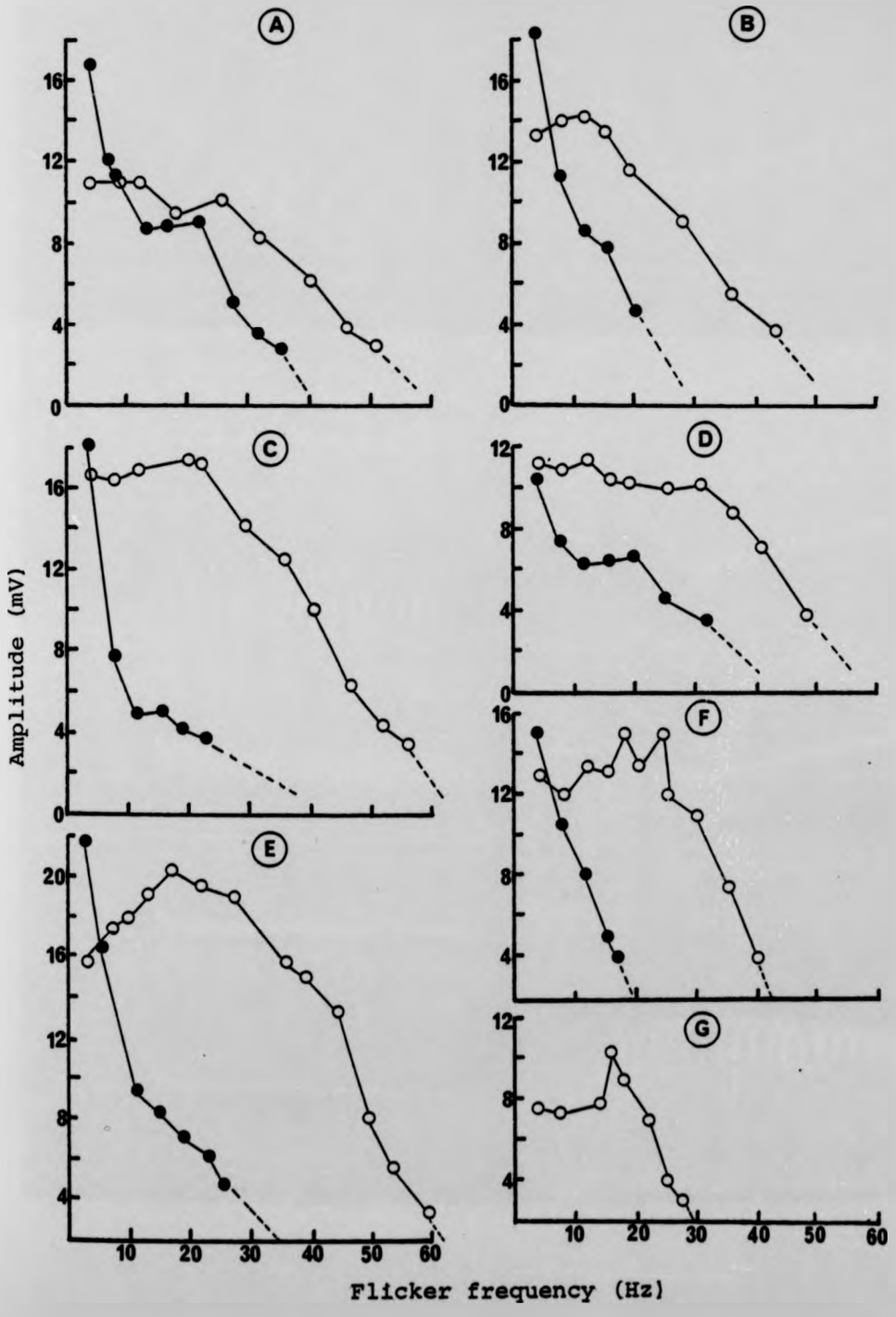

0
0·7
1·2
1·6
·2mv

Fig 5.11. Relationship between ERG amplitude and flicker frequency in light (○)+dark adapted (●) fish.



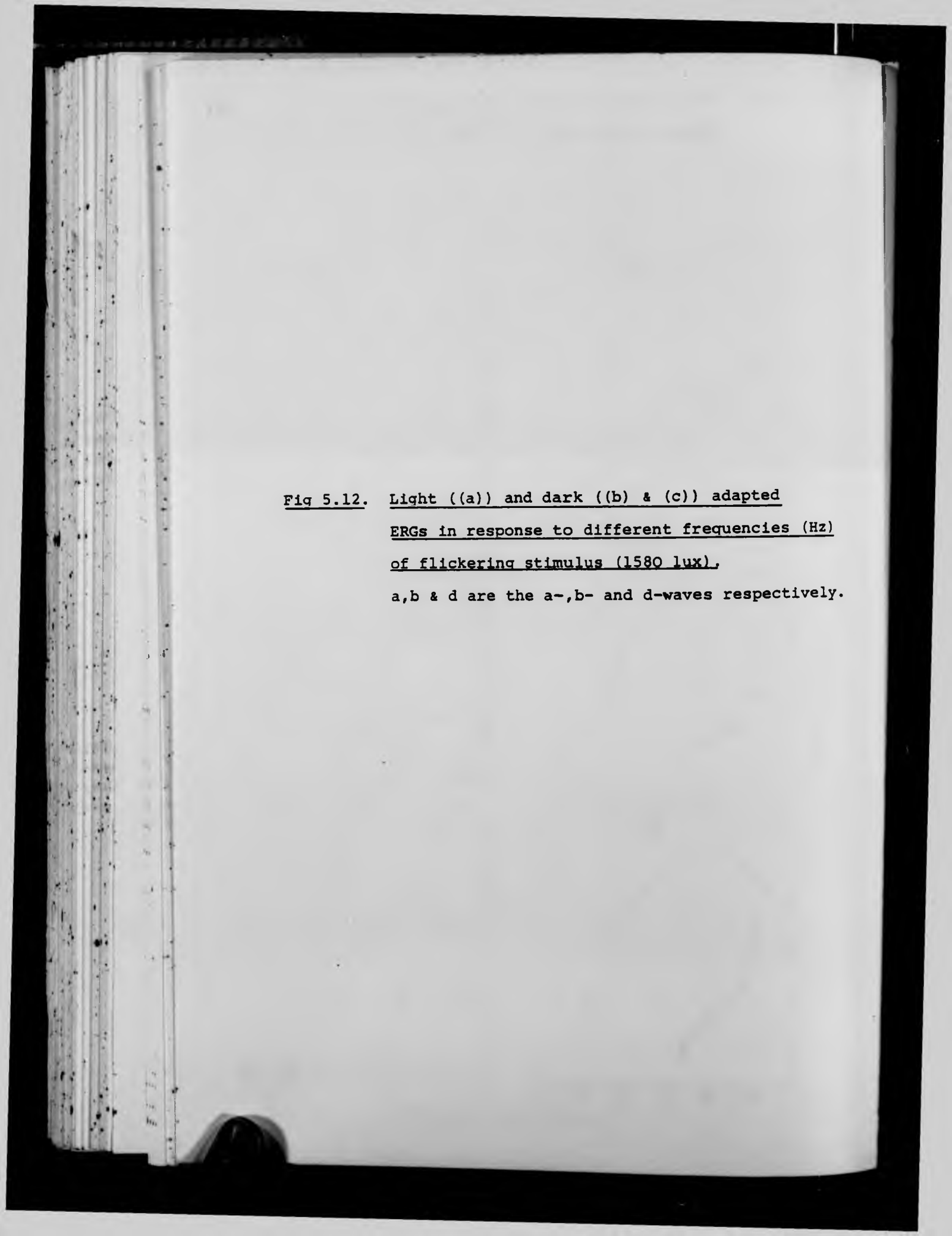
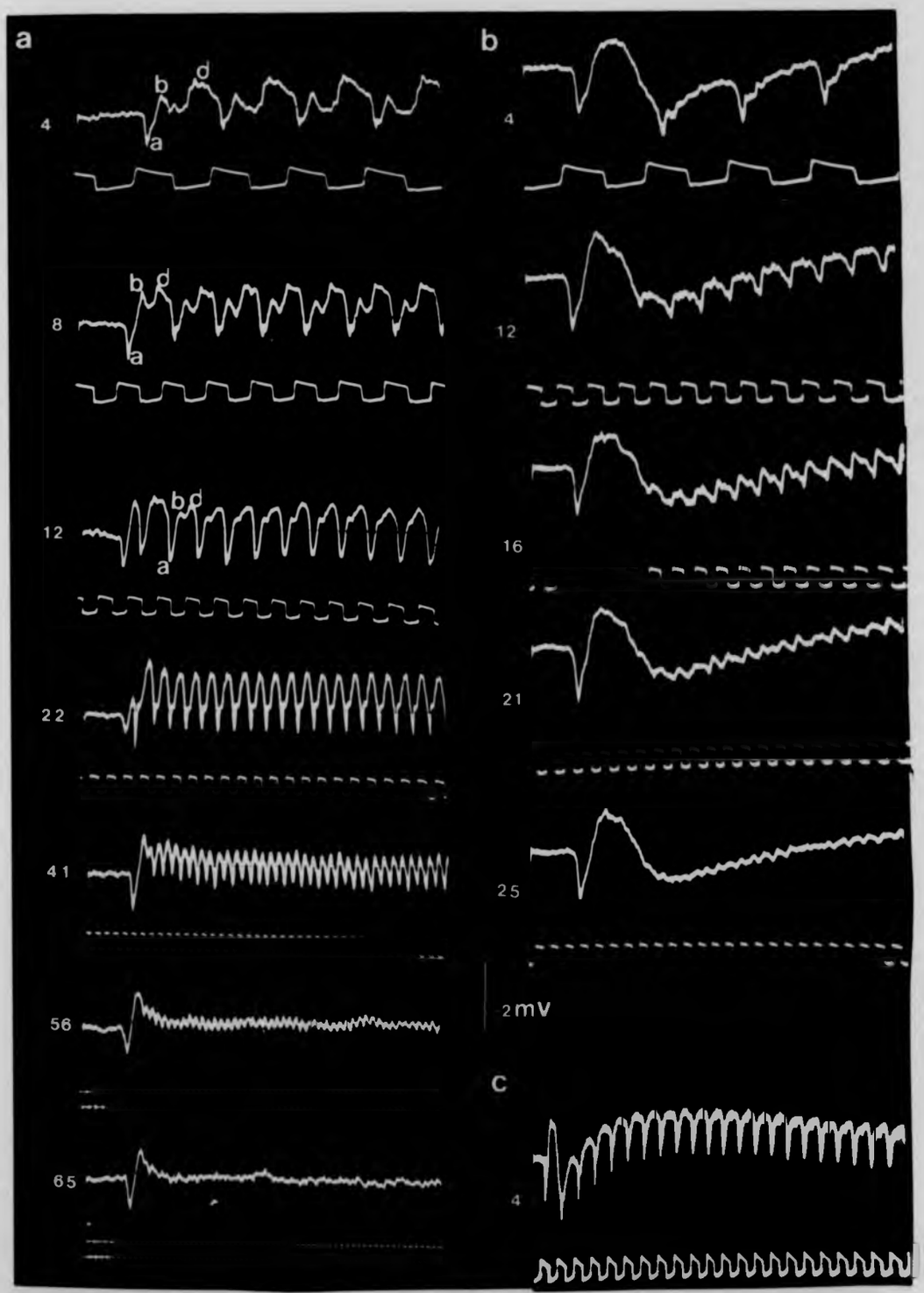


Fig 5.12. Light ((a)) and dark ((b) & (c)) adapted ERGs in response to different frequencies (Hz) of flickering stimulus (1580 lux).
a,b & d are the a-,b- and d-waves respectively.



c)) adapted

frequencies (Hz)

lux).

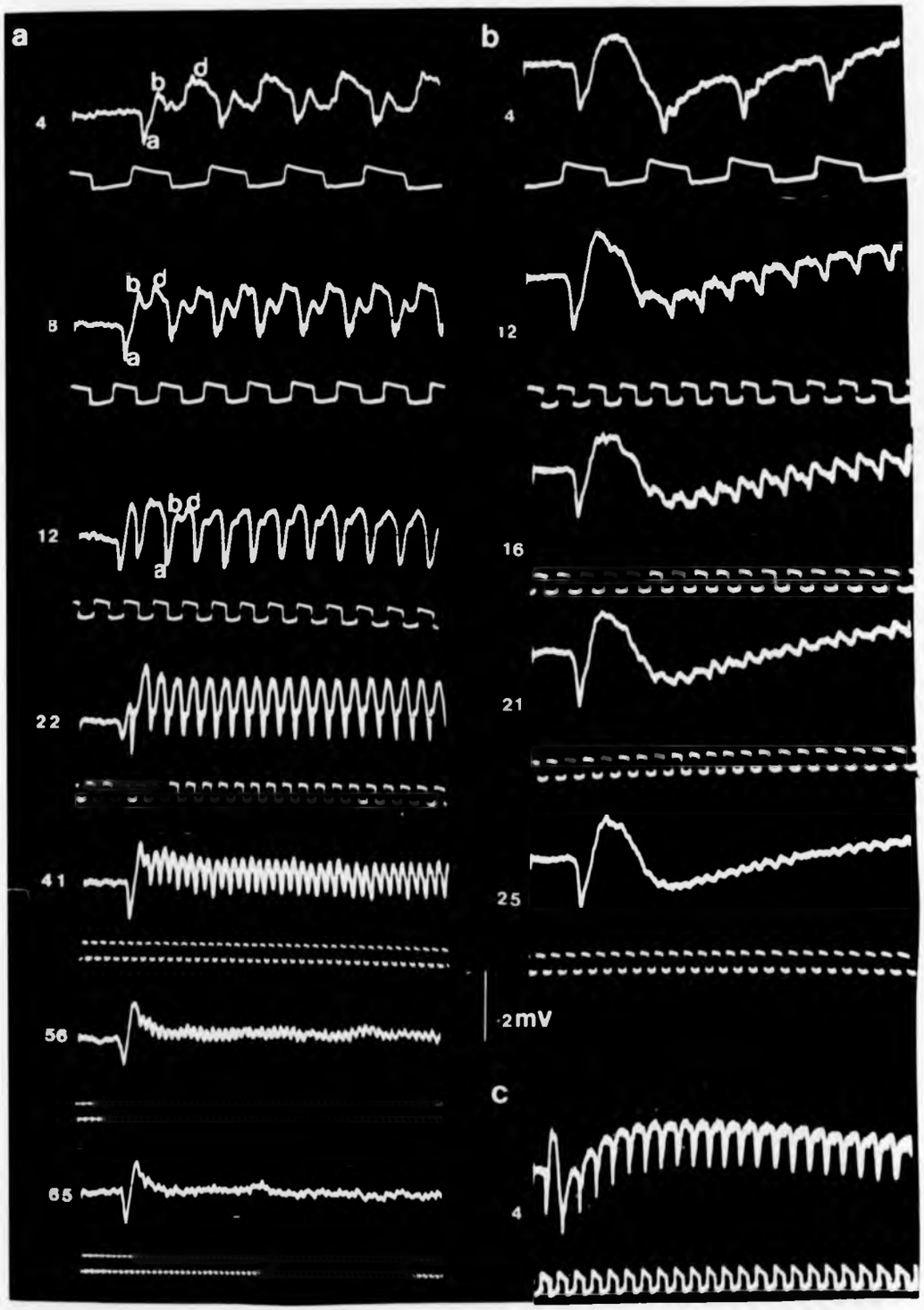
aves respectively.

c)) adapted

frequencies (Hz)

lux).

waves respectively.



12 - 18 Hz. Such a change, from a polyphasic response to a diphasic response, with a consequent amplitude peak at intermediate frequencies has been observed widely among vertebrates, including teleosts (eg: Tamura & Hanyu 1959) and Hanyu & Ali 1964). The dark adapted response, as shown in chapter four, is characterised by the lack of a d-wave. Consequently, the reponse is diphasic at all frequencies, resulting in a smooth amplitude/frequency relationship.

The flicker fusion frequencies for all light and dark adapted eyes are shown in table 5.I. On average they are higher by a factor of 1.8 in light, compared to dark, adapted eyes.

5.4 DISCUSSION

5.4.1 Scotopic spectral sensitivity. As the rainbow trout is a paired pigment species with a vitamin A_1 based extractable pigment whose λ_{max} is 503 nm and another extractable pigment based on vitamin A_2 with λ_{max} 527 nm (Munz & Beatty 1965), its scotopic spectral sensitivity maximum should lie between these two limits, with the exact position depending on the proportion of the two pigments in the retina. As fig 5.8 shows, this was the case, with the ERG action spectrum being well fitted by the absorption spectrum of the extracted visual pigment mixture (λ_{max} 514 nm).

Such an agreement is in some ways surprising as the absorption spectrum of a visual pigment as determined from the nomogram is not that of the pigment in situ (Dartnall 1953). The nomogram represents the absorbance of the pigment in a solution of low optical density ($D_\lambda < 0.05$ - Dartnall 1957), yet the optical density of rod visual pigments in the retinas of

TABLE 5.I Light and dark adapted critical flicker frequencies

Fish No	Dark adapted cff (herz)	Light adapted cff (herz)	dark adapted: light adapted
a	34	52	1 : 1.53
b	25	45	1 : 1.8
c	30	57	1 : 1.9
d	34	52	1 : 1.53
e	34	60	1 : 1.76
f	19	42	1 : 2.21
average	29.3	51.3	1 : 1.8

fishes is known to be fairly high. Maximum optical densities for many species of teleost from diverse localities have been ascertained (eg: Denton 1959, Denton et al 1971, Muntz 1973 & 1976b) and an average value of 0.6 for fresh water teleosts seems reasonable (Muntz 1975a). Such high optical densities would tend to broaden the visual pigment absorption considerably (Dartnall 1957). The solid line in fig 5.8 was obtained from nomograms and thus represents the fairly narrow absorption spectrum of a solution of visual pigment of low optical density. The absorption spectrum of a visual pigment with an optical density of 0.6 is shown as the dashed line on the same figure, and it can be seen to be too broad to fit the observed ERG action spectrum. This apparent anomaly can be explained by incomplete regeneration of visual pigment. The fish used in the electroretinographic determination of spectral sensitivity were dark adapted for only ninety minutes, which is not enough time for complete replacement of the visual pigment. Rushton (1959) noted that in frogs after ninety minutes dark adaptation the pigment density was only 0.2, and the spectral sensitivity measured from the ganglion cells was consequently narrower than after twenty-four hours dark adaptation, when the optical density was 0.7.

A similar correlation between the ERG scotopic action spectrum and the absorbance spectrum of the rod visual pigment has been noted in several other studies on teleosts (Crouzy & Ali 1966, Kobayashi & Ali 1971, Hanyu et al 1973 and Easter & Hamasaki 1973), although less good agreement has also been reported, usually with the long wavelength arm of the action spectrum being too broad to fit the visual pigment absorbance curve (eg: Burkhardt 1966, Witkovsky 1968, Thorpe 1973 and Cohen & Gruber 1977). A similar bad fit was observed by

Witkovsky et al (1973) when recording mass photoreceptor potentials from the isolated aspartate treated carp retina, and for single goldfish ganglion cell responses (Raynauld 1972). All these authors invoke residual cone contribution to the scotopic response to explain this broadening, probably present due to the high intensity of stimulus needed to elicit a measurable ERG response.

Easter & Hamasaki (1973) explain the good fit of the ERG data by the visual pigment curve in some, and the bad fit in other, species by differences in their cone:rod ratios. Wunder (1926) determined such ratios in twenty-four species of fish, including the carp, Cyprinus carpio, used by Burkhardt (1966) and the crucian carp, Carassius vulgaris, a species in the same genus as that used by Witkovsky (1968), both of whom found a mismatch between the visual pigment absorption spectrum and the ERG action spectrum. These fish had ratios of 12:38 and 7:32 respectively. Easter & Hamasaki (1973) state that these ratios are "probably" higher than those found in the three species of marine teleost they studied, whose pigment absorption spectrum and ERG spectral sensitivity agreed well, and that the b-wave, and consequently the action spectrum, therefore represent only rod activity. The higher proportion of cones in the species studied by Burkhardt (1966) and Witkovsky (1968) thus intruded into the scotopic action spectra, with a consequent mismatch between the spectral sensitivity and the visual pigment absorption spectrum. Unfortunately, Easter & Hamasaki (1973) made no actual count of the relative numbers of receptors in the species they used, which considerably weakens their argument.

The idea does, however, find some support from the present study. Although the rainbow trout cone:rod ratio has never been determined, Wunder (1926) did measure a ratio of 1:11 for the closely related brown trout. This was confirmed in the present

study for the rainbow trout, by counting the relative number of rod nuclei to cone parabaloids, using Abercrombie's (1946) correction, in sections of the eye used in chapter one. A ratio of 1:7 was obtained. The number of rod to cone nuclei was not compared directly as the cone muclei were much less visible due to their weak affininty for stains (Lyall 1957). Therefore, despite the fact that some cone nuclei were visible and easily distinguishable from those of rods, due to their size and position with respect to the e.l.m., it was by no means certain that this represented all the nuclei present in the section. However, since each cone contains one nucleus and one paraboloid, the count will not be affected by the procedure used here. Both the ratio found by this method and by Wunder (1926), reveal a very much higher number of rods relative to the cones than observed in either the carp or goldfish. This could explain the good fit between the action spectrum and the visual pigment apsorption spectrum.

That cones may contribute to the broadening of the action spectrum is also indicated indirectly by Kobayashi (1962), who, in his study of the spectral sensitivity of twenty species of teleost, observed the action spectra were broader in shallow water fishes, while they were narrower in deep water and nocturnal fishes. The greater number of cones in diurnal shallow water fish could presumably account for this broadening.

Yet high cone:rod ratios are not the only cause of such mismatches, as is indicated by the fact that Crouzy & Ali (1966) obtained a good match in goldfish, while Burkhardt (1966) failed to do so for the same species. Also Hanyu & Tamura (1978) obtained a bad fit in the ayu, Plecoglossus altivelis, but were able to rule out cone contribution, as the spectral sensitivity of fully dark adapted eyes and ones adapted to a one lux background were

identical. If cone intrusion was causing the mismatch, this would be relatively more significant with a white light background. Similarly, one can rule out cone contribution as a reason for the deviation of the ERG data points from the visual pigment absorbance in the deep sea fish Stenobrachius leucopsarus (O'Day & Fernandez 1976), as it has a pure rod retina. A possible reason for such a mismatch could be the broadening of the action spectrum due to the high optical densities of deep sea fish visual pigments. Finally, Bowmaker (1973) indicated that the absorption spectra of visual pigments in the intact retina are broader at longer wavelengths than those of visual pigments in solution as specified by the nomograms. Thus, the long wavelength mismatch between spectral sensitivity and visual pigment absorption spectra may be an artifact due to visual pigment extraction. This would not, however, explain why it occurs in some species and not in others.

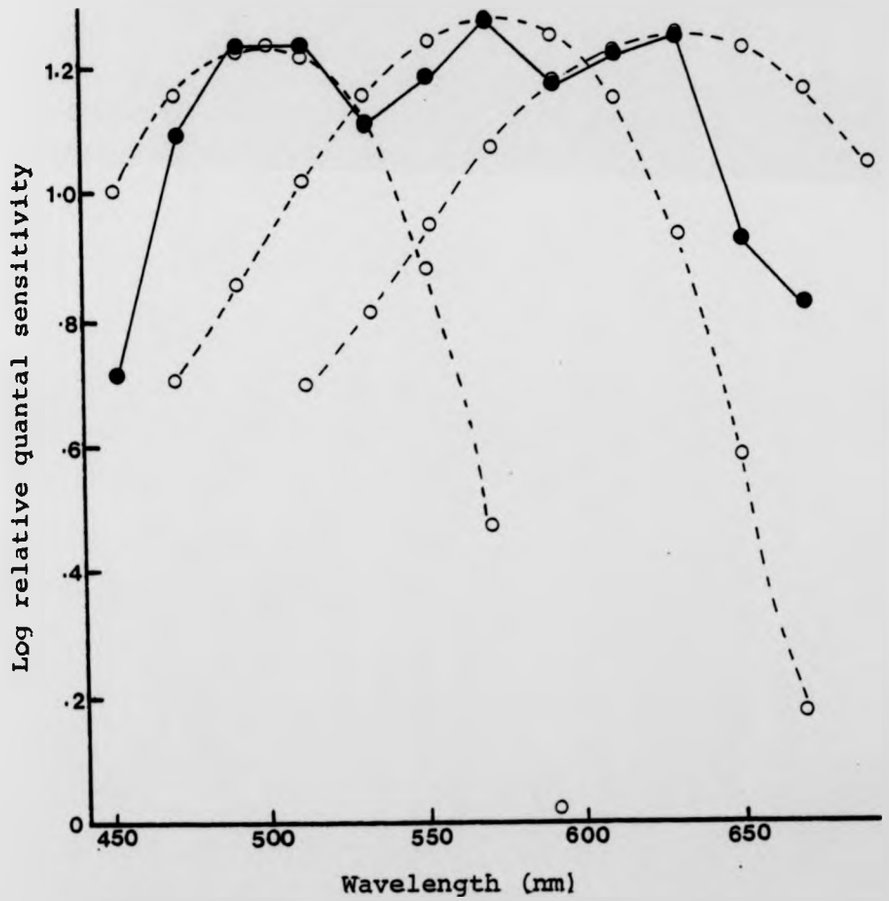
5.4.2 Photopic spectral sensitivity. Previous work has shown that the ERG photopic action spectrum in fishes can take one of two forms. It has been observed as either a smooth bell shaped curve, similar to the scotopic action spectrum, with its λ_{max} shifted towards longer wavelengths relative to the dark adapted function (eg: Burkhardt 1966, Cohen & Gruber 1977, Easter & Hamasaki 1973, Kobayashi 1962 and Witkovsky 1968), or, less frequently, it can be a more complex function with several distinct maxima (eg: Kobayashi & Ali 1971). Alternatively, the photopic and scotopic functions can be identical, indicating colour blindness (eg: Hanyu et al 1973). The rainbow trout shows a similar pattern to the pigmented brook trout (Kobayashi & Ali 1971), in that a smooth bell shaped scotopic action spectrum is transformed into a more complex function with three peaks on light adaptation.

Photopic vision has long been known to rely largely on the cones for receiving the visual stimulus. Consequently photopic action spectra are the result of some form of interaction between the outputs from the different cone populations. Thus, the scotopic action spectrum can usually be adequately explained in terms of visual pigments extracted from the rods, but due to the greater number of receptors and high degree of retinal integration involved, the same cannot be said for the photopic spectrum.

The absorption spectra of three visual pigments with λ_{max}s at 500 nm, 570 nm and 630 nm were constructed as described above (5.2.5), and plotted with the average ERG spectral sensitivity (fig 5.13). Since the A_1/A_2 ratio of the rods reflects the situation in the cones (Loew & Dartnall 1976), the absorption spectra are based on the A_1 pigment nomogram of Ebrey & Honig (1977), as the average A_1 content of the scotopic visual pigment extracts was 55%. Ideally a fitting procedure such as that available for scotopic pigments (503 - 527 nm), based on a mixture of both the A_1 and A_2 nomograms, should have been carried out. Yet even such manipulations would not have led to the complete agreement between the ERG action spectrum and the visual pigment absorption spectra. The hypothetical cone visual pigments do not explain the average photopic spectral sensitivity in any simple way. These complex interactions underlying photopic visual sensitivity will be discussed in greater detail in the following chapter.

5.4.3 Response to flicker. The critical flicker frequency (cff) varies enormously throughout the vertebrates, fusion occurring between three and one hundred and forty-three hertz (Ordy & Samorajski 1968) depending on the species, as well as a wide variety of other factors.

Fig 5.13. Average ERG photopic spectral sensitivity (●) and absorption spectrum of three hypothetical cone pigments (o).



The first parameter known to affect the cff is the state of adaptation, the response disappearing at a lower frequency when the animal is dark adapted (Schaternikow 1902). This observation has subsequently been confirmed in a variety of vertebrates (eg: Granit & Riddell 1934, Granit 1935, Dodt & Jessen 1961b and Ordy & Samorajski 1968) including teleosts (eg: Kobayashi 1962, Gramoni & Ali 1970b), and the present study shows that the same is true in rainbow trout. Such a decrease in cff on dark adaptation is presumably linked to the general slowing of the ERG response in the dark (see chapter 4). The ability to temporally resolve stimuli in the scotopic condition has been sacrificed in order to obtain maximum sensitivity.

Temperature and the intensity of the stimulus have also long been known to play a part in determining the precise cff. Thus, both an increase in temperature and intensity, within limits, serve to increase the frequency of fusion. This has been demonstrated in teleosts for temperature by Tamura & Hanyu (1959), Motakawa et al (1958), Hanyu & Ali (1963 & 1964) and Ali & Kobayashi (1967), table 5.II, and for intensity by Wolf & Zerrahn - Wolf (1936), Crozier & Wolf (1940), Tamura & Hanyu (1959), Kobayashi (1962), Hanyu & Ali (1963 & 1964), Protasov et al (1964), Ali & Kobayashi (1967) and Gramoni & Ali (1970b). Several other factors that may influence the cff such as stimulus size, retinal locus, wavelength and stimulus waveform, have been reviewed by Brown (1965).

All of these factors will cause variation in the frequency of fusion, which makes it difficult to compare the cffs of different species. This difficulty is further increased by variations in the cff due to different authors taking different subjective criteria to determine the point of fusion. A further

source of variation is that preparations differ widely in their noise levels. Thus, a preparation with a higher level of background noise will tend to give a lower level of fusion. Yet some real differences between species do exist. Such systematic variation is most easily uncovered if several species are investigated by the same author. The two most extensive studies on teleosts are those of Kobayashi (1962) and Ali and his co-workers (summarised by Gramoni & Ali 1970b). High cffs were found to be characteristic of diurnal or active fish, while low values were found in nocturnal or more sluggish animals. Although both authors come to the same conclusion, the range of values for the cffs are quite different. The total range in Kobayashi's fish was 15-32 Hz at 20°C, while at the same temperature Gramoni & Ali (1970b) recorded a cff of 38 Hz for Amia calva, the most sluggish fish, and 85 Hz for Salmo salar (Hanyu & Ali 1964), the most active (table 5.II). This serves to illustrate that cross study comparisons are difficult to make, as what in terms of its fusion frequency would be an active species in Kobayashi's study would be a sluggish one in the studies of Ali.

The rainbow trout, which in its life style is an active species, has, on average, a light adapted cff of 51 Hz at 14 - 17°C. At a corresponding temperature the salmon (Hanyu & Ali 1964) has a cff of 73.5 Hz, while in the more inactive goldfish the cff is 42 Hz. A comparable cff to that of the rainbow trout, at a similar temperature, is found in the pigmented brook trout, a species that is intermediate, in terms of cff, between the most active and most sluggish species. All these values, tabulated in table 5.II, refer to the maximum fusion frequency attained at a variety of intensities. The relationship between intensity of stimulation and cff was not, however, determined for the rainbow trout, and it is possible

TABLE 5.II Maximum cff observed in previous studies.

Authors	Species	5°C	10°C	15°C	20°C	25°C
Hanya & Ali (1964)	Salmo salar	47.7		73.5	(84.1)	95.8
Ali & Kobayashi (1967)	Lepomis gibbosus		51.1	(65.5)	79.9	
Ali & Kobayashi (1968)	Salvelinus fontinalis			(54.0)	68.0	
Hanyu & Ali (1963)	Carassius auratus	24.4		43.4	(55.3)	67.2
Gramoni & Ali (1970)	Amia calva				38.0	
Kobayashi (1962)	Trachurus japonicus				32.0	
Kobayashi (1962)	Carassius auratus				28.0	
Kobayashi (1962)	Sillago japonica				22.0	
Kobayashi (1962)	Lagocephalus lunaris				20.0	
Kobayashi (1962)	Halichoeres poecilopterus				18.0	
Kobayashi (1962)	Chrysophrys major				18.0	
Kobayashi (1962)	Stephanolepis cirrhifer				15.0	
Kobayashi (1962)	Fugu niphobles				16.0	
Kobayashi (1962)	Gymnothorax reticularis				15.0	

Values in brackets are determined by extrapolation based on the observation that the relationship between temperature and cff is linear (Hanyu & Ali 1963 & 1964). The value for S. fontinalis at 15°C is estimated from the relationship between L. gibbosus & S. fontinalis at 20°C.

that the maximum frequency of fusion may be higher than the cff determined here, moving the rainbow trout higher up the "activity scale".

A word of caution about the cff, as determined by the ERG, must be added. As a specific value it may have no biological significance. Firstly, the frequency of fusion observed is limited by the noise level of the preparation. Thus, the eye may still be responding to individual stimuli at higher frequencies, but the responses are masked by the baseline noise, although such low level responses can sometimes be retrieved using some form of averaging technique. Secondly, just because the retina responds to individual stimuli, this does not mean the information is necessarily directly used by the brain. In humans, for example, Riggs et al (1962) were able to record retinal responses up to 90 Hz but the subject reported all stimuli above 65 Hz as fused. Similarly, Schneider (1968) in rabbits, recorded the cff behaviourally and using the cortical potential. The cortical potential was found to respond to individual stimuli after the animal had behaviourally ceased to respond. Thus "the organism may possess information that is not used for cognitive processes," (Schneider 1968). The cff is thus best used as a comparative tool, as has been done by both Kobayashi (1962) and by Ali and his co-workers, taking due account of the diverse factors that may affect it.

CHAPTER 6 PHOTOPIC SPECTRAL SENSITIVITY DETERMINED BY A TWO CHOICE APPETITIVE TRAINING METHOD

6.1 INTRODUCTION

Walls (1942) stated that, "No fish is known not to have colour vision." Although this statement by its very nature is impossible to disprove, as this would mean testing a species in all conceivable experimental situations (see discussion), it is probably an oversimplification. Colour blindness, for several species, based on an absence of chromatic type S - potentials, has been suggested by Tamura & Niwa (1967) and Niwa & Tamura (1975). Similarly the absence of a Purkinje shift between scotopic and photopic ERG and ganglion cell action spectra in the skipjack tuna is indicative of a lack of colour vision (Hanyu et al 1973). The majority of deep sea fish probably also lack the ability to discriminate colours, due to the narrow spectral bandwidth of the available light.

In order to demonstrate colour vision unequivocally in an animal, one has to show that it is able to distinguish monochromatic stimuli on the basis of wavelength, independent of their relative intensities. This has been done using either (1) innate responses, such as unconditioned preferences for, or changed physiological responses to, particular wavelengths or (2) using learnt responses, such as training to choose between two or more monochromatic targets. Although much of the earlier work must be discarded, due to a failure to control brightness adequately, a definite ability to see colour has now been demonstrated in several teleosts, and on the whole it is generally accepted that the majority of fish do possess some form of colour vision (Warner 1931, Walls 1942, Herter 1953, Brett 1957, Viaud 1960 and Muntz 1974 for reviews).

Information pertinent to colour vision can be obtained

from three sources; electrophysiological recording from different levels of the visual system, microspectrophotometric analysis of the visual pigments, and psychophysical experiments. In order to gain a complete understanding of an animal's chromatic visual system, information from all these sources must be considered. In this way one can, theoretically, obtain an understanding of how the incoming information is processed at all retinal layers in order to produce the final response. The only species for which such comprehensive data are available is the goldfish.

In this species three individual cone pigments have been characterised microspectrophotometrically by Liebman & Entine (1964), Marks (1965), Svaetichin et al (1965), Harosi & MacNichol (1974) and Harosi (1976), and all the cell types in the retina as well as optic fibres and tectal units have been recorded from (Beauchamp & Lovasik 1973 and Yager & Thorpe 1970 for review). That goldfish can discriminate colours on the basis of hue and not brightness has been shown by McCleary & Bernstein (1959) and Muntz & Cronly-Dillon (1966). Action spectra have also been determined using the optomotor reaction (Cronly-Dillon & Muntz 1965), two choice appetitive training (Yager 1967 & 1968), classical conditioning (Schefner & Levine 1976, Beauchamp & Rowe 1977 and Powers 1976 & 1978), the dorsal light reaction (Powers 1976 & 1978) and electrophysiologically (Regan et al 1975 and Burkhardt 1966 & 1968). Other studies on the goldfish visual system include the effect of temperature (Thorpe 1971 & 1973 and Schellart et al 1974) and chromatic adaptation (Yager 1969 and Burkhardt 1968), spatial summation (Northmore 1977) and absolute visual sensitivity (Powers & Easter 1978a). This review is in no way comprehensive but serves to show the great volume of work needed on a single species in order to obtain a complete description of its visual system.

Apart from the electroretinographically determined spectral sensitivity curve described in the previous chapter, no data pertinent to colour vision are available for the rainbow trout. Therefore, as a comparison to the electrophysiological action spectrum, and as a further step toward understanding the trout chromatic system, its photopic spectral sensitivity was investigated behaviourally using two choice appetitive training.

There are two basic types of behaviour that can be used in determining an animals spectral sensitivity; those that are innate (reflex) and those which have to be learnt (training). As there are several reviews of these techniques, as they can be applied to vision, available (Blough & Yager 1972, Muntz 1974 and Northmore & Yager 1975) only a brief summary will be given below.

One of the most common innate behaviours used is the optomotor reaction (Grundfest 1932a, Cronly-Dillon & Muntz 1965 and Cronly-Dillon & Sharma 1968), which makes use of the fishes tendency to follow moving stripes, usually projected onto the side of a circular aquarium. Other innate behaviours that have been used are, the dorsal light reaction (Thibault 1949, Lang 1967, Silver 1974 and Powers 1976 & 1978), phototaxis (Kawamoto & Konishi 1952 and Blaxter 1964, 1968b & 1969), feeding (Blaxter 1964, 1968b & 1969), barrier avoidance (Blaxter 1964) and the light shock reaction (Northmore 1973).

Training techniques can be further subdivided into two categories; (1) Classical conditioning (also called Pavlovian or instrumental conditioning) involves relating a conditioned stimulus, in this case a visual cue, to an unconditioned stimulus, usually electric shock, which elicits an easily measurable response. Thus, for example, the administration

of electric shock leads to a decrease in respiration and heart rate or an increase in swimming activity, all of which are easily monitored. In this way, after training, the conditioned stimulus will also elicit the measurable response. Such techniques have been used by Bull (1957), Northmore (1973), Northmore & Muntz (1974), Powers (1976), Schefner & Levine (1976) and Beauchamp & Rowe (1977).

(2) In operant conditioning, on the other hand, a learnt response is maintained either by positive reinforcement or by avoidance of aversive stimuli. Of the many different forms this type of training can take (Blough & Yager 1972), the most commonly used in the determination of teleost spectral sensitivity is the two choice appetitive situation linked to some form of tracking procedure (Muntz & Northmore 1970, 1971 & 1973, Yager 1967, 1968 & 1969, Yager & Thorpe 1970, Thorpe 1971 & 73, Wainwright 1973 and Cameron 1974). The experiments described in this chapter are another example of such a procedure. Avoidance conditioning has been employed less frequently, but Tavolga & Jacobs (1971), for example, used a shuttlebox to determine the spectral sensitivity of Tilapia.

Although the above summary has emphasized the use of these techniques for determining spectral sensitivity, it should be remembered that they can also be used in the study of other visual functions such as: the ability to discriminate hue (Muntz & Cronly-Dillon 1966, two choice appetitive), absolute visual sensitivity (Powers & Easter 1978a & b, classical conditioning of heart rate and respiration), colour mixing (Oyama & Jitsumori 1973, shuttlebox) and spatial summation (Northmore 1977, classical conditioning of respiration).

The spectral sensitivity of the rainbow trout was investigated using two choice appetitive training for two

reasons. Firstly, Adron et al (1973) have shown that the rainbow trout can be conditioned to activate a lever in order to obtain food, and that they can learn to make a choice between two levers when only one of them gives a reward. Rainbow trout were thus likely to be easily trained. Secondly, four teleost species have been investigated by this method before. As the form of the action spectrum depends largely on the experimental situation (see discussion), the more species examined by one method, the greater the scope for interspecific comparison.

The only previous demonstration that rainbow trout may have colour vision again came from Adron et al (1973), who showed that the choice between two food levers, with only one delivering food, was greatly facilitated if the levers were of different colours. However, as the authors themselves pointed out, this differentiation could have been made using brightness cues. While the following determination of photopic spectral sensitivity cannot positively prove colour vision in the trout, which would require some form of hue discrimination experiment, a demonstration of more than one receptor mechanism would be strongly indicative of it.

6.2 MATERIALS AND METHODS

These experiments were performed on six fish over a period of two years (fish 1&2 - 12/77 to 3/78, fish 3 & 4 - 12/78 to 3/79, fish 5 & 6 - 11/79 to 2/80). During these periods fish were individually kept in aquaria (46 x 25 x 25 cm) surrounded on three sides by black plastic, and kept on a twelve hour daylength. Illumination was provided by a 100W bulb, 32 cm above each tank. A continual flow of water was maintained

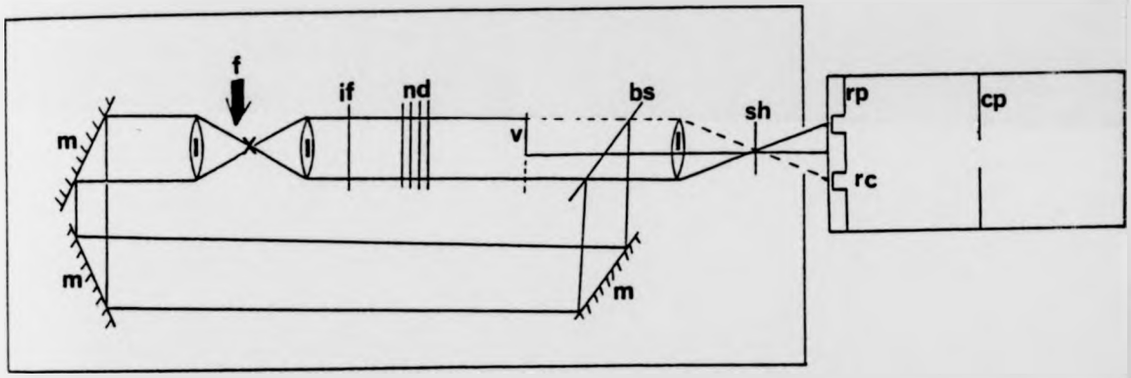
through the aquaria and additional oxygenation provided by an air stone in each. No record was made of temperature.

6.2.1 Apparatus. The basic apparatus has previously been described by Muntz & Northmore (1970 & 1973). It comprises; two panels, which were placed into the fish's home tank at the beginning of each experimental period, the optical system on a trolley, which could be positioned in front of each tank as required, and the logic circuitry and data recorder in an adjacent room.

The response panel, situated at the front of the tank, was made of black Perspex containing two round holes four centimeters in diameter, their centres 7.5 centimeters apart (fig 2 ,Muntz & Northmore 1970). These holes formed the openings of response chambers, six centimeters deep, the back of which was made of translucent Perspex onto which a stimulus light could be projected. Entry into a chamber was registered by the breaking of a photocell beam from a 22V, 0.5A pre-focused bulb. Tubifex worms could be delivered into the response chamber by an automatic dispenser as described by Northmore (1968). In order to initiate a trial the fish first had to interrupt photocell beams in the 5 x 6 entrance of the second black Perspex panel situated in the centre of the tank, nineteen centimeters from the response panel. During an experiment the tank was covered with a lid containing two 2.8W, 24V tungsten bulbs, giving approximately even illumination, thus keeping the fish light adapted.

The light source, split into the stimulus and background beam (fig 6.1), was a 24V, 150W tungsten iodide projector bulb with a colour temperature, determined as in chapter four, of 3540^{o}K, situated in a cooled mat black container. Stimulus intensity could be varied over a three log unit range in steps

Fig 6.1. Apparatus used to determine spectral sensitivity by two choice appetitive training.



m - mirror, l - lens, s - light source, if - interference filter, nd - neutral density filters, v - vane to determine side of stimulus, bs - beam splitter, sh - shutter (position of UDT optometer for calibration), rp - response panel, rc - response chamber, cp - centre panel.

of 0.2 log units, by the insertion into the light beam of any combination of four solenoid operated neutral density filters (0.2, 0.4, 0.8 & 1.6). The wavelength of the stimulus could be altered by the insertion of Balzer interference filters. During a trial the background (0.96 cd/m^2) was focused onto both response chambers, while a vane cut off the unwanted portion of the stimulus beam so that only one chamber was illuminated. At all other times both the stimulus and background were prevented from reaching the response panel by an electromagnetic shutter. The complete optical system was enclosed in a mat black casing, to eliminate stray light.

6.2.2 Training. The final aim of training was for the fish to choose the response chamber with the monochromatic stimulus, having previously set up the trial by swimming through the centre panel. The sequence of training can be split into five distinct phases.

(1) Initially only the response panel was put in the tank, and Tubifex worms dropped by hand in front of the panel. In this way the fish learnt to associate the panel with food.

(2) When the fish swam to the response panel as soon as it was put into the tank, after two to five days training, worms were delivered into the response chamber using the automatic feeder. After three to six days the fish would swim into the chamber to collect the reward. At this stage there was no stimulus light on the response chamber and the fish was fed for going into either one.

(3) During phases (1) and (2) when the fish was not being trained, the centre panel was left in the tank for several hours, until the fish eventually swam through it with no hesitation.

(4) The fish was finally put into the complete experimental set up with both panels and the lid. At this point the fish

freely entered the response chambers and swam through the centre panel, but had not yet learned to respond only to the response chamber with the stimulus, or to associate the centre panel with setting up a trial.

When the fish broke the photocell beam in the centre panel, presumably by random swimming, a white light stimulus was projected onto the back of one of the response chambers, the order of left/right presentation being determined by a modified Gellerman (1933) sequence. Following a correct response the fish was automatically fed one or two Tubifex worms, the stimulus and background were extinguished, and a magazine light, at the top of the response panel, came on for 2.5 seconds, after which a new trial could be initiated. After an incorrect choice, the stimulus and background light went off immediately, the fish were not rewarded, and another trial could be initiated straight away.

Initially the fish were very slow at this procedure, doing only two to four trials in two hours, responding randomly to either side. After daily training for about ten days the fish responded at a rate of up to eighty trials in less than one hour, choosing the illuminated response chamber with nearly 100% accuracy.

(5) Once this level of performance had been reached with a white light stimulus, monochromatic stimuli were substituted for the white light and phase (4) was repeated. Fish learned within a single experimental period to respond to the monochromatic light. At this point the tracking procedure (see below) was initiated.

Very early on in this study it was found that rainbow trout are very prone to exhibiting side preferences, the fish tending to go to the response chamber on one side only. Different

fish went to different sides, showing this was not due to any irregularity in the apparatus. Wainwright (1973) made a similar observation in the blenny. This behaviour allowed the fish to gain a reward on 50% of the trials, without having to make a discrimination. Such side preferences were broken by programming the machine not to terminate a trial until the fish has gone to the side of the stimulus. Initially the fish failed to respond if the stimulus was on the unpreferred side, but within one experimental period these side preferences were abolished.

6.2.3 Testing. An experimental period consisted of seventy to one hundred and fifty trials, depending on the speed of responding, with the first ten trials at the maximum intensity of stimulation. If fish responded correctly at least nine times, the tracking procedure was started.

This procedure is the same as that used by Muntz & Northmore (1970, 1971 a & 1974) for the rudd, and by Wainwright (1973) and Cameron (1974) for the blenny and perch respectively. Following two consecutive correct responses, the intensity of the stimulus was reduced by 0.2 log units, whereas after a single incorrect response, the intensity was increased by the same amount. In this way, the intensity of the stimulus was reduced to a point where the fish was unable to distinguish the stimulus reliably from the background. At this threshold level the fish started to make errors in its choice, resulting in an increase in stimulus intensity until the fish once more responded correctly. An example of such a tracking record is shown in fig 6.2a.

If P is the probability of a correct response, it follows that 1 - P is the probability of an incorrect response. P^2 is thus the probability of two consecutive correct responses. At threshold, the probability of the stimulus intensity increasing equals that of it decreasing. Thus the probability of two correct responses equals the probability of an incorrect response plus

the probability of a correct followed by an incorrect response, as both the latter cause an increase in intensity.

$$P^2 = 1 - P + (1 - P)$$
$$P^2 = 1 - P^2$$
$$2P^2 = 1$$
$$P = 0.707$$

Thus this form of tracking causes the intensity to oscillate around a threshold at which the fish responds correctly on 70.7% of occasions.

Every fish was tested at most of the fifteen wavelengths (391 - 669 nm) at least once, and on several occasions the threshold was determined more than once at the same wavelength at different times during the experiment, to check that the sensitivity did not change. The order of presentation of wavelengths was random and different for each fish, thus controlling for any improvement in tracking ability with time. The threshold of wavelengths presented at the beginning of the experiment and re-presented at the end showed little variation (see below). Only one wavelength per fish was tested every day. All responses were recorded on a paper tape recorder in an adjacent room.

The threshold of all six fish in response to a white light stimulus was also determined on several occasions in order to determine the Weber fractions. Fish 1 and 2 were both tested with backgrounds of 0.242 and 5.951 cd/m^2, while fish 3 and 4 and fish 5 and 6 were tested at just one background each, 3.461 and 0.964 cd/m^2, respectively.

In order to check that this method of tracking, calibration and analysis of results gives reliable results, the action spectrum of a human observer was also determined using the same apparatus, and compared to published results. The author

sat behind a tank, which had the black covering removed from the back, thus affording a clear view of both response chambers. The eyes, like the fish's, were free to move but were kept roughly on the same level as the stimulus. All lights in the room were off and the only diffuse illumination came from the lid of the tank, giving lighting conditions for the human observer similar to those experienced by the fish. Photocell beams in the response chambers were interrupted by a rod through a hole in the lid. The order of stimulus presentation is given with the results in fig 6.3.

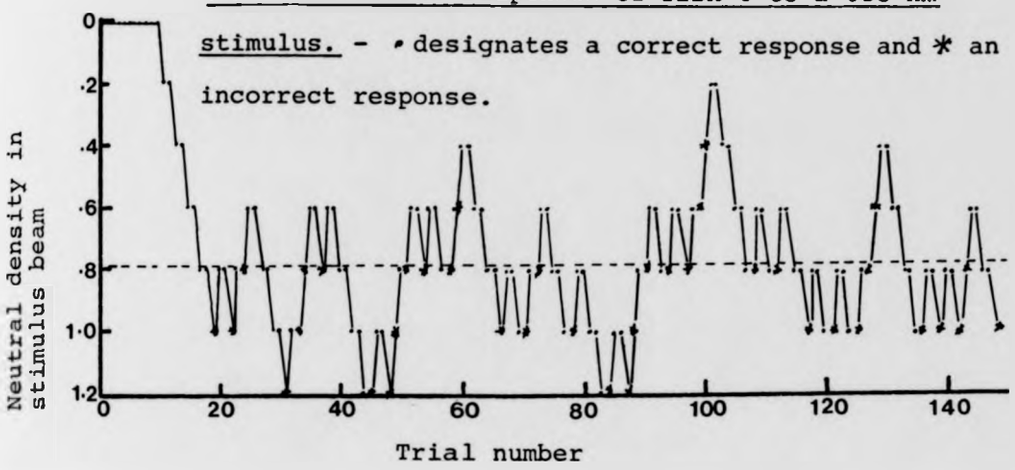
6.2.4 Analysis of results. Thresholds obtained by the above tracking procedure were determined using probit analysis as described by Finney (1962). Details of such analysis applied to tracking data derived from turtles can be found in Muntz & Sokol (1967) and Muntz & Northmore (1968). The same procedure has also been applied to teleosts by Muntz & Northmore (1970 & 1973), Wainwright (1973) and Cameron (1974). Briefly the analysis proceeds as follows. The percentage of correct responses at each intensity is calculated (fig 6.2b) and expressed as a preliminary probit value (Pc). This value, which represents the probability of a correct response, must then be corrected using Abbott's formula, as the fish will have a 50% chance success rate (P') even when the stimulus is below threshold. The corrected probit (Pd) is given by:

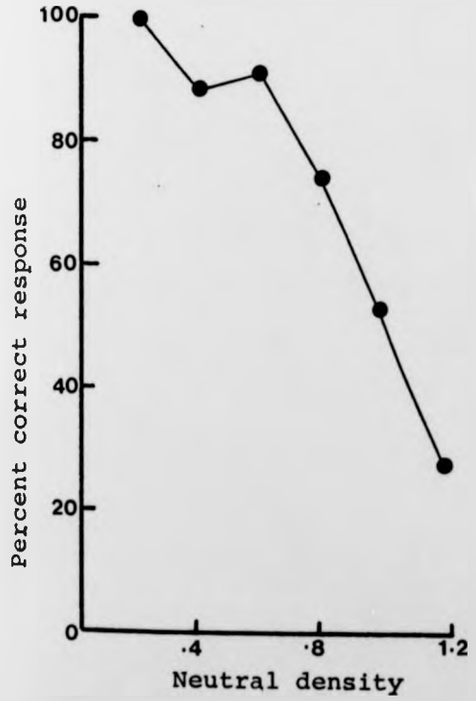
$$Pd = \frac{Pc - P'}{1 - P'} = \frac{Pc - 0.5}{0.5}$$

This corrected probit, which represents the probability of detecting the stimulus, can then be plotted against intensity, and an estimated straight line fitted to give a first approximation of the psychometric function. Probit analysis then iteratively fits a better line by regression until the best fitting equation

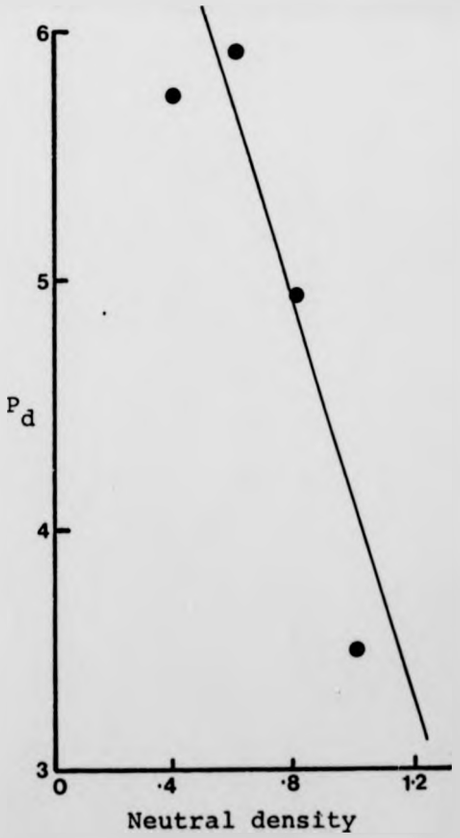
Fig 6.2.

(a) Behavioural responses of fish 6 to a 650 nm stimulus. - • designates a correct response and * an incorrect response.





(b) Psychometric function of the same data.



(c) Fitted regression line to the same data

(regression formula y=8.094-4.024x)

for the experimental data is derived. From this the threshold (Pd = 0.5) can be determined. Fig 6.2c shows this function for the tracking data shown in fig 6.2a. All calculations were performed using a computer program originally written by D.P.M. Northmore.

6.2.5 Calibration. (1) Monochromatic stimuli. Thresholds to monochromatic stimuli can be converted to relative retinal quantal sensitivity exactly as outlined in chapter five. The UDT optometer was placed at the focal point shown in fig 6.1. No further correction was necessary for the aquarium glass, water or Perspex as these are effectively chromatically neutral for the wavelengths used (Muntz & Northmore 1970).

(2) White Light. The intensities of the background and stimulus incident on the response chamber were measured using an SEI spot photometer. In this way, thresholds in terms of neutral density could be converted into candles/m^2. As the background and the stimulus light have the same spectral composition, the Weber fractions ($\Delta I/I$) could be determined directly, by dividing the white light threshold (ΔI) by the background intensity (I) without further corrections.

6.2.6 Pigment extraction. The visual pigment was extracted using digitonin, as described in chapter three, and subjected to analysis as outlined in chapter five. In this way the λ_{max} and percentage vitamin A_1 based pigment was determined for five fish, whose behavioural spectral sensitivity had previously been determined. The other fish died prematurely and could not be used for pigment extraction as it was only discovered several hours after death.

6.3 RESULTS

6.3.1 Human spectral sensitivity. The action spectrum of the human observer, shown in fig 6.3, has been corrected for wavelength dependent absorption by the lens (Wyszecki & Stiles 1967). As no attempt was made by the observer to fixate the target on the fovea and the eye was free to move, it is likely that the discrimination was at least partially done in the more sensitive peripheral retina, although the target only subtended an angle of 4.96^{o} on the eye. Thus no correction was made for the foveal macular pigment. The resulting action spectrum shows three distinct maxima at 452,534 & 626 nm, with the short wavelength maxima being the most, and the long wavelength peak the least, sensitive. The absorption spectra of human cone pigments (Bowmaker & Dartnall 1980) are superimposed on the action spectrum for comparison (see discussion).

6.3.2 Rainbow trout spectral sensitivity. Fig 6.4 shows the action spectra of six fish arbitrarily shifted along the ordinate to facilitate separation of individual curves. On several occasions more than one threshold was obtained at one wavelength for an individual fish. These thresholds and their ranges are given in table 6.I. The average range of thresholds in an individual for one wavelength is only 0.287 log units. The points on fig 6.4 represent the averages of these thresholds. The average spectral sensitivity for all six fish, obtained as in chapter five, (fig 6.5) shows three distinct areas of high sensitivity at 391 - 473, 490 - 573 and 650 - 669 nm.

While there is some variation between the individual action spectra, they are of the same general form, all showing the minimum sensitivty at 626 nm with the maximum at the short wavelengths. All fish also show a secondary maximum at intermediate wavelengths between 530 - 570 nm. Unfortunately,

TABLE 6.I. Thresholds and ranges for wavelengths at which more than one threshold was obtained.

Fish	Thresholds (Neutral density)					Range
1	391 .352	.642	.542			.29
	410 1.0	.978	.755			.245
	433 1.217	1.364	1.17			.194
	452 1.249	1.124				.125
	471 .848	1.552	1.39	1.08	1.456	.704
	491 .858	1.106	1.189	1.481	1.123	.623
	513 1.055	1.190	1.186			.135
	534 1.102	1.852	1.281	1.334	1.20	.75
	549 1.312	1.430	1.188			.242
	573 1.254	1.352				.098
2	391 .362	.215	.69			.475
	410 .593	.443	.198	.715		.517
	433 1.168	1.135	1.266	.986	.72	.546
	452 1.071	1.19	1.196			.125
	471 1.347	1.025	1.04			.322
	491 .886	.780	.833			.106
	513 1.301	1.178	1.011	1.154	1.219	.290
	534 1.097	.93				.167
	549 1.243	.747	1.339	1.107		.592
	573 1.300	1.233				.067
	609 .746	.626	.674			.120
	626 .819	.653	.424			.395
3	493 .589	.597				.008
	549 1.405	1.46				.055
	609 .643	.72				.077
	626 .775	.305				.47
	650 .678	1.00				.322
4	549 1.231	1.174				.057
	626 .177	.405				.228

average: 0.287

Fig 6.3. Human spectral sensitivity in a two choice situation corrected for lens absorption (●).
(○) absorption spectra of three human cone pigments (Bowmaker & Dartnall 1980).
The order of stimulus presentation is given below each wavelength.

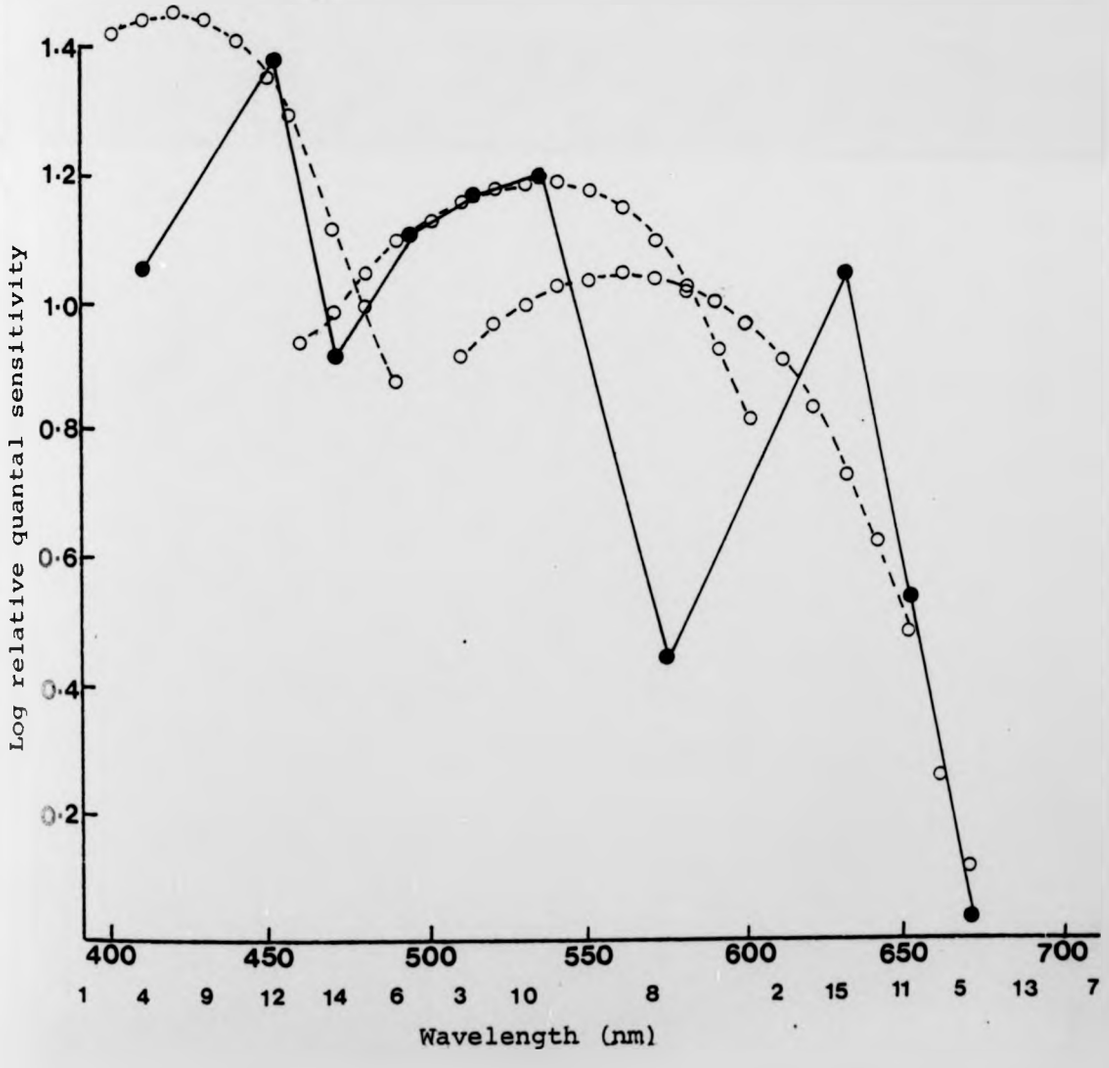


Fig 6.4. Rainbow trout photopic spectral sensitivity determined using two choice appetitive training for six individuals.

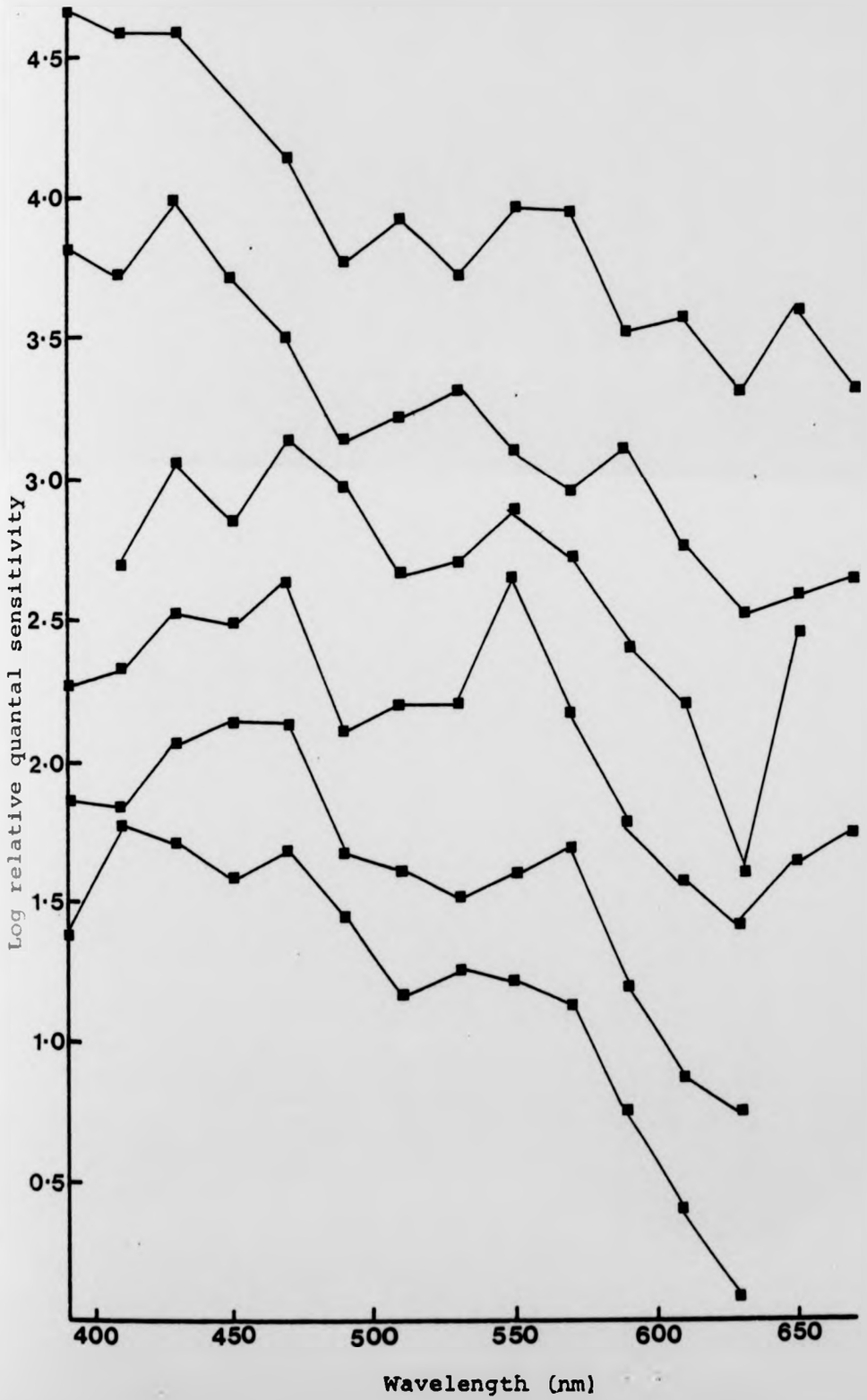
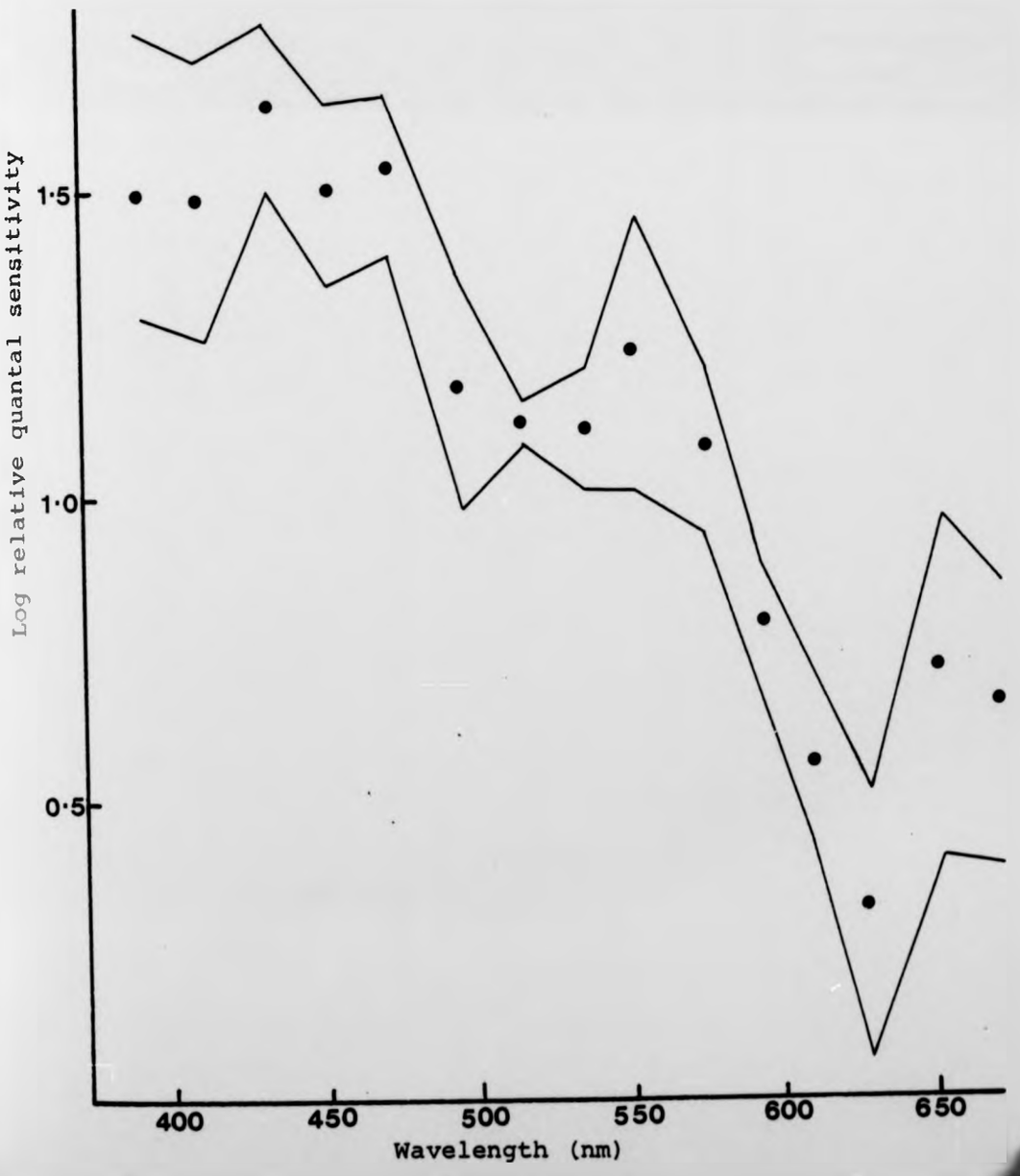


Fig 6.5. Average photopic action spectrum determined by two choice behavioural training - showing 95% confidence limits.



the threshold at 650 nm and 669 nm was only determined for four fish. These wavelengths were not tested in the first two fish (1 & 2), as it was wrongly assumed they would not respond beyond 626 nm due to the low sensitivity at this wavelength. This decreased number of data points makes the exact position and form of the long wavelength maximum somewhat uncertain. Wavelengths beyond 669 nm could not be tested as, at these wavelengths, the interferences filters diminished the intensity of the light to such an extent that the fish were no longer able to respond.

Table 6.II shows the λ_{max} and percent vitamin A_1 based visual pigment in five of the six fish. As on average 72% of the pigment was A_1 based, all hypothetical cone pigments in the following discussion are determined using the Ebrey & Honig (1977) nomogram for A_1 pigments.

Table 6.II Visual pigments extracted from five fish used in the psychophysical determination of spectral sensitivity.

FISH	1	2	3	4	5	AVERAGE
λ_{max}	510.8	510.4	508.5	509.0	515.4	510.8
$\%VP_1$	74	75	81	79	52	72

6.3.3 Rainbow trout Weber fractions. The Weber fractions for all fish at the various intensities of background illumination are shown in table 6.III. Thresholds to white light can be seen to be very variable, as is reflected in the individual variation in Weber fractions, ranging from 0.15 - 1.58 within one individual. The average range of thresholds for an individual at any one intensity of background illumination was 0.509 log units, nearly twice the variation found for monochromatic stimuli. Large variations in Weber fractions were also noted by Muntz & Northmore (1970), 0.055 - 0.21 for the rudd, and Wainwright (1973), 0.258 - 0.937 for the blenny. The smallest intensity difference between the stimulus and the background that each fish discriminated, that is its lowest Weber fraction, is given in column four of table 6.III. There is no consistent variation of Weber fractions with the intensity of the background.

6.4 DISCUSSION

Psychophysically and electroretinographically determined spectral sensitivities are most useful in combination with other physiological data, such as the absorption spectrum of individual visual pigments. With such information, models of receptor interaction can be proposed to account for the observed action spectra. These can then be used as a basis for comparison to other species. Such models and the difficulties inherent in interspecific and even intraspecific comparisons, as well as tentative models to account for the observed rainbow trout action spectra, will be outlined below.

6.4.1 Factors affecting the form of action spectra. The action spectrum is by no means a constant feature. Many factors may influence its form. These can be classified into two

TABLE 6.III. Weber fractions ($\Delta I/I$) determined behaviourally.

Fish	Background Intensity (cd/m^2)	Weber fractions	Average Weber fractions	Minimum Weber fractions
1	0.242	.165 .089 .213	0.156	0.089
2		.282 .562 .282 1.510	0.659	0.282
1	5.951	.15 1.58 1.15	0.96	0.15
2		.456 .977	0.717	0.456
3	3.461	1.56 .653 .432	0.882	0.432
4		.375 .248 .402 .473 .084 .045	0.271	0.045
5	0.964	.089 .059	0.074	0.059
6		.047 .148	0.098	0.047

categories, which can loosely be termed "environmental" and "experimental". The latter include such things as various characteristics of the stimulus light and the particular response that is being measured, while environmental factors are not connected directly to the experimental design, although they do include such things as temperature and daylength which can be determined by the investigator.

Environmental factors. These include;

(1) Age - Blaxter (1968b & 1969) demonstrated a clear dependence of the shape of the action spectrum of both plaice and herring larvae, on the age of the individuals. Similarly Gramoni & Ali (1970) cite some unpublished results in which the photopic spectral sensitivity of yearling brook trout was found to be different from that of two year olds. Such age dependent changes may be related to changes in the relative numbers of, or connections between, the various receptors.

(2) Temperature - Thorpe (1971 & 1973) showed that the shape of the goldfish photopic action spectrum and the extent to which chromatic adaptation altered the shape of this psychophysical curve depended on temperature.

(3) Season - Using the optomotor response Cronly-Dillon & Sharma (1968) found the photopic spectral sensitivity of the female stickleback to have two maxima at 510 & 594 nm. In the summer the 594 nm peak was most sensitive and the 510 maximum less so, while in the winter the reverse was true.

(4) Sex - The same authors observed that although both the male and female stickleback had maxima at 594 nm, the male short wavelength maximum was displaced by eight nanometers from the female peak, to 502 nm.

(5) Phase of the moon - Lang (1967), using the dorsal light reaction as an index, observed that the sensitivity of the guppy to white light and 432 nm and 582 nm stimuli changed with the

lunar cycle.

The ratio of vitamin A_1 to vitamin A_2 based visual pigments in the rods of paired pigment fish, such as the rainbow trout, is not constant. It undergoes changes depending on such factors as age, temperature, photoperiod, light intensity, diet and habitat (Beatty 1975 for review). Loew & Dartnall (1976) have shown that in the rudd these changes are reflected in the A_1:A_2 ratio of cones, and the same is probably true for trout. Thus the first three of the above factors, age, temperature and season, may exert at least part of their effect, in paired pigment species, by changing the ratio of A_1 to A_2 based visual pigments in the retinas of such fish. Although such a theory can be ruled out to account for the effect of season found by Cronly-Dillon & Sharma (1968), as season only caused a change in relative sensitivity of the peaks and not an actual shift in their position, it is nevertheless possible that in other species season may affect spectral sensitivity. If the A_1:A_2 ratio is important in determining photopic spectral sensitivity, one might expect that the action spectrum would be affected by other factors, such as daylength, which alter the pigment ratio. Evidence contrary to this was obtained by Muntz & Northmore (1970), who found that the photopic spectral sensitivity of the rudd determined using two choice appetitive training, was not affected by changes in daylength, while the λ_{max} of the extractable visual pigment varied in the expected manner. Similarly, results from the electroretinographic action spectrum of Rana temporaria suggest that changes in the A_1:A_2 ratio affect the rods but not the cones (Muntz & Reuter 1966). This apparent anomaly between the results obtained by Muntz & Northmore (1970) and Muntz & Reuter (1966) and those of Loew & Dartnall (1976) can not be easily explained, and the role of

the A_1:A_2 ratio in determining photopic spectral sensitivity remains uncertain.

If the pigment ratio does help determine the spectral sensitivity, the action spectra obtained in any one study will be specific to that population of fish. Thus it would perhaps be desirable always to state the ratio of A_1 to A_2 based visual pigment in any investigation of fish spectral sensitivity.

A second consequence of this variability in pigment content is that individuals within a population will often vary in their ratios, although the percentage of A_1 based pigment in the extracts from both the fish used in the electroretinographic and behavioural determination of spectral sensitivity varied only slightly. Bowmaker et al (1975) also showed that the λ_{max} of rhodopsin from individual frogs can vary by as much as eight percent. This difference in pigment composition between individuals could result in individual differences of spectral sensitivity. Variation in the pigment ratios might help explain the relatively large individual variation of electroretinographic action spectra compared to the constancy of individual behavioural spectral sensitivities, as Northmore & Muntz (1970) demonstrated an effect of pigment ratios on the electroretinographic spectral sensitivity but failed to do so for the behaviourally determined action spectrum (Muntz & Northmore 1970).

Experimental factors. These are probably more important than the above environmental factors in determining the form of the action spectrum, as altering the experimental design can radically change the form of the action spectrum within a species. This has been most clearly demonstrated in two species of teleost, the rudd and the goldfish. The spectral sensitivity of the rudd has been determined behaviourally in four different experimental situations (Muntz & Northmore 1970, Northmore & Muntz 1974 and

Northmore 1973) and each time a different action spectrum was obtained (Muntz 1975b and Northmore 1973 for summaries). Similarly, various determinations of goldfish spectral sensitivity using different behavioural criteria (Cronly-Dillon & Muntz 1965, Yager 1967, Schefner & Levine 1976, Beauchamp & Rowe 1977 and Powers 1976 & 1978) have all resulted in different forms of spectral sensitivity curves. These differences can be caused by either the stimulus conditions or the behaviour itself.

Various stimulus parameters that could affect the form of the action spectrum include;

(1) Size - Northmore & Muntz (1974) obtained different action spectra for the rudd using classical conditioning depending on the diameter of the moving bars of light the fish had to respond to.

(2) Position - Lang (1967), using the dorsal light reaction as a measure of sensitivity, found the middle and ventral parts of the retina to differ in their spectral sensitivities. Similar results were obtained by Thibault (1949) also using the dorsal light reaction. The different action spectra obtained for the rudd using overhead stripes (Northmore & Muntz 1974) and spots of light presented in front of the fish (Muntz & Northmore 1970) may also be due to stimulus position. This finds support from the fact that the visual pigment composition of the rudd varies with position on the retina (Muntz & Northmore 1971b). The teleost retina may also contain the area centralis, an area analogous to the human fovea, and depending on if this is involved in a particular discrimination, the spectral sensitivity will change. Differential pigmentation of yellow corneas, if present, will also affect the form of the action spectrum, depending on where the stimulus strikes the cornea. Muntz (1974) reviews such position related differences in sensitivity in greater detail.

(3) Shape - The above mentioned difference between the action spectra obtained by Muntz & Northmore (1970) and Northmore & Muntz (1974), could be due to the use of a round stimulus compared to a rectangular one.

(4) Adaptation - Both the spectral composition and intensity of adapting illumination are likely to affect the spectral sensitivity. At extremes, the photopic and scotopic function will usually be different, and monochromatic adapting light will suppress complete populations of receptors. Such differences will be obvious from the various texts and nobody would try and compare, for instance, an action spectrum obtained with a white light background and one with a red background, or a light to a dark adapted action spectrum, and be surprised to find that they are different. However, lesser differences in intensity of the background and the colour temperature of the adapting bulb, which are less obvious, may still have an effect on the spectral sensitivity.

Such differences in the stimulus conditions cannot, however, always account for the observed variations in action spectra. Powers (1978) was able to reject all the above factors in accounting for the observed differences between the various studies of goldfish spectral sensitivity, on the grounds that the differences were not consistent throughout all studies. Thus, for example, two studies which used round stimuli gave maximum sensitivity at opposite ends of the spectrum, and the largest and smallest stimuli used both gave peak sensitivity at long wavelengths, thus indicating neither stimulus shape or size could be responsible for the observed differences in spectral sensitivity. Similarly, for the rudd, Muntz & Northmore (1974) noted that the stimulus used by Northmore (1973), in an unlearned orientation response, was nearly the same as that used by these authors in a determination of spectral

sensitivity using classical conditioning, yet the two methods resulted in different action spectra. Thus, the many differences in the form of the action spectra which cannot be explained by differences in stimulus condition are thought to be caused by differences in the behaviour studied. Such response dependency is not specific to determinations of spectral sensitivity using learnt behaviours, as the same was found by Blaxter (1964, 1968b & 1969) using innate behaviours, such as feeding and phototaxis. Furthermore, physiological results also vary depending on, for instance, the specific level of the visual system recorded from. Muntz (1971 & 1974) has summarised evidence from many vertebrates showing such response dependency of the action spectrum and it is now generally accepted that the form of an animal's spectral sensitivity depends to a large extent on the response studied.

This dependence can be explained in terms of the different retinal mechanisms involved in the various behaviours. Thus, Northmore (1973), Muntz (1974) and Northmore & Muntz (1974) summarise evidence for the rudd showing that for the two choice discrimination situation independent receptor action explains the data best, while some of the results using classical conditioning are best described assuming some form of inhibitory interaction between receptors. Therefore, not only is incoming information analysed differently by different species, it is also processed differently within one species, depending on the behaviour used. This often results in action spectra from different species determined by the same methods, being more similar than the spectral sensitivity of one species determined in several different ways. Thus, most determinations of spectral sensitivity using two choice appetitive training tend to have a high blue sensitivity, while blue sensitivity is

much reduced with classical conditioning. Therefore, in order to make interspecific comparisons meaningful, action spectra determined using the same method, in this case two choice appetitive training, must be compared. Fig 6.6 shows the action spectrum of the rainbow trout together with all previous determinations of teleost spectral sensitivity using this form of training. The basic pattern of all five can be seen to be the same.

6.4.2 Form of the trout action spectrum. In common with all the previous determinations of teleost spectral sensitivity using the two choice appetitive situation, the rainbow trout shows three areas of maximum sensitivity, with the highest sensitivity in the blue region of the spectrum. The occurrence of three such processes, one in the blue, one in the green and one in the red region of the spectrum, while not proving that the trout has colour vision, is strongly indicative of it. If, as is generally assumed, these different maxima are due to different cone populations, the primary condition for colour vision, namely the existence of more than one receptor type, has been fulfilled.

All previous teleost action spectra using this form of training have resulted in very high sensitivity at the shortest measurable wavelengths (approx 400 nm), which can not be explained by the absorption spectrum of any known visual pigment. Muntz & Northmore (1970) obtained some measurements of sensitivity at 377 nm and found these to be even higher than at 406 nm. It is interesting to note that the previous study with the lowest blue sensitivity is that of Cameron (1974) on the perch, a species that has a yellow lens. Several reasons for this high blue sensitivity have been proposed. Yager (1967) suggested that it could be explained by:

(1) Uncertainty about the shape of the visual pigment absorption

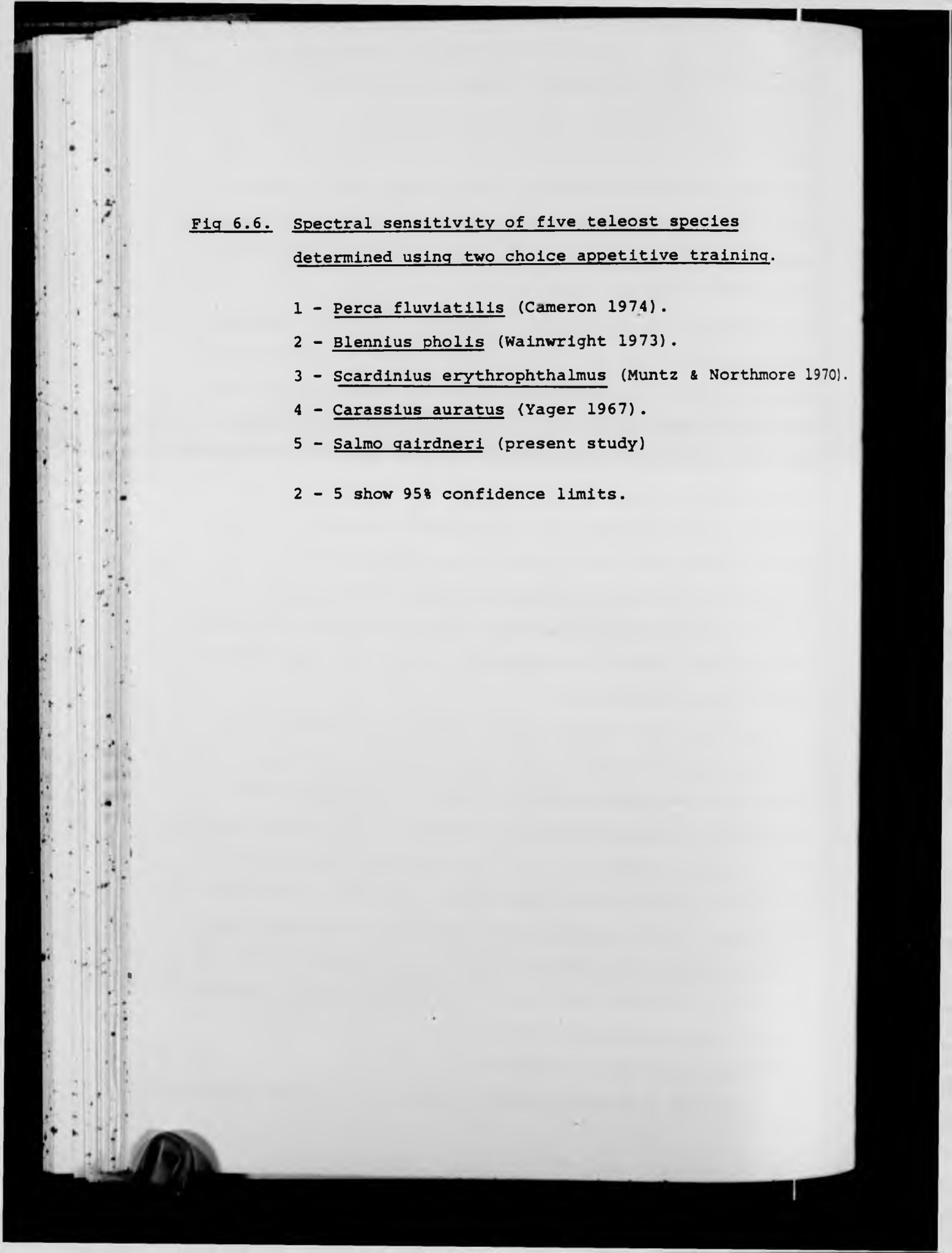


Fig 6.6. Spectral sensitivity of five teleost species determined using two choice appetitive training.

1 - Perca fluviatilis (Cameron 1974).

2 - Blennius pholis (Wainwright 1973).

3 - Scardinius erythrophthalmus (Muntz & Northmore 1970).

4 - Carassius auratus (Yager 1967).

5 - Salmo gairdneri (present study)

2 - 5 show 95% confidence limits.

eost species

titive training.

974).

1973).

(Muntz & Northmore 1970).

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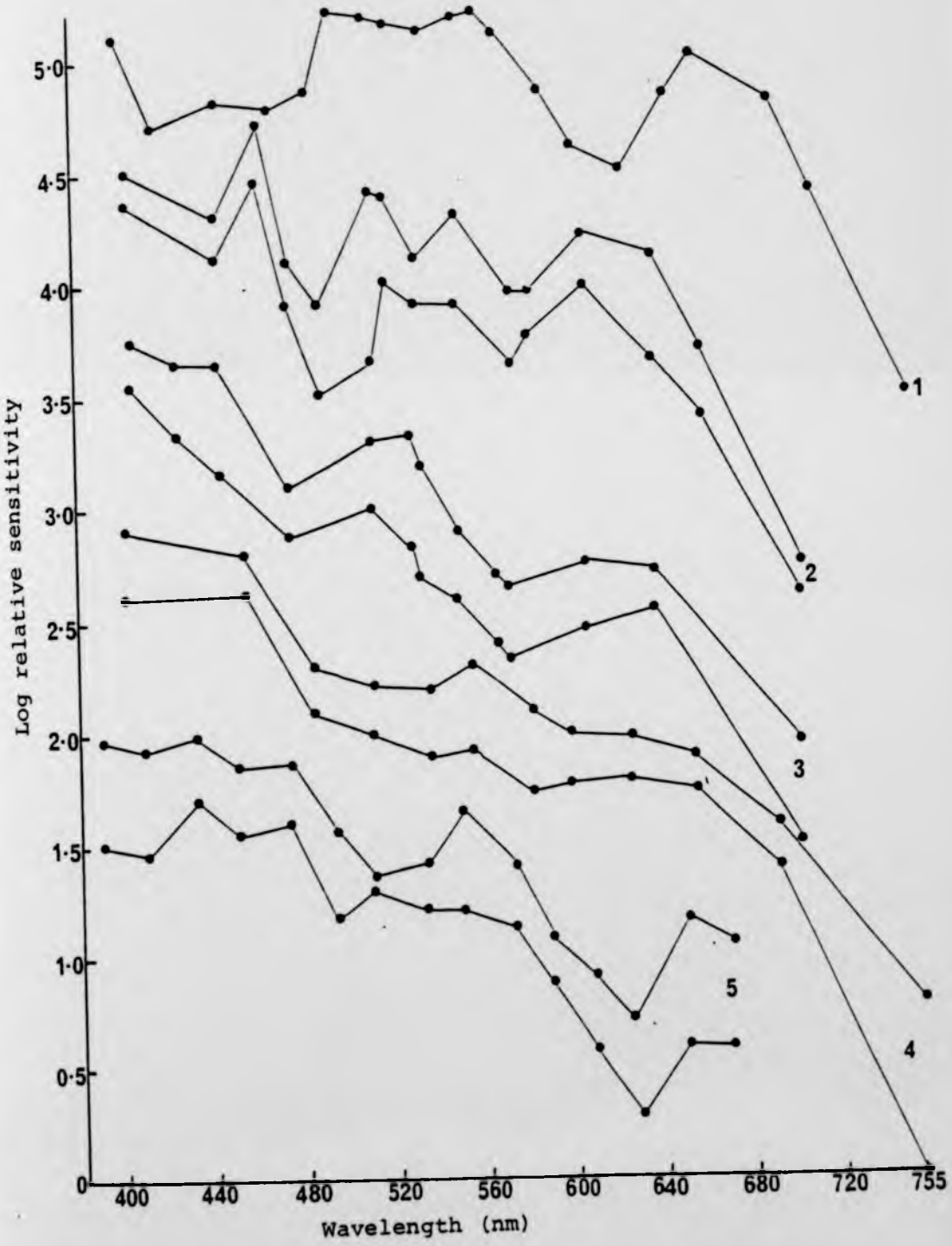


spectra at short wavelengths. The sensitivity may be higher at these wavelengths than at present assumed.

(2) Fluorescence of either the ocular media or the optical system between the light source and the eye.

(3) Scatter of short wavelength light.

These possibilities are discussed in detail by Muntz & Northmore (1970), who suggest two further alternatives.

(4) Conversion of the visual pigment to a thermally stable photosensitive photoproduct, which absorbs maximally at these short wavelengths. Cameron (1974) suggests this might be metarhodopsin II.

(5) As the tungsten backgrounds tend to contain more long wavelength radiation, red receptors will tend to become more adapted than shorter wavelength ones.

Perhaps surprisingly the present data do not show such high short wavelength sensitivity to the same extent. Sensitivity was only maximal at the shortest wavelength in one out of the six fish (fish 5, fig 6.4). Maximum sensitivity usually occured around 430 nm and the points of the average action spectrum are quite well fitted by the absorption spectrum of a visual pigment with a λ_{max} of 440 nm (see below).

6.4.3 Models to explain the form of the action spectrum. We have a detailed knowledge of the stimulus incident on the retina and the psychophysical and electroretinographic response of the fish. The question that must now be asked is, how does the former give rise to the latter? In order to answer this, the action spectrum must be explained in terms of the underlying visual pigments' absorption spectra. As shown in the previous chapter, for scotopic vision this is often a simple matter, as only a single class of receptors underlies the visual response. The photopic response, on the other hand, is

usually more complex as it is mediated by the cones, of which there are normally three distinct populations. Thus, the photopic action spectrum is determined by some form of interaction between these receptors. Such relationships between the receptors can best be described in terms of models, of which there are two basic types.

The effective amount of light absorbed by any one receptor is given by the expression,

$$I_\lambda (I - 10^{-D\lambda}),$$

where I_λ is the quantum intensity of the light and D_λ is the optical density of the visual pigment. Thus the sensitivity at any wavelength, S_λ, is some function of this,

$$S_\lambda = f(I_\lambda (1 - 10^{-D\lambda})).$$

For photopic vision the optical density is effectively less than 0.1. The above expression therefore simplifies to,

$$S_\lambda = k(I_\lambda . A_\lambda)^n,$$

where A is the absorbance of the visual pigment in that receptor, k is a constant and n is a power transform. In the simplest case n = 1, such that,

$$S_\lambda = k(I_\lambda . A_\lambda).$$

In the following description of the various models, for the sake of brevity, the sensitivity of each receptor type given by the above equations, will be designated by B_λ, G_λ, and R_λ respectively, where B is the blue receptor, G is the green receptor and R the red receptor.

(1) Independent model. The threshold at any wavelength in this model, also termed the envelope model, is determined by the receptor most sensitive at that wavelength. Thus the sensitiivity, S_λ, can be expressed as,

$$S_\lambda = k_1 . B_\lambda$$

$$\text{or} \quad S_\lambda = k_2 . G_\lambda$$

$$\text{or} \quad S_\lambda = k_3 . R_\lambda$$

In such a model, the peaks of the action spectrum must be of the same dimensions as the visual pigment absorption spectrum and their maxima coincident with the pigments λ_{max}. Muntz & Northmore (1970) consider this form of model to explain the spectral sensitivity of the rudd determined by two choice appetitive training.

(2) Interactive models. As the name implies this involves some form of interaction between the different cone types. Such interaction can be either additive or inhibitory in nature.

(i) Additive. At any wavelength, the threshold depends on the summed inputs from the various receptors, such that,

$$S_\lambda = k_1.B_\lambda + k_2.G_\lambda + k_3.R_\lambda .$$

This form of interaction implies that the individual peaks of the spectral sensitivity curve will be broader than the absorption spectrum of any one visual pigment, and will tend to be less well defined than predicted by the envelope model. Such a model has been used to fit the action spectrum of the goldfish determined using two choice appetitive training (Yager 1967 & 1969, Thorpe 1971) and the dorsal light reaction (Powers 1978), but a close inspection of Yager's (1967) data, especially at the extreme long and short wavelengths, reveals it probably does not fit the data as well as is claimed.

(ii) Inhibitory. Sperling & Harwerth (1971) were not able to explain their data for the rhesus monkey by either of the above models, which led them to propose a third, involving inhibition between the red and green receptors. Thus,

$$S_\lambda = k_1.R_\lambda - k_2.G_\lambda$$

$$\text{or} \quad S_\lambda = k_3.G_\lambda - k_4.R_\lambda$$

$$\text{or} \quad S_\lambda = k_5.B_\lambda .$$

The threshold depends on the mechanism that is most sensitive at that wavelength. With such an interaction, the red and

green peaks would tend to be narrower than the visual pigment absorption spectra, while the blue peak should be fitted perfectly by the visual pigment.

A more complex form of this type of model was fitted to the action spectrum of the rudd determined by classical conditioning (Northmore & Muntz 1974). All the above models have assumed that the sensitivity is a straight forward function of the receptors sensitivity, but the data of the above authors was better fitted by a power function.

$$S_\lambda = k_1.B_\lambda^{\,p} + k_2.G_\lambda^{\,p} - k_3.R_\lambda^{\,p}$$

where $p = 0.6$.

A similar model has also been used by Cameron (1974) to explain the action spectrum of the perch.

As noted above (6.4.1), the form of the spectral sensitivity curve depends not only on the species but also on the experimental conditions. Consequently, the interactions between receptors, and hence the models used to describe these interactions, also differ as reflected in the above summary. Such differences must eventually be described by differences in the underlying neurophysiological reactions. Thus the diffuse stimulus used by Northmore & Muntz (1974) may cause the antagonistic surround of ganglion cells to be activated resulting in a lower sensitivity and inhibitory interaction, whereas the surround may not be activated in the two choice discrimination situation (Muntz & Northmore 1970) due to the smaller size of the stimulus, resulting in independent receptor action (6.4.1).

In order to explain any form of action spectrum, determined behaviourally or electrophysiologically, in terms of the interactions between the underlying visual pigments with complete certainty, a detailed knowledge of the absorption

spectra of these pigments is required. The only reliable way of specifying these pigments precisely is by microspectrophotometry (MSP). Perhaps surprisingly, until recently data on teleost cone visual pigments obtained in this way have been scarce. The first such study was carried out by Hanaoka & Fujimoto (1957), who characterised the pigments of the carp. Subsequent studies include those of Liebman & Entine (1964), Marks (1965), Svaetichin et al (1965), Laufer & Millan (1970), Liebman (1972 & 1973), Harosi & MacNichol (1974), Stell & Harosi (1976), Leow & Dartnall (1976), Harosi (1976), Loew & Lythgoe (1978), MacNichol et al (1978) and Levine et al (1979 a & b).

These cone pigments generally have λ_{max}s ranging between 450 nm and 625 nm. Absolute theoretical ranges of such pigments are discussed by Lythgoe (1979, pp 63-65). Unfortunately, the cone visual pigments of the rainbow trout have not been characterised in this way. Ali & Wagner (1980) noted that the brook trout had green cones with a λ_{max} of 530 nm, and that blue and red cones were also observed, but furnish no further details. Liebman (1972) investigated the rainbow trout rod pigment using MSP but no mention is made of cones.

In the absence of such data, it may be tempting to assign λ_{max}s to the three presumed cone pigments coincident with the maxima of the action spectrum. Unfortunately such a practice has several problems. In agreement with the response dependency of the spectral sensitivity described above, the ERG and behaviourally determined action spectra have their maxima in quite different areas of the spectrum. As it is not possible that different visual pigments underly each different determination of spectral sensitivity, the original assumption must be faulty. This variability in the position of the maxima is,

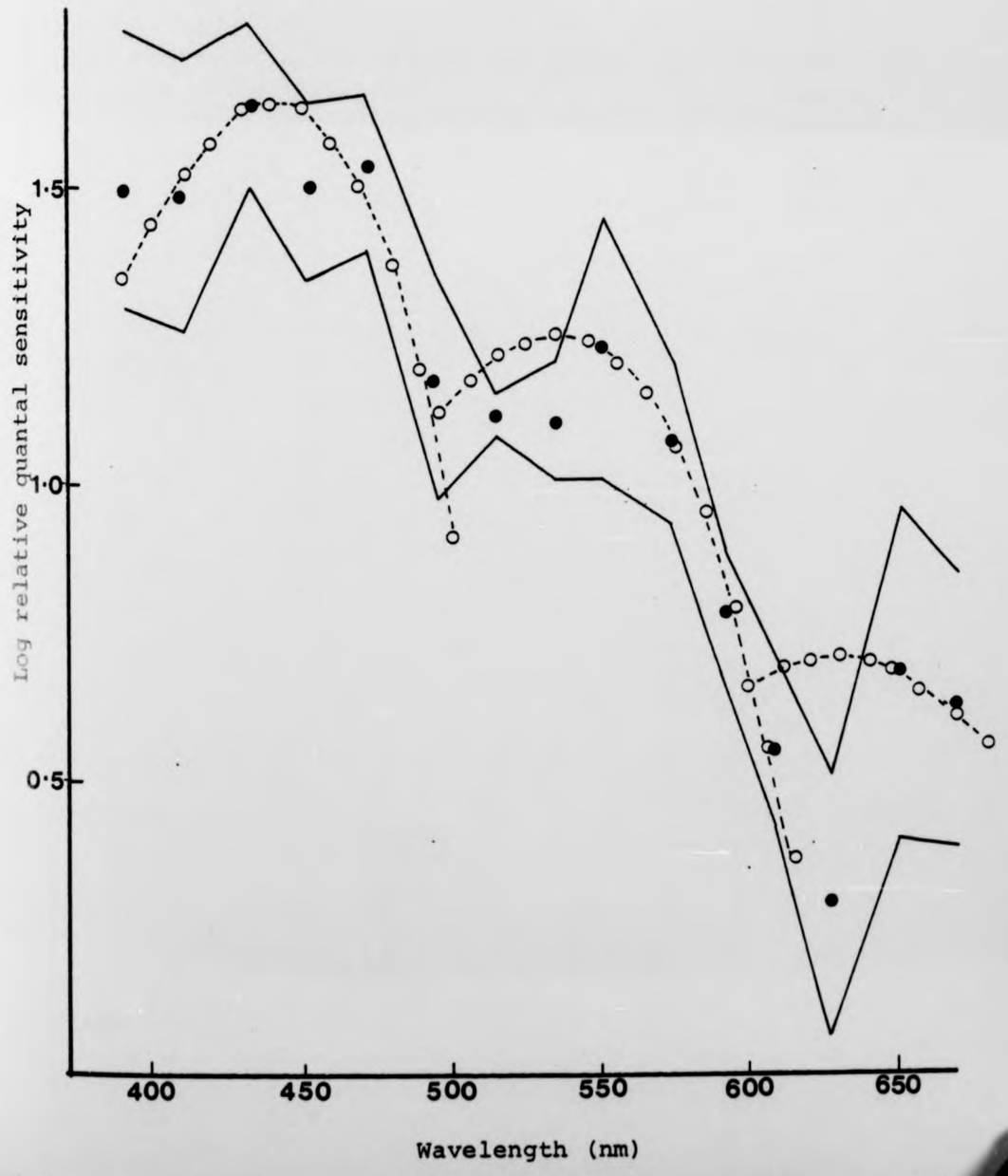
as described above, almost certainly due to different interactions taking place within the retina. Thus, any form of inhibitory interaction between cones will tend to shift the maxima from that of the visual pigment. In fact, any two pigments could interact to produce a third maxima not dependant on its own pigment. Such "pseudo-pigments" or "ghost cones" are discussed in greater detail by, for instance, Naka & Rushton (1966).

Yet even in the absence of specific data on rainbow trout cone pigments some observations about the underlying interactions can be made. There are two basic types of predictions about the underlying visual mechanisms that can be made without a knowledge of the visual pigments, concerning the form of the action spectrum and the fish's response to white light. The former will be discussed here, while the latter is dealt with in a later section, (6.4.5).

Different models of receptor interaction make different predictions as to the form of the spectral sensitivity, which have already been alluded to above. If the receptors are acting independently, as suggested by the envelope model, the maxima of the action spectrum will coincide with the λ_{max} of the visual pigments, and the widths of the action spectrum maxima and the visual pigment absorption spectra will be of the same dimensions. Thus, the templates of the absorption spectra of the three visual pigments placed on the action spectra would form an envelope describing the data, although there may be some summation at the "troughs" in the action spectra,causing a slight mismatch. Additive or inhibitory interactions, on the other hand, would result in peaks of the action spectrum, not necessarily coincident with the λ_{max} of the visual pigment and respectively broader and narrower than the pigment absorption spectrum.

Fig 6.7 shows the absorption spectra of three hypothetical

Fig 6.7. Average action spectrum of rainbow trout determined using two choice appetitive training. (●) - fitted by eye to the absorption spectra of three hypothetical cone pigments (○).



cone pigments, with λ_{max}s at 440, 535 & 630 nm, determined from the Ebrey & Honig (1977) nomogram as described in chapter five, fitted by eye to the average behavioural spectral sensitivity. Up to a wavelength of 600 nm, the data, especially the descending arms of the blue and green processes, could be fitted by such an envelope model, but the "dip" between the green and the red peak is too sharp and the red peak too narrow to be fitted by such a simple system. Thus, some form of inhibitory red - green interaction is implied. Similar models with independent blue receptor action and inhibitory interaction between the green and red cones has previously been suggested by Sperling & Harwerth (1971) for the rhesus monkey, Northmore & Muntz (1974) for the rudd and Cameron (1974) for the perch (see above). Such inhibitory red - green interactions have also been well documented electrophysiologically, especially at the level of the ganglion cells. The mismatches between the data and the expected values at the shorter wavelengths is sufficiently small to be explained by the variability of the individual action spectra.

Such variability itself presents a problem, in that it might be more valid to fit models to the individual curves as opposed to the averages. This is not so much of a problem for the behavioural data, as individual action spectra are fairly similar, having peaks in the action spectra in the same regions and of the same general shape. This is not so for the individual spectral sensitivity curves determined using the ERG as a measure of sensitivity.

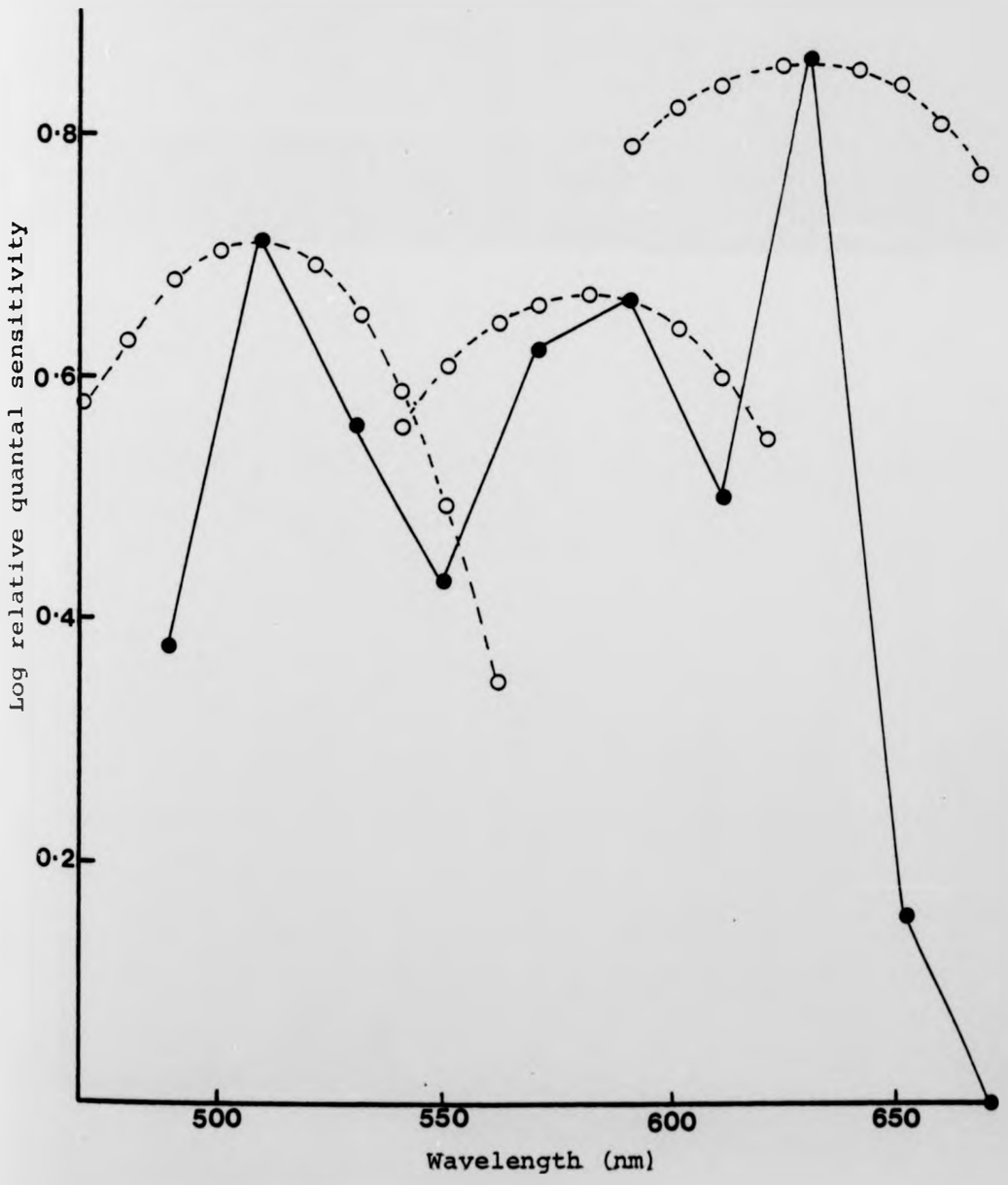
As can be seen from fig 5.9, individual spectral sensitivity curves determined electroretinographically differ quite markedly in the positions of their maxima, resulting in the peaks of the average curve being much less pronounced than those of individuals. Therefore, although fig 5.13 shows, by fitting hypothetical

pigments (λ_{max} 500, 570 & 630 nm) to the average curve, that the peaks in the action spectrum are narrower than the absorption spectrum of the pigments, the degree of mismatch is much greater if individual curves are considered. An example of this is given in fig 6.8, where the action spectrum of fish 119 is matched to three visual pigments of λ_{max} 510, 580 & 630 nm. The extreme narrowness of the peaks in these ERG determined spectral sensitivity curves implies some form of inhibitory interaction between all receptors.

Thus, the action spectrum determined using two choice appetitive training may depend on independent receptor action at shorter wavelengths and some form of inhibitory interaction between the red and green processes at the longer wavelengths. The ERG determined spectral sensitivity, on the other hand, appears to be determined by inhibitory interactions at all wavelengths. This is backed up by the positions of the maxima in these two determinations of spectral sensitivity. Most fresh water teleosts have three cone pigments absorbing maximally around 455, 530 & 625 nm. Therefore, if the rainbow trout has pigments similar to these, and if the models proposed for the action spectrum determined behaviorally is valid, one would expect the maxima to occur at around 455 and 530 nm, with the red peak shift to a wavelength beyond 625 nm. If, on the other hand, the interactions are inhibitory, as suggested for the ERG determined action spectrum, the λ_{max}s of these pigments would not coincide with the peaks of the spectral sensitivity curve. In both cases these expectations are fulfilled.

6.4.4 Comparison of behaviourally & electroretinographically determined action spectra. Both the shape and position of the maxima of these two action spectra differ. Such disagreement between behaviourally and electrophysiologically

Fig 6.8. Action spectrum of fish 119 determined electroretinographically (●) - fitted to the absorption spectra of three hypothetical cone pigments (○).



determined action spectra is not uncommon. The photopic action spectra of the rudd (Muntz & Northmore 1970) and goldfish (Yager 1967), determined by the two choice appetitive methods, for example, do not resemble the corresponding curves when the ERG is used as a measure of sensitivity (Northmore & Muntz (1970) and Burkhardt (1966) respectively). Furthermore Thorpe (1973) has shown that the psychophysical and ERG spectral sensitivities of the goldfish are not affected in the same way by temperature and chromatic adaptation.

As described above, the behavioural action spectrum of the trout can be explained by largely independent receptor action, while the ERG spectral sensitivity may depend largely on inhibitory interactions. The occurrence of these different mechanisms and the consequent difference in action spectra can be explained in terms of the different intensities of stimulus employed. The maximum intensity of stimulation that has to be used in the determination of the ERG response is far greater than that used in the behavioural determination, since an animal can respond behaviourally at intensities well below those that elicit a b-wave. At such low levels of stimulation only one receptor, the most sensitive, is stimulated, and consequently the receptors behave independently. As the intensity of stimulation is raised, however, more than one receptor is activated and there is scope for inhibition between the receptors. Thus, low level behavioural thresholds can be described by an independent model, while the higher intensity ERG thresholds depend on receptor interaction. In this connection it is interesting to note that receptor interaction is only postulated for the behaviourally determined spectral sensitivity at the long wavelengths, at which the intensity necessary to elicit a behavioural response is the highest.

6.4.5 Response to white light (Weber fractions). The large

amount of variability in the threshold to white light (average range 0.509 log units) compared to that for monochromatic stimuli (average range 0.287 log units), would seem to indicate that the fish find it easier to discriminate on the grounds of wavelength than brightness. This may be explained by the fact that colour vision depends on the relative activation of different populations of receptors, and changing the colour changes the extent to which different spectral classes of receptor are activated. Thus, if a fish views two different coloured targets, each one will activate the receptors differently. The same is true when discriminating between a white light and a coloured target. White light, on the other hand, stimulates all receptors and two white light targets only differ in the amount of receptor output they cause and not in the type of receptor output. It is thus quite probable that a discrimination between two targets is more easily made if the targets cause differential receptor activation rather than just differences in the amount of output from the same receptors.

Table 6.III shows that four fish at some time obtained a high level of brightness discrimination, with Weber fractions of less than 0.1. It is likely that all rainbow trout have the ability to achieve this degree of discrimination but do not always fulfill their potential, due to the greater difficulty of the task.

Using the ERG to obtain Weber fractions (chapter 4) resulted in values ranging from 1.80 - 3.99, very much higher than those psychophysically determined (0.45 - 0.045). As noted previously, this large discrepancy is due to the fact that the ERG is not an indication of the animals actual sensitivity. The fish will respond behaviourally to intensities that no longer elicit a measurable b-wave.

For similar reasons to those outlined above (6.4.1), it is advisable only to compare Weber fractions from determinations using the same experimental situation. Weber fractions for teleosts using two choice appetitive training have only been determined twice previously. Muntz & Northmore (1970) for the rudd obtained values ranging from 0.055 - 0.21, and Wainwright (1973) obtained somewhat higher values of 0.258 - 0.937, for the blenny. The results for the rainbow trout are more similar to those of the rudd, as six of the eight lowest trout Weber fractions fall within the range of the rudd's Weber fractions, with only two extreme values within the range of those of the blenny. The reason for this may again be found in the experimental situation. As Wainwright (1973) pointed out, due to the experimental design, rudd were able to make a simultaneous discrimination between the illuminated and unilluminated response chambers, as were the trout in this study. The blenny however, was not able to do this since the stimuli were further apart and they had to remember one side while looking at the other.

As with the spectral sensitivity determinations, other factors apart from the experimental situation will also affect brightness discrimination. Hester (1968), for instance, using classical heart rate conditioning on goldfish, showed that fish size (age), temperature, position of image on the retina, target size and level of adaptation all affected the ability to discriminate brightness.

The response to white light can also be useful in helping to determine what interactions between the chromatic mechanisms underlie the action spectrum. The various models of receptor action, outlined above, make different predictions about the sensitivity to tungsten white light compared to the sensitivity

to monochromatic stimuli, when both are superimposed on the same tungsten white light background, because the former is a broad band stimulus and will tend to stimulate all types of receptor, while the latter will only affect receptors sensitive over a restricted area of the spectrum. Thus, if the receptors are acting in an additive manner, the white light threshold would be lower than that to monochromatic light, as the level of receptor output needed for the stimulus to be responded to will be reached at a lower intensity with white light. Similarly, independent receptor action will result in a lower white light threshold, as white light provides more effective quanta than the narrow bank monochromatic stimuli. If, on the other hand, the receptors are acting in an inhibitory manner, the threshold to white light will tend to be the higher as there will be less inhibitory interaction with monochromatic stimuli, as fewer receptor types are stimulated.

For the independent model one can make precise predictions as to the value of the increment threshold to white light if the monochromatic threshold on the same background is known. In order to do this the number of effective quanta in each type of stimulus must be determined, as it is the number of quanta, not the energy, that underlies the threshold. This can be calculated for the white light stimulus by multiplying the absorption spectrum of the visual pigment assumed to underlie the threshold by the radiant emittance curve for a tungsten source of known colour temperature ($3540^{\circ}k$). The integral of these values over wavelength is an indication of the number of quanta absorbed by the receptor. A corresponding value for the monochromatic stimulus is obtained by multiplying the above product of visual pigment absorption and tungsten source radiation at each wavelength by the transmission of the interference filter

at that wavelength. The integral of this against wavelength again gives the effective number of quanta in the monochromatic stimulus (Muntz & Northmore 1970, for further details).

As independent receptor action has only been proposed for the shorter wavelengths of the psychophysically determined action spectrum, only these will be considered. The visual pigments underlying the thresholds at these wavelengths will be assumed to be the ones that gave the best fit to the data, absorbing maximally at 440 & 535 nm. If, as predicted by the envelope model, the threshold at any wavelength is determined solely by the receptor most sensitive at that wavelength, the difference between the number of effective quanta in the two types of stimuli should be the only cause for a difference in threshold. Thus, the difference between the value for the effective number of quanta obtained above, should be the same as the difference between the behaviourally determined thresholds for monochromatic and white light stimuli. Monochromatic stimuli of 433 & 534 nm were chosen for comparison to white light as they most nearly coincided the the λ_{max} of the two visual pigments. Table 6.IV gives the expected difference between the white and monochromatic light thresholds and the values actually obtained at these two wavelengths. The agreement between the predicted and observed differences is quite close and probably as good as can be expected considering the assumptions that have been made. The threshold of fish 3 to white light is probably unrepresentative, as discussed above (6.4.4), and if this value is excluded (value in brackets) agreement is even better. As the additive model would predict larger differences between the thresholds, it seems that the psychophysical action spectrum at wavelengths up to approximately 600 nm may be explained by independent receptor action.

TABLE 6.IV. Observed and expected differences between white and monochromatic light thresholds.

Fish	1 Lowest white light threshold	2 Thresh to 433	Difference 1 - 2	3 Thresh to 534	Difference 1 - 3
1	2.8	1.25	1.55	1.33	1.47
2	2.37	1.05	1.32	1.01	1.36
3	1.088	0.855	0.233	1.0	0.088
4	1.932	0.901	1.031	1.0	0.932
5	2.176	1.22	0.956	1.08	1.906
6	2.27	1.3	0.97	1.0	1.27
average difference			1.01 (1.165)		1.17 (1.226)
expected difference			1.293		1.279

The uncertainty as to the exact interactions, and the visual pigments underlying these, at the long wavelength region of the psychophysical action spectrum, do not allow a similar predication to be made about the threshold in this area. A similar analysis can also not be performed on the ERG determined threshold, as the Weber fractions (chapter 4) and photopic response to monochromatic stimuli (chapter 5) were determined in different experimental situations.

Thus, even without a knowledge of the rainbow trout's cone pigments, one can be fairly certain that the spectral sensitivity determined using two choice appetitive training depends on independent receptor action at short wavelengths and on red-green inhibition at longer wavelengths. The ERG action spectrum, on the other hand, seems to be determined by largely inhibitory interactions.

6.4.6 Human spectral sensitivity. In common with other studies in which the human action spectrum has been determined using a two choice apparatus previously used to determine fish spectral sensitivity (Yager 1967, Wainwright 1973 and Cameron 1974), maximal sensitivity was attained at the blue end of the spectrum. All these studies have in common the fact that the eye was free to move, and the spectral sensitivity is thus probably largely that of the extrafoveal region. That such extrafoveal stimulation results in high short wavelength sensitivity was demonstrated by Weale (1953) and Yager (1970) substantiated that it was not the result of experimental error by showing that if the subject fixated the target on the fovea, the high blue sensitivity disappeared and a more normal foveal spectral sensitivity was obtained. It is perhaps interesting to note in this connection, that in the most detailed study of human visual pigments up to date by Bowmaker & Dartnall (1980), the only three blue cones found were situated in the peripheral retina.

Prior to this study by Bowmaker & Dartnall (1980), only thirteen individual human receptors had been investigated using MSP (Marks et al 1964, Brown & Wald 1964 and Wald & Brown 1965). As their study obtained records from forty-eight receptors it was felt to be the most representative, and absorption spectra of individual cone types determined by Bowmaker & Dartnall (1980) were used for comparison to the human action spectrum. These absorption spectra, corrected for optical density are shown with the action spectrum in fig 6.3. The observed mismatch between these absorption spectra and the spectral sensitivity demonstrates that a simple envelope model, such as that proposed by Stiles (1959), does not explain this human action spectrum. In view of what has been said above (6.4.1), this is not surprising considering the different methods employed.

The present data show three clear maxima, at around 450, 530 and 620 nm. Exact positions of these peaks cannot be determined due to the large separation of individual points. These compare well to the maxima found by Sperling & Harwerth (1971), with an equivalent background. Both curves clearly show "Sloan's notch" at around 580 nm, as do the data of Cameron (1974). A similar form of red-green inhibitory interaction, as described by Sperling & Harwerth (1971) (6.4.3), could account for the shifted red peak of the action spectrum in the present study, but the short wavelength peak is too narrow tobe explained by the independent receptor action proposed by these authors.

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