The circadian control of retinomotor movements in a teleost fish, the glowlight tetra (*Hemigrammus erythrozonus*)

A Thesis submitted by

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Abstract

Retinomotor movements are the movements of cones, rods, and retinal pigment epithelium (RPE) melanosomes associated with the transitions between light and darkness. They are most prevalent and strongest in fish and amphibia, less in reptiles, and birds, and least in mammals. During the light phase, cones, rods, and RPE melanosomes occupy positions different to those found during the dark phase. Not only do retinomotor movements occur as a result of changes in the ambient light levels, but they are also under endogenous control as shown by their rhythmic continuation in constant darkness where cones, rods, and RPE melanosomes move to light adapted positions during 'expected' day and to dark adapted positions during 'expected' night.

This work commences by outlining the basic rhythms of both cone and RPE melanosome movements during a normal light/dark cycle and in constant darkness, in an equatorial teleost fish, the glowlight tetra (*Hemigrammus erythrozonus*). Both the cone and RPE melanosomes movement rhythms persist far longer in constant darkness than in any other species examined to date which is interpreted as being due to strong circadian drive of retinomotor movements in the glowlight tetra. Further, after reviewing retinomotor literature, it is suggested that the glowlight tetra and other equatorial fish may have relatively more robust circadian drive than species from more temperate regions.

The relatively long duration of retinomotor movements in constant darkness afforded a good opportunity to study circadian organisation of these movements

in more detail. For example, the light control of cone and RPE melanosome movements were examined. The light control of cone movement was found to be poor in response to light exposure and dark placement in the middle of the dark and light phases respectively. In comparison, strong light control of RPE melanosome movement was found during light in the middle of the dark phase, but not to a dark pulse in the middle of the light phase. However, more light control of cone movement was recorded at two other times during a full cycle of cone movements suggesting that there is a circadian rhythm in the light control of cone movements. Although these intervals of light control of cone movements were relatively short in comparison to the period of the cone rhythm, the rhythm of cone movements in glowlight tetras entrained to a 180° phase shifted light/dark cycle in a similar time found in other animals.

As the glowlight tetra is an equatorial species, there would be little need for oscillators governing retinomotor movements to be sensitive to photoperiod. However, the rhythm of cone movement was found to entrain well to all 24 hour light/dark cycles with photoperiods varying from 1-14 hours. Models using a one or two-component oscillator were able to explain the mechanism by which the rhythm of cone movements in the glowlight tetra could entrain to the different light cycles. Further, following entrainment to most light/dark cycles, the free-running period remained at 24 hours.

Another ocular rhythm, the formation and degradation of horizontal cell spinules, was examined in the glowlight tetra. In constant darkness, the rhythm of spinule dynamics showed signs of damping within the first 24 hours. This was interpreted

as representing less strong circadian drive than that for both cone and RPE melanosome movement. In comparison to both the cone and melanosome movements, spinule dynamics was affected by both light exposure and dark placement in the middle of the dark and light phases respectively. Following exposure to light in the middle of the dark phase, spinule formation followed a time course similar to all other species examined to date.

Retinomotor movements, in particular the movements of both cones and RPE melanosomes, were also examined to determine whether they played a possible role in regulating visual sensitivity as determined by the dorsal light reaction (DLR). Retinomotor responses in the glowlight tetra and the zebrafish (*Danio rerio*) were examined in this series of experiments. During light exposure in the middle of the dark phase, RPE melanosomes but not cones, undergo retinomotor movements in the glowlight tetra whereas both cones and RPE melanosomes show retinomotor movements in the zebrafish. When fish are exposed to light in the middle of the light phase, no retinomotor movements are seen in both species of fish. However, under both conditions of light exposure in the middle of the light and dark phases, there was a similar change in sensitivity as measured by the DLR in both species. Thus, it was concluded that it was unlikely that retinomotor movements have a role to play in sensitivity control.

Chapter 1-General Introduction

Rhythmic behaviour is widespread. Plants open and close their leaves at the same time each day. Desert rats remain underground throughout the daylight hours and emerge in the dark to forage for food, retreating before the onset of light. The sleep/wake cycle occurs in most animals. Seasonal migration is seen in many birds while some animals such as hamsters, chipmunks, squirrels and many others hibernate in winter. It is now known that these and many other physiological processes are not merely a result of changes in the ambient environment such as day/night or seasonal variation. Rather, they are under the control of endogenous clocks which govern numerous physiological functions.

The use of internal clocks to control specific behaviours clearly confers some advantages to the individual by signalling the time of day to, for example, avoid predators, capture prey or conserve energy (Refinetti, 2000). Most plants open their leaves during the day so that the sunlight's energy can be used more effectively for photosynthesis and close them at night. If the nocturnal desert rat responded only to the day/night changes and did not have a clock influencing its behaviour, it would not return underground until daylight, leaving itself exposed to predators.

Other less obvious but important physiological functions are also under clock control. For example, metabolic rate, body temperature, heart rate, blood pressure, and urinary excretion are governed by clocks in various species (Refinetti and Menaker, 1992; Plaza et al, 1993; Rashotte et al, 1995; Anava et al, 2002). The bulk of this study concerns one such rhythm; the clock controlled rhythms of

retinomotor movements in the eye of a teleost fish, the glowlight tetra (*Hemigrammus erythrozonus*).

In the first part of this introduction, general characteristics of the glowlight tetra are described briefly before a more detailed description of its retina and retinomotor movements is given. Next, the field of 'biological rhythms' is introduced. There is a long history to the study of retinomotor movements yet no workers have interpreted their findings in the context of the wider field that makes up 'biological rhythms'. It is hoped that by introducing this topic simply, those who have studied retinomotor movements may benefit not only by the studies undertaken here, but also by reinterpreting their own work.

1.1 General characteristics and habitat of the glowlight tetra

The glowlight tetra (*Hemigrammus erythrozonus*) is a teleost (bony fish) belonging to the family Characidae, which is a part of the order Characiformes. Characiformes are mostly carnivores with well defined teeth, almost always have a body that is scaled, and have an adipose anal fin as well as a pelvic fin. Members of this order, like the glowlight tetra, are usually small and often extremely colourful. The Characidae are a large and diversified family containing the potentially dangerous piranhas (*Serrasalmus*), the South American tetras (*Hemigrammus*), a blind cavefish in Mexico (forms of *Astyanax mexicanus*) a species that dwells in Brazil (*Stygichthys typhlops*), and a species that has dispersed into the southwestern United States of America (*Astyanax mexicanus*).

The glowlight tetra (Figure 1.1) is a freshwater fish with a maximum size of 3.3cm and can survive in shallow waters with a pH ranging from 6.0-8.0 in a tropical climate ranging from 24-28°C (Fishbase, 2004). Of most importance to this piece of work is the geographical distribution of the glowlight tetra.

Fig 1-1 Glowlight tetra
Photograph of glowlight tetra. Note its relatively colourful appearance, scaled body, and adipose anal fin typical of most characiformes.

The glowlight tetra is an equatorial species. In standard textbooks its distribution is given variously as ''the Guineas and adjacent regions of the Amazon'' (Axelrod et al, 1985), "North-eastern parts of South America'' (Mills and Vevers, 1989), or simply "Guyanna" (Paysan, 1975). Thus, the glowlight can be considered to originate in Guyanna which stretches from 1°N to 9°N latitude. This distribution can be narrowed by concentrating on the recorded findings. All recorded collections in museums have been in creek tributaries of the Potaro River (British Museum of Natural History, Californian Academy of Science), which begins at the Ayangana Mountain Range in the North Rupunni Savannah and extends 140 miles to the Essequibo River. A range of the co-ordinates for the Potaro River is given by those at either end of the river as it does not wind to any great extent. At Ayanganna, the co-ordinates are 5°10'N latitude and 59°29'W longitude. The Essequibo River runs to 1°N lattitude and 58°W longitude and although there are

no recorded findings this far south, it seems reasonable that glowlight tetras may inhabit these regions. Thus, a narrower range of distribution for the glowlight tetra is 5°10'N latitude and 59°29'W longitude to 1°N and 58°W.

The geographical distribution of the glowlight tetras is important in the context of this work as it allows a fairly accurate determination of the day-light hours to which fish are exposed during their lifetime. Using the above distributional range, we would expect the glowlight tetras living closer to the Ayangana Mountain Range (5°10'N latitude) to experience the most variation in day-light hours during a year. In 2003, the maximum and minimum values in sunrise and sunset times at Ayangana are given in the table below.

Table 1.1. Variation in sunrise and sunset times at Ayangana during 2003.

Earliest sunrise at 06.45	Sunset at 19.10	Day-light hours = 12h25m	June 2nd
Latest sunrise at 07.17	Sunset at 19.10	Day-light hours=11h53m	January 30
Earliest sunset at 18.41	Sunrise at 06.50	Day-light hours = 11h51m	November 20
Latest sunset at 19.13	Sunrise at 07.10	Day-light hours=12h03m	March 5

(Table values taken from the U.S. Naval Observatory)

Thus, during 2003, fish inhabiting waters near the Ayangana Moutain Range experienced daylight hours that varied from 11h51m to 12h25m, a variation of only 34 minutes. If glowlight tetras do inhabit the Essequibo River (1° at it most southern point) as speculated above, then the variation in daylight hours during a year will be less than 34 minutes.

The experience of constant day-lengths throughout the glowlight tetras life history is a theme that will reoccur throughout this thesis and is one reason why the glowlight tetra was chosen for study.

1.2 The teleost retina

To date, there have been no anatomical studies describing either the glowlight tetra (*Hemigrammus erythrozonus*) eye. In fact, an extensive literature search revealed only 3 studies involving glowlight tetras. All 3 were behavioural studies and involved glowlight tetra inspection of various chemicals and whether fish could correctly guess chemicals released by possible predators (Brown and Godin, 1999; Brown et al, 1999; Brown et al, 2000). However, there is a plethora of ocular anatomical studies in various other teleosts which has allowed the general description of both the structure of the eye and retina (Nicol, 1989). As the glowlight tetra is a teleost, its structure fits the general description given below (personnel observation).

Lying directly inside the choroid is the retina, which is a projection of the brain. It can be divided into ten layers, eight of which consist of different cells and two

consist of structures which look like membranes under light microscope although they are not. From the sclera to vitreous direction, these layers are; 1, the pigment epithelial layer. 2, the photoreceptor layer. 3, the outer limiting membrane. 4, the outer nuclear layer. 5, the outer plexiform layer. 6, the inner nuclear layer. 7, the inner plexiform layer. 8, The ganglion cell layer. 9, the nerve fibre layer.10, the internal limiting membrane (Figure 1.2).

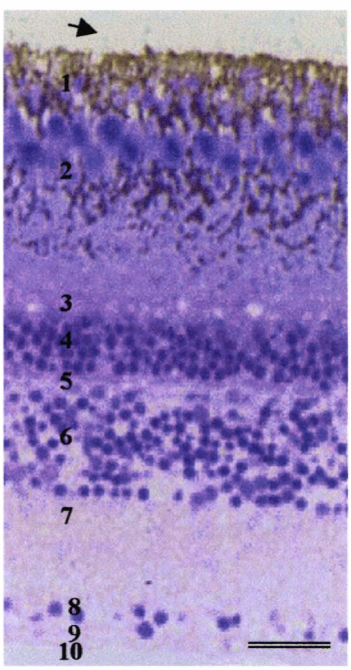

Fig 1.2 Glowlight tetra retina
Transverse section of glowlight tetra retina (Bruch's membrane is missing). Here, the arrow gives the position where Bruch's membrane is normally found;1,pigment epithelial layer; 2, photoreceptor layer; 3, outer limiting membrane; 4, outer nuclear layer; 5,outer plexiform layer; 6, inner nuclear layer; 7, inner plexiform layer; 8, ganglion cell layer; 9, nerve fibre layer; 10, internal limiting membrane. Bar=25μm.

1.2.1 Bruch's membrane

In teleosts, Bruch's membrane is a trilaminar structure. The outer layer is the basement membrane of the choriocapillary endothelium, collagen fibrils make up the medial layer and the inner layer is composed of the basement membrane of the pigment epithelial cells (Braekevelt, 1985a). In most fish, the retina is avascular and metabolic exchange occurs across Bruch's membrane. There are phagocytic

cells between Bruch's membrane and the pigment epithelial cells that are also present at the interface between photoreceptors and epithelial cells (Braekevelt, 1985a).

1.2.2 The pigment epithelial layer

Pigment epithelial cells consist of a basal portion and apical finger-like processes that extend inwards amongst photoreceptors. The basal part is usually hexagonal in shape and forms a regular array. This region also contains the nucleus, mitochondria, tubular endoplasmic reticulum, and lysosomes. There are several kinds of lysosomes which destroy packets of discs derived from the outer segments of photoreceptors (Braekevelt, 1974, 1982, 1985a and b; Collin et al, 1996a and b). Melanin granules, or melanosomes, are located in the pigment cells and are capable of retinomotor movements (see below). During the day, they move inward into the process of the pigment cell and during the night, concentrate in the basal region of the cell (Burnside and Nagle, 1983).

1.2.3 The photoreceptor layer

This layer consists of parts of photoreceptors of which like in most vertebrates there are two types; rods and cones. Situated in this layer are the outer and inner segments of both types of photoreceptors, which are connected by a narrow eccentric-connecting stalk containing a modified cilium. The outer segment consists of a series of lamella sacules, which are a series of closely spaced sacs

that lie parallel to one another and usually perpendicular to the long axis. These sacs, also called discs, are formed by invaginations of the plasma membrane and it is in the membrane of these that the visual pigments are found. In rods, these discs have lost the continuity with the outer plasma membrane and are free floating within the outer segment whereas in cones, the discs retain their continuity with the membrane from which they arise by infolding. Lamellae of the outer segments are continually renewed by new discs being added at the base of the segment and old discs discarded at the tip in discrete packets. In many teleost cones there is also an accessory outer segment which is a ciliary structure arising from the inner segment close to the connecting cilium. It is connected to the outer segment by a series of fine processes and contains fine granular and filamentous material and its function is yet to be established (Engström, 1963a). The inner segment of the photoreceptor is made up of an ellipsoid containing mainly mitochondria and the myoid. The myoid, which is vitread to the ellipsoid, extends to the outer limiting membrane (OLM) and comprises ribosomes, vesicles, rough and smooth endoplasmic reticulum, Golgi bodies, and microtubules (see Nicol, 1989; Wagner, 1990; Locket, 1999 for reviews).

The continuation of the photoreceptors lies outside the photoreceptor layer. The nucleus is situated vitread to the OLM in the outer nuclear layer (see below). Following the nucleus in a vitread direction, is the inner fibre (axon) which contains mainly microtubules and leads to the terminal pedicle in the outer plexiform layer (see below) and contains synaptic vesicles and ribbons (see Nicol, 1989; Wagner, 1990; Locket, 1999 for reviews).

1.2.4 Rods

In general, rods far outnumber cones and are slender with a cylindrical outer segment. However, depending on species, there is some variation in shape and distribution. For example, catfish have a single layer of rods with long stout outer segments while in other species rods are more numerous and have short and thin outer segments. Often, in shallow water species, rods have varying myoid length as well as being staggered in depth, which allows a greater number to be packed into the photoreceptor layer. In many deep –water species, rods are layered regularly in several tiers which is thought to improve sensitivity in dim light (Munk, 1966; Locket, 1980). An accessory rod outer segment has been recognised only in some fish and is much less prominent than in cones. Rod synaptic terminals are spherical and called spherules and unlike cone terminals, they lack basal filaments (Locket, 1999).

1.2.5 Cones

Cones are usually both fewer and bulkier than rods having a shorter and tapering outer segment. There are several kinds of cones such as single, double, triple, and very rarely quadruple as in some cyprinids (Engström, 1963b). Teleosts generally have single and double cones. Double cones consist of two equal or unequal cones. If they are of equal size and indistinguishable morphologically, they are referred to as twin cones whereas when they are not equal, the larger or longer one is referred to as the principle cone and the smaller as the accessory cone. If each

cone of the twin cone contain the same pigment type, they are termed identical twins otherwise they are termed non-identical twins. It is thought that twin cones are formed by the fusion of two single cones (Engström, 1963a and b; Wheeler, 1982). The functional significance of double cones is not well understood. As they are usually associated with animals that inhabit deeper waters (Boehlert, 1978) and code for luminosity rather than colour vision in birds (Maier and Bowmaker, 1993), double cones are thought to enhance sensitivity. There is also circumstantial evidence that double cones are involved in motion detection in both birds (Campenhausen and Kirschfeld, 1998) and fish (Levine and MacNichol, 1982). When there are several cone types present, cones that are longer and more tapering contain longer wavelength pigment than shorter and less tapered cones. Single cones also show morphological variation with some species having long, short and miniature sized cones (Engström, 1963b). The synaptic terminals of cones are bell-shaped, called pedicles, from which basal filaments or telodendria may arise (Wagner, 1990).

In most species it is usual to find that the single and double cones are arranged in a particular order to give a structured mosaic appearance. In a very common mosaic type, displayed by the glowlight tetra, a single cone is surrounded by four double cones giving a square mosaic (Engström, 1963a and b) (Figure 1.3). There are also regional differences in cone patterns over much of the retina. For example, the above square mosaic may only exist in the inferior retina whilst the superior retina contains a different pattern (Ali and Anctil, 1976). Cone patterns may also differ depending on whether the retina is in a state of light or dark adaptation (Kunz, 1980, 1983).

As well as cone patterns, there are regional variations in densities, shapes and proportion of cones which extend to bipolar and ganglion cells. High concentrations of cones will give regions of good acuity or resolution and are known as areae (Ali and Anctil, 1976).

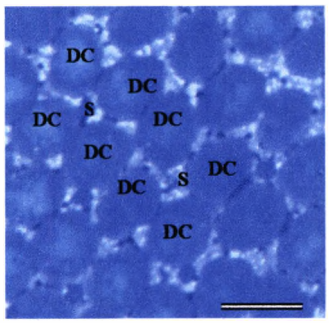

Fig 1.3 Tangential section of glowlight tetra retina

Tangential section of glowlight tetra retina at the cone ellipsoid level showing a cone mosaic where a central single cone (S) is surrounded by 4 double cones (DC). Bar = 25μm.

1.2.6 The outer limiting membrane

The outer limiting membrane is situated slightly scleral to the line of cone nuclei although some cone nuclei can overlie this membrane. It is easily seen and is the dividing line between the myoids and the nuclei of photoreceptors. It is made up of tight junctions between Müller cells and the photoreceptors (Ali and Anctil, 1976).

1.2.7 The outer nuclear layer

This layer consists of the nuclei of photoreceptors where rod nuclei, which are smaller, are located vitread to the larger cone nuclei, which are positioned close to the outer limiting membrane (Nicol, 1989).

1.2.8 The outer plexiform layer

The synaptic terminals of both rods and cones, and processes of both horizontal and bipolar cells which make contact with them, are situated in this layer. Photoreceptor terminals show invaginations and receive processes from both horizontal and bipolar cells at central and lateral regions. For example, in goldfish cone pedicles, most of the central processes come from small bipolar cells while horizontal cells are responsible for the lateral processors (Stell, 1972). Interplexiform cells (see below) form a diffuse pathway in this layer and make synapses with mainly cone type horizontal cells although some synapses with bipolar, cone and rod terminals are seen (Wagner, 1990).

1.2.9 The inner nuclear layer

This is composed of three distinct layers of neural cell bodies and nuclei. The outer layer, the most sclerad, comprises horizontal cells, the middle layer consists of bipolar cells and the inner, most vitread, contains amacrine cells. Amongst the amacrine cells, interplexiform cells are widely distributed. These layers are not

mutually exclusive and some mixing of neurons is seen between layers (Nicol, 1989).

In teleosts, the layer of horizontal cells consists of more than one sublayer of cells depending on the species. For example, in the carp there are two layers (Hidaka et al, 1986), whilst the goldfish (Stell et al, 1975) has three layers of horizontal cells. Horizontal cells are usually flat or cuboidal with large nuclei, smooth fibrillar cytoplasm and generally form a lateral spread of interconnections in the outer plexiform layer (Nicol, 1989). They are classified according to whether they connect with rods or cones. In goldfish and carp for example, there are three types of horizontal cell types that connect with cones and only one type that connect to rods. The goldfish horizontal cells are classed as H1, H2, and H3 in order of increasing diameter of the dendritic tree, decreasing size of cell body and decreasing density of cone contacts. H4 contact only rods. H1, H2, and H3 cells have specific connectivity with different type cones. In the goldfish, H1 cells contact red, blue, and green-sensitive cones. H2 cells only contact green, blue, and UV-sensitive cones while the H3 cells contact blue and UV-sensitive cones (see Djamgoz and Wagner, 1987 for review). Horizontal cells can also be classified according to their electrophysiological responses (Stell and Lightfoot, 1975) (see chapter 7).

Bipolar cells have slender cell bodies, round nuclei and dendrite ramifications that can be elaborate (Sherry and Yazulla, 1993). Teleosts have large and small bipolars. Large bipolars extend scleral to the cone pedicle to synapse with mainly rods but also a few cones and in carp and goldfish these have been termed M

(mixed) bipolars, whereas small bipolars synapse with cones only, do not reach the pedicle and are called C (cone) bipolars (Scholes, 1975). In goldfish, mixed bipolars are further subdivided; subtypes a1 and b1 contact rods and red-sensitve cones while a2, b2, and b3 contact rods and both red- and green-sensitive cones (Stell et al, 1977). Further subdivision is given by the termination of axons in the inner plexiform layer; those that terminate in sublaminar a are called a-bipolars while those that terminate more vitread in sublaminar b are called b-bipolars to give Ma, Mb, Ca, and Cb type bipolars. Nine different cone bipolar types have been identified according to their terminations in either sublaminar a or b, in the goldfish (Sherry and Yazulla, 1993). Functionally, these cells can be further catorgorized according to their response to light, as bipolar cells in sublaminar a give hyperpolarizing responses (known as ON-centre bipolars) and those in sublaminar b give depolarizing response to light stimuli (OFF-centre bipolars) (Stell et al, 1977).

Amacrine cells are larger than bipolar cells, lack axons, and their processes terminate in the inner plexiform layer. Generally, amacrine cells form extensive and wide interconnections within the inner plexiform layer where they have presynaptic and postsynaptic links with bipolars as well as other amacrine cells and presynaptic links to ganglion cells (Wagner and Wagner, 1988 for review).

Interplexiform cells are distributed amongst amacrine cell bodies. Their processes extend into both the outer and inner plexiform layer. Most input to these cells occurs in the inner plexiform layer and the output takes place in the outer plexiform layer and mainly onto cone type horizontal cells (Wagner, 1990).

Interplexiform cells can be classified according to their transmitter; dopaminergic, GABAergic, glycinergic, and serotonergic subtypes exist. Of these, the most prominent are the dopaminergic types which direct most of their output to H1 type horizontal cells and are thought to play a role in light adaptation (Wagner and Wulle, 1992).

1.2.10 The inner plexiform layer

The inner plexiform layer is a dense network of fibres that runs both horizontally and vertically to give an appearance of a meshwork and comprises bipolar axonal cell processes, amacrine cell processes, interplexiform cell processes, and ganglion cell dendrites. Here, the ganglion cell dendrites make contact with both bipolar and amacrine cell processes.

1.2.11 The ganglion cell layer

Ganglion cell bodies are found in this layer. There are several types of ganglion cells, which are classified according to the size of their soma, the size of their dendritic fields and where they terminate in the inner plexiform layer. For example, in the goldfish four types of ganglion cells were distinguished according to the size of their soma and dendritic field and another fifteen types could be further subdivided according to their stratification in the inner plexiform layers (Hitchcock and Easter, 1986). In the zebrafish, 11 different morphological types of ganglion cells could be identified (Mangrum et al, 2002). In light of such

variation in morphological types amongst different species of fish, one would expect there to be several types of ganglion cells in the glowlight tetra also. Ganglion cell dendrites are postsynaptic to amacrine and bipolar cell processes and their output is to the optic nerve (Wheeler, 1982).

1.2.12 The nerve fibre layer

Between the ganglion cell layer and the internal limiting membrane, the ganglion cell axons form a distinct layer known as the nerve fibre layer. These fibres converge in the region of the optic disc to form the optic nerve. In some species, the retinal ganglion cell axons form discrete fascicles that lie within the nerve fibre layer in effect forming multiple optic nerve heads (Herrick, 1941; Ali and Anctil, 1976; Douglas et al, 2002).

1.2.13 The internal limiting membrane

This 'membrane' separates the nerve fibre layer from the vitreous humour and vitreal vessels. It consists of a homogenous hyaline-like substance and the inner ends of the processes of Müller cells.

1.2.14 Müller cells

Müller cells span the retina from the outer limiting membrane to the retinal-vitreo boundary. Their elongated fibres extend vertically to provide a skeleton framework that supports the retina. Their perikarya are situated in the inner nuclear layer (Nicol, 1989). In many avascular teleost retinae, the cell cytoplasm contains numerous mitochondria and their cell processes are rich in glycogen suggesting that Müller cells also have a nutritive function (Nicol, 1989).

1.3 Retinomotor movements

Retinomotor movements are movements of rods, cones, and retinal pigment epithelial (RPE) melanosomes that accompany the diurnal light/dark changes. They are strongest in fish and amphibia, less in reptiles, and birds, and least in mammals. Extensive tables giving the prevalence of retinomotor movements among various vertebrate groups have been constructed (Ali, 1971; Ali and Wagner, 1975). The study of retinomotor movements began well over a century ago and by 1915, Arey (1915) wrote a considerable review on the subject. Since then, interest in retinomotor movements has waned as shown by the relatively few and sporadic publications (for more recent reviews see Burnside and Nagle, 1983; Wagner et al., 1992a; Cahill and Besharse, 1995).

During the day, or light phase, cones constrict their myoids to position the cone ellipsoid close to the outer limiting membrane (OLM). At the same time, rods

elongate their myoids positioning their ellipsoids nearer to Bruch's membrane close to the processes of the RPE cell. While this is occurring, melanosomes within the RPE cells disperse and accumulate vitread in the long apical, microvillus-like projections of the RPE cells. The dispersal of melanosomes shields the rods from light (Figure 1.4). The reverse is seen during night. The cones elongate their myoids to position their ellipsoids closer to Bruch's membrane while the rods constrict their myoids positioning the rod ellipsoids near the outer limiting membrane. Meanwhile, the RPE melanosomes accumulate at the base of the RPE cells (Figure 1.5). In general these changes, either going from light to dark or the reverse, take between 20 – 30 minutes. Other factors such as temperature, developmental stage, stress, and light intensity can all effect this rate (Ali, 1975 for review).

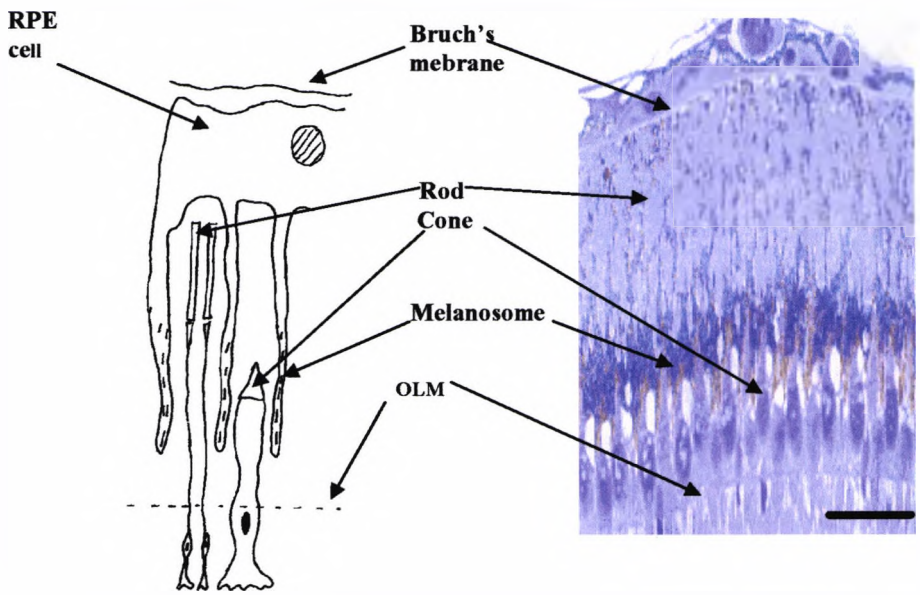

Fig 1.4 Light adapted teleost retina
Diagram and micrograph of a teleost retina showing the position of cones, rods, and RPE melanosomes when light adaptated. In the micrograph, bar = 25μm.

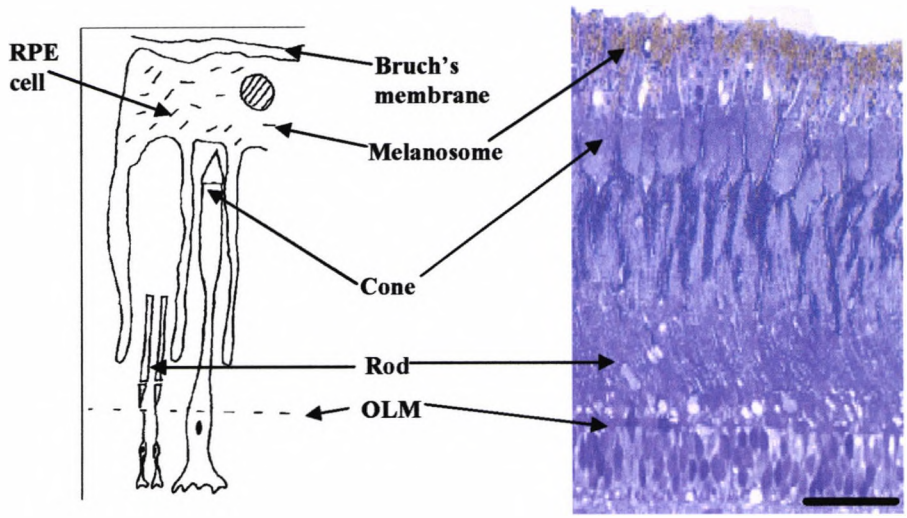

Fig 1.5 Dark adapted teleost retina
Diagram and micrograph of a teleost retina showing the positons of cones, rods, and RPE melanosomes when dark adapted. In the micrograph, bar =25μm.

Very brief flashes of light, as short as 0.0025 seconds, have been found to cause full light adaptive retinomotor movements, indicating that light can easily over-ride the dark signal and once light exposure signals the light adaptation process, the full process will complete irrespective of external light stimuli. Thus, the signal to light adapt is all that is required to trigger the events leading to full light adaptation (Muntz and Richard, 1982; Wagner and Douglas, 1983).

Comparison of the absorption spectra of known photoreceptor pigments with action spectra of retinomotor movements show that in a cichlid fish, the blue acara, rods trigger light adaptive retinomotor movements and that green-sensitive

cones trigger dark adaptative movements (Kirsch et al, 1989; Wagner et al, 1993). Meanwhile, RPE melanosome movements in trout exhibit action spectra, which indicate regulation by a rod pigment (Ali and Crouzy, 1968). In the monochromatic catfish, however, cone contraction is modulated directly by the red-sensitive cones (Douglas and Wagner, 1984).

The function of retinomotor movements has led to much speculation. A popular early idea concerned space utilisation and the creation of two separate functional retinas. During the light phase, the cone ellipsoids are positioned near the OLM and during darkness the rod ellipsoids are positioned near the OLM and thus optimum capture of light by cones during photopic conditions and rods during scotopic conditions will ensue (Herzog, 1905; Exner and Januschke, 1906; von Studnitz, 1952). Another idea that also received much attention was that during the light phase the RPE melanosomes contribute to photopic acuity by situating themselves between cone outer segments and absorbing scattered rays refracted from cone ellipsoids, oil droplets, or paraboloids (Garten, 1907). A more generally accepted argument to the purpose of the RPE melanosome migration is that it provides a movable shield for the rod outer segments which would ensure shielding during bright lights and allow exposure of rods during scotopic conditions (Herzog, 1905; Exner and Januschke, 1906; Back et al., 1965; Douglas, 1982b). It has also been suggested that photoreceptors change length in order to modify their electrical responses (Burnside and Nagle, 1983). As both cones and rods are shortest in light conditions where they are expected to be active, elongation of the photoreceptor might decrease the magnitude of the response

conducted from the outer segment to the synaptic terminal thus shutting off the photoreceptor at the appropriate time of day.

Adult fish that contain both rod and cone photoreceptors first develop cones in young larvae. During this stage of development, cones and RPE melanosomes remain in their fully light adapted positions. Rods appear later and with their appearance come the first signs of retinomotor movements. The absence of RPE movements in rod-free larval retinas is consistent with the suggestion that in adults, retinomotor movements serve to regulate the amount of incident light striking rod outer segments (Herzog, 1905; Exner and Januschke, 1906; Back et al., 1965; Douglas, 1982b) but does not exclude the notion that they may serve to save space.

1.3.1 Mechanisms of retinomotor movements

1.3.1.1 Cone contraction and elongation

Many studies have shown that actin filaments, myosin filaments and microtubules all play a part in retinomotor movements (see Burnside and Nagle, 1983 for review). As the vitread half of the cone is fixed to its cellular neighbours in the outer nuclear layer, shortening of the cone takes place in the scleral half where the cone is free. This 'free' half consists of the outer segment, the ellipsoid, and the myoid region which extends into the space between the RPE and the outer limiting membrane and is where most of the shortening takes place.

Cones contain actin filaments that are longitudinally orientated, arranged in bundles, originate at the base of the outer segments and extend to the myoid region. Fewer filaments can be observed extending around the nucleus to the axon and further into the pedicle (Burnside, 1976 a and b). Thicker myosin filaments are found predominantly in the vitread region of both light and dark adapted cones as well as in the myoid region (Burnside, 1978). Longitudinally orientated microtubules have also been located in the myoid and axon of the cone (Burnside, 1976a; Ferrero et al, 1979).

Cytochalasins inhibit actin polymerization by capping the growing filament end and thus have been used to identify actin-dependent motile processes (Burnside, 1976a). Colcihine disrupts microtubules allowing microtubule-dependent motile processes can be identified. It is thought to do this by binding with free microtubules which then caps the free assembly end and thereby prevents assembly (Burnside and Nagle, 1983).

Using cytochalasins to block the action of actin shows that cone contraction requires actin filaments and it is thought that myosin is also involved (Burnside, 1976a; Warren and Burnside, 1978; O'Connor and Burnside, 1982). Both calcium and ATP are required for cone contraction indicating an actin-myosin mechanism similar to that found in skeletal muscles (Burnside et al, 1983). Further support for an actin-myosin mechanism was shown by using fragments of skeletal muscle myosin to block access of native myosin to cone actin filaments during cone contraction (Porrello et al, 1983). Myosin filaments are thought to interact with actin filaments using a sliding mechanism commonly associated with skeletal

muscles (Burnside, 1978). Actin filaments are organized into two opposing sets (Burnside, 1978). In the 'free' scleral half, filaments have proximally directed arrowheads while in the fixed vitreal half, most actin filament arrowheads are distally, although some are proximally, directed. It has been proposed that in the vitreal half during light, myosin is activated and bridges opposite polarity actin filaments which increases their overlap and thus shortens the cone. During darkness, the myosin cross-bridging could disengage to allow the actin filaments to glide past each other (Burnside, 1978).

Inhibition studies using colchicine show that cone elongation is dependent on microtubules (Warren and Burnside, 1978). Colchicine, however, has no effect on actin filaments or cone contraction, while cytochalasins have no effect on microtubules or cone elongation (Warren and Burnside, 1978; Burnside et al., 1983). Thus, cone elongation requires microtubules and constriction needs actin. The mechanisms of microtubule contribution to cone elongation is yet to be established although one explanation is that elongation occurs via a sliding redistribution of microtubules along the length of the myoid in regions where they closely overlap (Warren and Burnside, 1978).

1.3.1.2 Rod contraction and elongation

As in cones, retinomotor movements in rods are a result of shortening and lengthening of the myoid region. The arrangement of actin filaments is very similar to that seen in cones, although they only extend as far as the myoids at the level of the OLM (O'Connor and Burnside, 1981, 1982; Klyne and Ali, 1980).

Microtubules have also been noted in the myoid region (O'Connor and Burnside, 1981). Microtubule inhibition studies using colchicine have shown that microtubules are not required for rod elongation, while actin inhibition using cytochalasins indicates that actin is important. Elongation is thought to involve actin filament assembly and possibly the cross-linking of these filaments into rigid bundles (O'Connor and Burnside, 1981), while inhibition studies have also been used to deduce that actin-myosin mechanisms are involved in rod contraction as in cone contraction (O'Connor and Burnside, 1982). Although the exact role microtubules play in rod retinomotor movement is not clear, it has been reported that overall microtubule length is greater in the elongated myoid (light adapted) in comparison to the shorter dark adapted myoid, suggesting microtubules are assembled during rod elongation and disassembled during contraction (O'Connor and Burnside, 1982).

1.3.1.3 RPE melanosome movement

In RPE cells, actin filaments are associated with the plasma membrane, the membrane surrounding pigment granules, and the zonula adherens (Burnside, 1976a and b). Using inhibition studies, it was found that actin filaments are required for both light adaptive movements of RPE melanosomes and to maintain melanosomes in their light adapted positions (Burnside et al, 1983). Abundant microtubules have also been noted orientated parallel to the lines of pigment movement in the apical projections of RPE cells. Their role in pigment transport is not well understood but they are required for pigment transport in both directions (Burnside et al, 1983).

1.4 Biological rhythms

Aschoff (1981) describes biological rhythms as "recurrent events in a biological system". That is, biological processes that occur more than once (usually many times) in an organisms lifetime. The rate at which biological rhythms happen varies tremendously. They may take place every fraction of a second as seen in the electrical activity of many neurons in the central nervous system which exhibit a constant rhythm of firing or as little as once a year as in the hibernation of a ground squirrel.

Biological rhythms can be regarded as oscillations or recurring cycles of events which graphically are portrayed as repeating curves in which the biological parameter is plotted against the time of day (Figure 1.6). A cycle, which can be used synonymously with the terms oscillation and rhythm, is formally defined as a sequence of events that repeat themselves through time in the same order and at the same interval. The period of the cycle is the time it takes to complete one full cycle and is usually measured from either peak to peak or trough to trough. The frequency of the cycle is the number of cycles per unit of time (the inverse of the period), while the amplitude of the cycle is the maximum intensity of the response indicated by the height of the peak. The phase of the cycle is an arbitrarily chosen part of the cycle as in the ascending or descending portion of the cycle. It can also be used to describe the positional relationship between two or more cycles. For example, a person waking at dawn and sleeping at around dusk can be said to be in phase with the natural light/dark cycle.

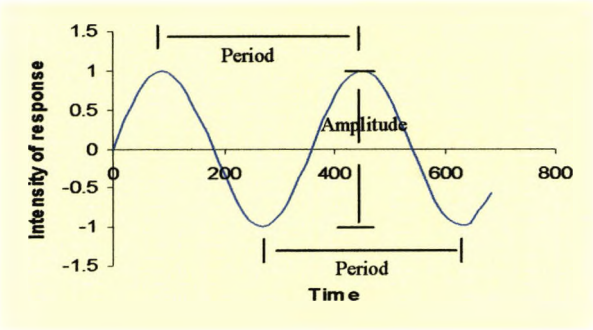

Fig 1-6 Biological cycle
Graphical representation of a typical cycle seen in a biological rhythm

Biological rhythms are usually classified according to their period. Circadian (from the Latin *circa diem,* about a day) rhythms are biological rhythms that occur about once every 24 hours, the most obvious circadian rhythm being the human sleep/wake cycle. Circadian rhythms are widespread in the animal and plant kingdoms, ranging from activity/non activity rhythms in hamsters to the daily movement of leaves in plants. Regular daily oscillations in activity have been recorded under controlled conditions in a variety of animals which include insects (Tomioka et al, 1997), fish (Reynolds et al, 1976), reptiles (Refinetti and Susalka, 1997), birds (Aschoff and von Saint Paul, 1973; Ebihara and Gwinner, 1992), and mammals (Tapp and Natelson, 1989; Kennedy et al, 1994; Refinetti, 1999). Many physiological functions also show daily rhythms in various species including eating and drinking (Plaza et al, 1993; Zucker, 1971), metabolic rate (Anava et al, 2002), heart rate and blood pressure (Smith et al, 1987), urinary excretion (Plaza et al, 1993), blood hormone concentration (Maywood et al, 1993), bioluminescence (Morse et al, 1994), and body temperature (Rashotte et al, 1995; Refinetti and Menaker, 1992; Weinert and Waterhouse, 1998) for example.

Ultradian rhythms are rhythms with periods less than 24 hours and usually less than 18 hours. Heart rate and breathing are the two most obvious ultradian rhythms. An example of an ultradian rhythm with a longer period of about 1 hour

is the production of carbon dioxode by birds and mammals (Stupfel et al, 1995). The several stages involved in sleep repeat themselves every 90 minutes and thus make up a well-known utradian rhythm (Luce, 1971).

Biological rhythms with a period longer than 24 hours and usually longer than 30 hours are known as infradian. Typical infradian rhythms are circalunar and circatidal rhythms. Circalunar rhythms closely follow the different phases of the moon that make up a lunar month, which has a period of 29.5 days.
Adult mayflies (*Povilla adusta*), for example, appear in large numbers in East Africa only around the time of full moon and it is thought that the moon induces a rhythm of emergence from the water, while increased mating occurs during new moon in Javanese frogs (*Rana cancrivora*) and during full moon in Javanese toad (*Bufo melamosticus*) (Cloudsley-Thompson, 1980). The human menstrual cycle is probably the most well known circalunar cycle with an average period of 29days (Refinetti, 2000). However, because there is a variation between both the period length and synchronization between individual women, this may indicate either the artificial lights of modern living have caused the desynchronization or that the menstrual cycle is not a circalunar rhythm.

Circatidal rhythms are typical infradian rhythms that closely follow the movement of the tides. The moon and its different phases affect tides so that circatidal rhythms are partly circalunar. The fiddler crab has bursts of activity at the time of low tide (Palmer, 1991) while the flatworm, *Convoluta roscofensis*, migrates to the surface of the sand during the day-time low tides and then disappears into the sand as the tide rises and also at night (Saunders, 1977).

Beside circalunar and circatidal rhythms, there are infradian rhythms with even longer periods such as circannual rhythms which have periods of about one year. A common example is reproduction in many species. In both hamsters and voles, the gonads regress during the winter making these animals unable to reproduce (Gaston and Menaker, 1967; Dark et al, 1983). Canadian beavers remain inactive inside their lodges during the winter even though they do not hibernate (Refinetti, 2000).

1.5 Biological clocks

The obvious question is what is actually driving such biological rhythms. Are they merely a response to a variation in the external environment, such as the changes in ambient lighting as one goes from daylight to night or are they the result of an internal signal (a 'clock') which directs the organism to change behaviour?

The existence of biological clocks governing rhythmic function can be shown by placing an organism under constant conditions, with no change in the external environment, and looking for the persistence of the rhythm. Rhythms that continue in the laboratory under constant conditions are called free-running. Early proponents of the exogenous theory claimed that it was impossible to have constant conditions, as there would always be hidden external forces (the magnetic or electric field for example) driving the rhythm. In 1729, a Frenchman named de Mairan (1729) found that plants placed in constant darkness continued their daily rhythm of leaf movement. A century later, de Candolle (1832)

measured the period in continuous darkness of leaf movement in *Mimosa* to be not exactly 24 hours. Over one hundred years later, Erwin Bünning (1930) determined that the leaf movements of the common bean *Phaseolus* oscillated with a period of 25.4 hours in constant darkness. He was the first to understand the significance of this; the period of the free-run was different to 24 hours seen in natural conditions.

The free-running period has been determined in unicellular organisms (Roennenberg and Morse, 1993), mollusks (Page et al, 1997), crustaceans (Palmer, 1976), insects (White et al, 1992), reptiles (Refinetti and Susalka, 1997), birds (Oshima et al, 1987), and mammals (Zucker, 1971, Maywood et al, 1993, Kennedy et al, 1994) including humans (Pollak and Wagner, 1994). In all cases, it was found to differ from 24 hours being either greater or less depending on the species being examined. This is strong evidence for a biological clock as all the hidden external forces such as magnetic and electric fields must oscillate with a period of 24 hours, the Earth's period of revolution. Further experiments to remove any other possible external force have been undertaken by placing humans in either underground bunkers or in space (Colin et al, 1968; Gundel et al, 1997) and the free-running period was still found to differ from 24 hours.

As the free-running period is usually close to, but not exactly, 24 hours, the term 'circadian' (about a day; see above) is usually used. This term applies to rhythms with a period of about 24 hours that persist in constant conditions and should be distinguished from those rhythms observed in natural conditions that are extinguished in constant conditions and termed 'diel' (occurring every 24 hours).

Although circalunar, circatidal, and circannual rhythms all show free-running under constant conditions, most research has been focussed on understanding circadian clocks, and to a lesser extent ultradian clocks, as examples of these are most abundant and usually easier to observe than rhythms with longer period.

1.5.1 Properties of a biological clock

Biological clocks have the following properties;

1/ they all show the ability to free-run as described above.

2/ they are temperature compensated as no clock is of any use if it varies with temperature. Man-made clocks incorporate built in mechanisms to compensate for temperature changes and the same situation applies in nature. This is quite unusual as most biological processes are temperature sensitive with their rates doubling or tripling with every 10°C rise in temperature, while for the same change in temperature, biological clocks will remain almost constant (Sweeney and Hasting, 1960).

3/ they can be 'entrained'. Biological clocks can usually be reset by external environmental cues. Aschoff (1981) named these *Zeitgeber*s, (German for "time giver"). The most common Zeitgeber is light. A Zeitgeber can entrain the biological rhythm to maintain a constant relationship between the two (Johnson et al, 2003). In the absence of day-night cycles, daily temperature changes and social cues can also act as Zeitgebers.

4/ all biological clocks are under genetic control. By changing certain clock genes, the free-running period can be altered (Ralph and Menaker, 1988).

1.5.2 Clock model

A simplified model of a biological clock can be constructed using only three components;

1/ a detector is required to receive the information given by the Zeitgeber.

2/ a central oscillator which generates its own rhythm. Information from the Zeitgeber for entrainment is passed onto the oscillator from the detector.

3/ outputs which are a translation of the oscillator generated rhythm into overt rhythms (Fig 1.7).

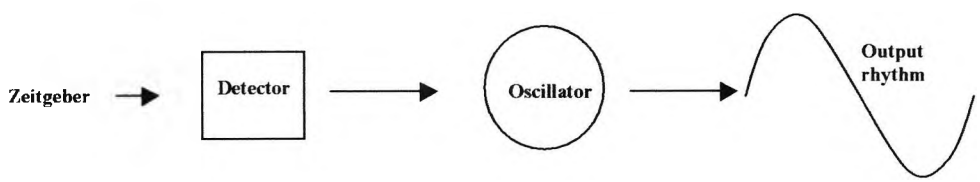

Fig 1-7 Clock model
Diagram showing Zeitgeber and a simplified model of a clock consisting of detector, oscillator and overt output rhythm.

1.6 Entrainment

Any biological clock governing a circadian rhythm must be able to be reset by the external environment or Zeitgeber (entrainment). If a biological clock with a free-running period τ is to be entrained by the Zeitgeber with a period T, then the free-running period must be changed to match that of the Zeitgeber. The amount of time that needs to be adjusted each day is given by the equation

$$\Delta\Phi = \tau - T \quad \text{(Pittendrigh, 1981)}$$

where $\Delta\Phi$ denotes the shift in the biological clock mechanism (phase shift of the circadian rhythm being expressed). Thus, if the period of the clock is 23.3 hours (a fast clock), it will need to be delayed by 42 minutes ($\Delta\Phi$ =-0.7 hours) each day.

There are two ways of doing this; either make the clock run faster or slower or by resetting the clock daily. Changing the speed of the clock is called the 'parametric' or 'continuous' model of entrainment. The underlying assumption is that the magnitude of the Zeitgeber has a proportional and continual effect on the biological clock. Aschoff (1960) proposed that acceleration and deceleration of the free running period by daily changes in the light intensity could allow the biological clock to adjust its period continuously to that of the Zeitgeber.

Daily resetting of the clock, known as the 'non-parametric' or 'discrete' model, constitutes a more successful theory for the entrainment of some organisms (Pittendrigh and Menis, 1964; Pittendrigh and Daan, 1976a, b, and c; Pittendrigh, 1981). Here, the Zeitgeber, the light/dark cycle consists of repetitive light pulses. The basic premise is that an entrained biological clock is in equilibrium with the light/dark cycle when each pulse falls at that phase in which it elicits a phase shift that is equal to the difference between τ (free running period) and T (Zeitgeber period). In nature, the Zeitgebers are the dawn and dusk transitions and as these are discrete time cues, the model has been named such. In the laboratory, dawn and dusk can be substituted by pulses of light (see below) (Johnson, 1999).

1.6.1 Transients

Immediately after exposure to a new light schedule the biological rhythm will undergo temporary changes. These are usually phase advances or phase delays and cause lengthening or shortening of the rhythm. These temporary changes are called transients and usually last for several days before the rhythm becomes stable (Palmer, 1976).

1.6.2 After-effects

The free-running period of a circadian rhythm can be affected by the history of the light/dark cycle to which the animal has been exposed. Entrainment to light/dark cycles with different periods can change the free-running period of the biological rhythm and the resulting alterations in the period length are called after-effects (Daan and Pittendrigh 1976b). For example, an animal that is entrained to a light/dark cycle with a period of 21.5 hours will have a shorter free-running period than an animal entrained to a 24-hour day. After-effects are temporary but can last for many months before disappearing.

The length of the photoperiod can also affect the length of the free-running period. In sparrows, the free-running period of locomotor activity in constant darkness following entrainment to a LD 8:16 cycle was found to be greater than following entrainment to a LD16:8 cycle (Binkley and Mosher, 1986).

1.7 Phase response curves

Biological clocks are not equally sensitive to Zeitgeber time–cues during the 24-hour day, with light stimuli causing more of a phase shift at some time points than at others. Pittendrigh (Pittendrigh and Bruce, 1957, 1959; Bruce and Pittendrigh, 1958; Pittendrigh and Minis, 1964;Pittendrigh and Daan, 1976 a-c) found that animals maintained in constant darkness could have their activity rhythm phase shifted consistently by using brief pulses of light (either one hour or 15 minutes duration). As the rhythm expresses the state of the biological clock, the phase shift in the rhythm represents a resetting of the clock and the magnitude and the direction of this phase shift depends on the time (relative to the animal's clock) that this pulse is given. Thus, for each animal examined, the effect of light pulses given at various times around a 24-hour period will cause either a phase delay, phase advance, or have no effect. This can be shown graphically by a 'phase response curve' (PRC) (Figure 1.8).

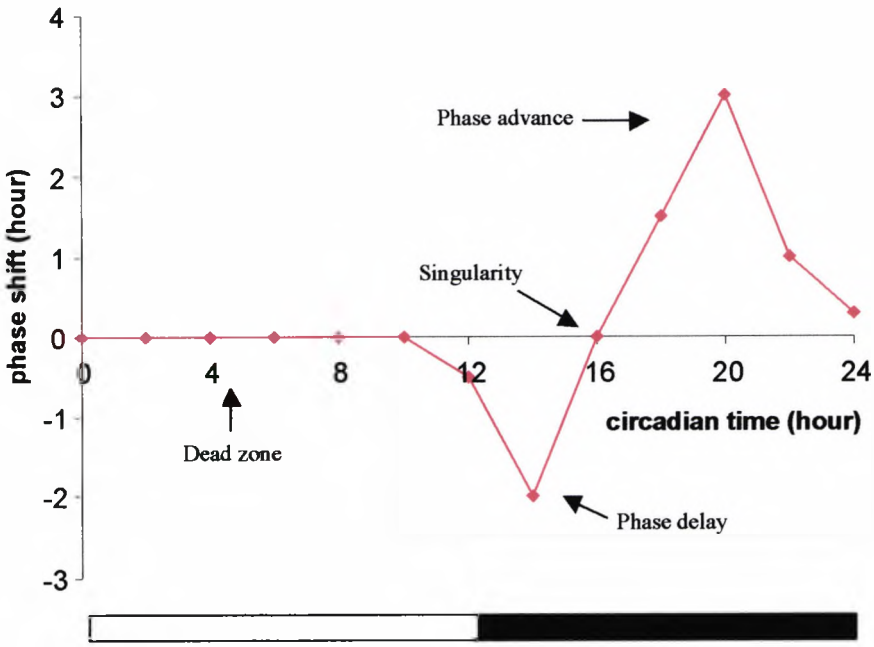

Fig 1-8 Diagram of a typical phase response curve

Diagram showing a typical phase response curve (PRC) in response to a pulse of light. A brief pulse of light given at various times throughout the day will cause phase shifts of the biological rhythm depending on what time of the day the pulse is given. Here, a pulse of light given at circadian time 14 (ct14; see below*) will cause a phase delay of 2 hours while a pulse of light given at ct20 will advance the biological rhythm by 3 hours. The 'dead zone' is so called because a pulse of light given here will neither advance nor delay the rhythm. * Circadian time gives the internal clock time of the species being investigated. 1 circadian hour is given by the period of the rhythm being investigated divided by 24 ($\tau/24$).

PRC curves have been determined for many species (DeCoursey, 1960; Daan and Pittendrigh, 1976a and b; Summer et al, 1984; Rosenberg et al, 1991; Sharma, 1996) and found to differ mainly in amplitude and timing, yet share common elements. Generally, delay phase shifts occur when the light pulse is given early in the subjective night (that is, the time in the animal's clock that would normally correspond to darkness if a light-dark cycle were present) and around dusk. When

a light pulse is given late in the subjective night at around dawn, an advance phase shift will occur. A 'dead zone' is where little or no phase shifting takes place and usually occurs during the middle of the subjective day. The 'dead zone' may be the result of a change in sensitivity of the biological clock or represent a decrease in the sensitivity of detecting the light pulse (Pittendrigh, 1981). Little or no phase shifting also occurs around the middle of the subjective night in an area called the 'singularity'. There is some confusion to the exact reason for the lack of phase shifting seen at the 'singularity'. One popular idea is that it is due to a change from delay phase shifts to advance phase shifts while another explanation is that light is causing the oscillator to stop (Figure 1.7).

Lifestyle differences can explain variations in the PRC. Mostly, nocturnal animals tend to have longer delaying portions as they usually have free-running periods less than 24 hours, which indicates a fast clock that needs to be delayed daily for entrainment to occur. Similarly, diurnal animals tend to free-run with periods longer than 24 hours, indicating their clock is running slow, which would require daily advancing of the clock and thus explains the larger portion of advance seen in diurnal animals' PRCs (Pittendrigh, 1981).

PRC curves can be subdivided into 'type 1' and 'type 0'. Type 1 usually displays relatively small phase shifts and has a continuous transition between delays and advances, whereas a type 0 PRC shows large phase shifts (Johnson, 1999 for review). PRC curves can also be constructed using dark pulses on animals kept in constant light. This reverses the pattern seen in the light and is referred to as 'mirror image' (Johnson et al, 1989).

1.7.1 Phase response curves and entrainment

Phase response curves are used to understand the mechanism by which the biological clock is entrained to the light Zeitgeber on a daily basis (Pittendrigh, 1981). This is best explained by the following example. An animal with a free-running period of 23.3 hours must entrain to the natural 24-hour light/dark cycle. As

$$\Delta\Phi = \tau - T \text{ (see above)},$$

to entrain to the Zeitgeber, the animal must delay its clock each day by 42 minutes (23.3 – 24 = -0.7 hours). Examination of the PRC in Figure 1.9 shows that a light pulse given at CT13 can achieve this (a light pulse at CT15 would also give this delay but at this point entrainment is unstable as it is too close to the advance portion of the PRC). Therefore, entrainment in this situation can occur by giving a pulse of light each day at CT13, which would cause the 42-minute delay required. If a light pulse were given every 24 hours, the animal's rhythm would advance, delay, or remain unaffected until the light pulse hit the exact 42- minute delay point required for entrainment.

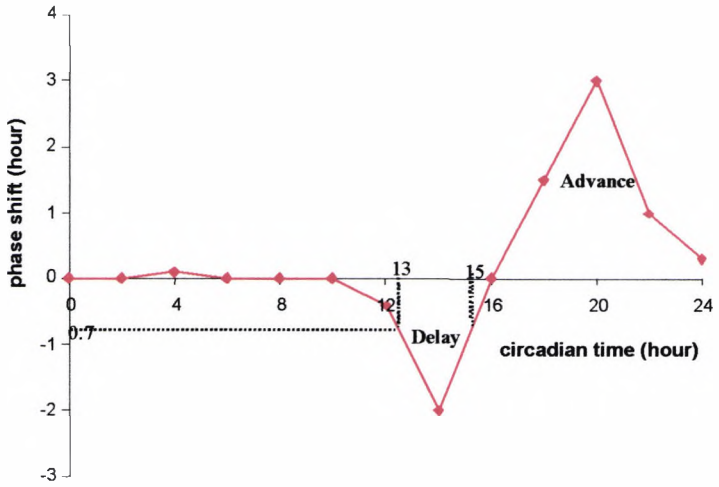

Fig 1-9 Effect of a light pulse

PRC showing that a pulse of light must be given at ct = 13 to delay the phase by 0.7 hours.

Pittendrigh (1965) successfully applied such PRCs to the eclosion activity rhythm of *Drosophilia*. Many subsequent experiments have confirmed that a single short light pulse is all that is required for entrainment (Rosenwasser et al, 1983; Joshi and Chandrashekaran, 1985; Kennedy et al, 1989; Minors et al, 1991; Boulos et al, 1996; Sharma and Chandrashekaran, 1997; Wei and Lee, 2001).When a full light/dark cycle is present, the above mechanism can still be used to understand entrainment. The extra light is considered to be unnecessary and will be placed in the 'dead' zone. (The position of the 'dead' zone varies according to the length of the free-running period). However, parts of the phase 'delay' and phase 'advance' portions of the curve will be stimulated. The overall effect will be given by determining the summation of the areas under the graph, which have been stimulated by the light.

The amplitude of the PRC gives the range of period that the organism can entrain to, which in most animals and plants, is between 18-30 hours (Brady, 1979) and outside of this range, the organisms will free run. Sometimes a smaller period may appear to entrain the organism. For example, a 12-hour period consisting of 6 hours of light and 6 hours of dark (LD 6.6) will not affect the organism, as it will remain entrained to a 24-hour period. This is called 'frequency demultiplication and occurs when the Zeitgeber has a period that occurs 2 or 3 times every 24 hours (Pittendrigh, 1981).

PRCs have also been used for other purposes such as to study the photopigments involved in setting the biological clock (Thresher et al, 1998) and also to determine the effects of chemicals on the biological clock (Inouye et al, 1988). One explanation of why circadian rhythms have a period that is not exactly 24 hours relies on using PRC's. Pittendrigh and Daan (1976b) showed that if τ (free-running period) and T (Zeitgeber period) are not equal, then there will only be one point on the PRC that can give stable entrainment. That is, the biological clock will always have a specific phase relationship to the environment.

1.8 Clock mechanisms

A biochemical loop can form the simplest biological oscillator. This is a series of biochemical reactions that repeats itself at a constant rate. For example, if structure X produced substance Y, and substance Y feedbacks on structure X to inhibit its own production, then a biological oscillator will be established (Figure

1.9). The period of this oscillator will be the time required to produce enough of Y to reach its threshold for acting back on X, plus the time needed for Y to be metabolized to concentrations that are below threshold. This period must of course be around 24 hours and these reactions must be independent of temperature. To fulfill the requirements of a clock, this oscillator must also be capable of Zeitgeber entrainment and an overt rhythm must be measurable.

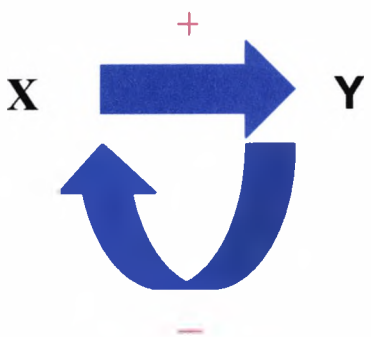

Fig 1-10 A biochemical loop that could form the basis of a biological oscillator
Diagram showing a simple biochemical loop where substance X produces Y which in turn feedsback on X inhibiting its production and thus forming a simple biological oscillator.

Genetic studies on the fruitfly *Drosophila* have revealed a molecular clock using such a biochemical loop. A single gene has been discovered which controls the period of free-running in *Drosophilia* and has therefore been called the *period* (*per*) gene (Konopka and Benzer, 1971). The protein product of the *per* gene, the protein PER, has been localized in many different tissues of the fly's body and initially found to be produced rhythmically in the brain (lateral neurons) and eyes (Siwicki et al, 1988), although it was later shown to also be produced rhythmically in nearly all tissues of the fly's body (Plautz et al, 1997). A

biochemical loop is thought to operate where PER inhibits its own production (Hardin et al, 1992). The *per* gene transcribes *per* mRNA which allows the production of PER protein. In the cytoplasm, the levels of PER increase until the protein enters the nucleus where it inhibits the production of further *per* mRNA by binding to the promoter region of the *per* gene. As the levels of *per* RNA are now being reduced, the levels of PER protein will be reduced until all PER production has ceased. After levels of PER protein in the cytoplasm have declined significantly, the *per* gene will no longer be inhibited and transcription of mRNA will commence for another cycle (Hardin et al, 1992).

Although such a simple biochemical loop involving the *per* gene and its protein product explains how the oscillator may function, there is no explanation for either entrainment by the Zeitgeber or how this oscillation would give a period of 24 hours (transcription, translation and movement into the nucleus usually only take minutes). It is now known that there are more clock genes involved although a biochemical loop is still operating. A different single gene, *timeless (tim)* (and its protein product TIM) which controls the free-running period in *Drosophila,* acting in a similar manner to *per*, has also been discovered (Seghal et al, 1994). After transcription, PER and TIM bind in the cytoplasm to form a PER/TIM complex before entering the nucleus to suppress the production of *per* mRNA and *tim* mRNA. As TIM protein is stable in darkness and very rapidly degenerates in light, it is thought to play a role in receiving the Zeitgeber signal to reset the clock (Seghal et al, 1995). Another clock gene, *cryptochrome (cry)* is thought to be involved in the mechanism which causes TIM to degenerate rapidly in light possibly by acting as a photopigment (Emery et al, 1998).

Neither PER nor TIM nor PER/TIM can bind to DNA to inhibit the transcription of *per* and *tim* mRNA. Other genes *clock* and *cycle (cyc)* and their protein products CLOCK and CYCLE (CYC) play a role in the inhibition of *per* and *tim* mRNA transcription (Lee et al, 1998). CLOCK and CYC bind together to form a complex which binds to the promoter regions of both the *per* and *tim* genes and activates the production of both PER and TIM. When the resultant PER/TIM complexes begin entering the nucleus, these bind to the CLOCK/CYC complexes to inhibit the transcription of both *per* and *tim* genes (Lee et al, 1998). Thus, a biochemical loop operates where PER/TIM indirectly inhibit their own transcription by inhibiting CLOCK/CYC which in turn inhibits the transcription of *per* and *tim*. This process needs to be delayed to give a period oscillation of about 24 hours and the gene *doubletime* (*dbt*) contributes to this delay by phosphorylation of free PER in the cytoplasm causing it to be degraded (Kloss et al, 1998).

Clock genes have also been identified in the unicellular algae *Chalmydomonas* (Mittag and Wagner, 2003), the fungus *Neurospora* (McClung et al, 1989) and in mammals (Ralph and Menaker, 1988; Vitaterna et al, 1994). As the clock mechanism from insects to mammals has remained conserved, orthologous mammalian genes to *Drosophila* have been found; 3 mammalian Period genes (*mPer1, mPer2, mPer3*) (Albrecht et al, 1997), *Clock* (Antoch et al, 1997), BMAL1 which is the mammalian equivalent of *cyc* (Honma et al, 1998), 2 Cryptochrome genes (mCry1 and mCry2) (Kume et al, 1999), and Casein Kinase I epsilon (CKI E) which is the equivalent to *dbt* (Vielhaber et al, 2000).

1.9 The Suprachaismatic nuclei (SCN)

In mammals, a "master" clock that drives locomotor rhythms resides in the suprachiasmatic nuclei (SCN), a distinct group of about 10,000 cells located in the hypothalamus of the brain. Complete destruction of the SCN destroys any circadian rhythms (Stephen and Zucker, 1972; Moore and Eichler, 1972; Stetson and Watson-Whitmyre, 1976; Ruby et al, 1989; Warren et al, 1994). SCN tissue has been found to show rhythmicity both *in vivo* and *in vitro* (Kurumiya and Kawamura, 1988; Satinoff et al, 1993). Transplanted SCN grafts restore rhythmicity following previous destruction of the SCN and the resulting period of the rhythm is dependent on the donor SCN not the recipient (Ralph et al, 1990;Saitoh et al, 1991; Sollars et al, 1995). *In vitro* studies of the electrical activity of single cells in SCN slices or cell cultures from rodent brain have shown that different cells have different periods of oscillation and that the overall period of oscillation generated by the SCN is the average of the period of the various cells (Welsh et al, 1995; Liu et al, 1997; Honma et al, 1998; Herzog et al, 1998). In each cell of the SCN, mammalian clock genes form biochemical loops to generate an intrinsic rhythm to the SCN cell with a period determined by the gene *Clock* (Herzog et al, 1998).

The SCN receives photic information from the retina of both eyes. Thus, the circadian rhythm of hamsters that have had their eyes removed is not affected by light stimuli (Foster et al, 1991; Freedman et al, 1999; Yamazaki et al, 1999). Recent evidence suggests that this information is not mediated by either rods or cones. Mice that are totally blind because of total loss of rods and cones show changes in circadian rhythm in response to light (Foster et al, 1991) and similarly,

transgenic mice with total lack of rods and cones also show a circadian response to light (Lucas et al, 1999; Freedman et al, 1999; Lucas et al, 2001) suggesting a novel photoreceptor and photopigment other than that found in rods and cones being used for generating the signal to the SCN.

There are three major pathways that take information to the SCN. The best known is the retino-hypothalamic tract (RHT), a fibre bundle that connects a small population of retinal ganglion cells directly to the SCN. In the mouse, only 0.1% of ganglion cells project to the SCN (Provencio et al, 1998). Usually, but not always, the RHT fibres project to the ventrolateral section of the SCN (Moore and Lenn, 1972; Moore, 1973). Following lesions to the hamster RHT, circadian rhythms could no longer be reset by light, yet in constant darkness the rhythms continued to free-run, suggesting that the RHT is responsible for conveying the circadian information from the eyes to the SCN (Johnson et al, 1988). The geniculo-hypothalamic tract (GHT) is an indirect afferent pathway that carries photic information from the lateral geniculate nucleus of the thalamus to the hypothalamus. It projects from the intergeniculate leaflet of the thalamus to the ventrolateral section of the SCN (Pickard, 1982) and is thought to play an auxiliary role in entrainment to the light-dark Zeitgeber as well as having a possible role in entrainment mediated by non-photic stimuli such as motor activity (Harrington and Rusak, 1988; Rusak et al, 1989). The third major afferent pathway is the raphe-hypothalamic tract which projects from the raphe nuclei to various parts of the brain including the SCN (Moore et al, 1978) and may also be involved in entrainment modulating the SCN response to non-photic stimuli (Mintz et al, 1997; Mistlberger et al, 1998). Three additional input pathways to the

rat SCN have also been recorded although their functions are unknown (Moga and Moore, 1997).

There are several efferent projections from the SCN with the main ones being ipsilateral and contralateral projections within the nuclei themselves (Moore, 1996). Another major projection is to the subparaventricular zone of the anterior hypothalamus from which there are projections to the superior cervical ganglion sympathetic neurons which in turn innervate the pineal gland (Moore, 1996). Via this pathway, the SCN regulate the circadian rhythm of pineal secretion of melatonin (see below). Other efferent pathways from the SCN include projections to the anterior hypothalamus, the thalamus, the lateral and dorsal medial hypothalamus, the IGL, the stria terminalis and lateral septal nucleus (Moore, 1996).

1.10 The pineal gland and melatonin

In mammals, the pineal gland is under direct control of the SCN and is not an independent oscillator. However, in birds, reptiles, amphibia and fish, the pineal gland also appears to contain an additional "master" clock that controls the rhythms of locomotor activity. In the house sparrow, pinealectomy abolishes circadian rhythms of locomotor activity and body temperature in animals kept in constant darkness (Gaston and Menaker, 1968). After transferal of donor pineal to a host house sparrow, the period of the resultant behavioural rhythms matched that of the donor sparrow, showing that the pineal does indeed contain its own clock (Zimmerman and Menaker, 1979). The degree to which the pineal controls

circadian rhythms varies not only between different classes of vertebrates, but also within closely related species such as birds. In comparison to the house sparrows, in pigeon (Chabot and Menaker, 1992) and quail (Underwood, 1994) pinealoctomy and removal of both eyes is required to eliminate circadian rhymicity. In lizards, pinealectomy will either abolish the rhythm, modify the period of the rhythm, cause splitting into several rhythmic components or do nothing depending on the species being examined (Underwood, 1977; Underwood, 1983; Janik and Menaker, 1990). Examinations of fish have revealed similar results. In the lamprey (*Lampetra japonica*) (Tabeta, 1986; Morita et al, 1992) and the catfish (*Heteropneustes fossilis*) (Garg and Sundararaj, 1986) pinealectomy causes breakdown of circadian rhythms whereas removal of the pineal organ altered the free-running period in the lake chub (*Couesius plumbeus*) (Kavaliers, 1979) and caused splitting of free-running activities in the white sucker (*Catostomus commersoni*) (Kavaliers, 1981).

The hormone melatonin (*N*-acetyl-5-methoxytryptamine) is the major product of the pineal gland. The first step in the biosynthesis of melatonin is the conversion of trytophane to 5-hydroxytrptophane involving the enzyme tryptophane hydroxylase (TPOH). Next, 5-hydroxytrophane is converted to serotonin via the enzyme amino acid decarboxylase. Serotonin is then converted to melatonin via the action of two enzymes. Arylalkylamine *N*-acetyltransferase (AA-NAT) converts serotonin to *N*-acetylserotonin and the second enzyme, hydroxyindole-O-methyltransferase (HIOMT), methylates *N*-acetylserotonin to produce melatonin (Dubocovich, 1988). In mammals, two melatonin receptor subtypes have been isolated, cloned and sequenced (Mel_{1A} and Mel_{1B}) while in many non-mammalian

vertebrates an additional third subtype Mel$_{1C}$ exists (Liu et al, 1995; Reppert et al, 1995; Reppert, 1997). All receptor subtypes are coupled to an inhibitory G-protein, reduce adenylate cyclase activity and also appear to be pharmacologically very similar.

Melatonin is rhythmically secreted from the pineal organ into the bloodstream under direct control of the SCN in mammals in contrast to lower vertebrates where its rhythmic release is under the guidance of the pineal clock (Cassone et al, 1993). It is also produced rhythmically in all vertebrate retinae (see below). In culture, isolated non-mammalian pineal organs maintain a rhythm in melatonin release as seen in birds (Binkley et al, 1978), lizards (Menaker and Wisner, 1983), and fish (Cahill, 1996; 1997; Falcon et al, 1989; Gern and Greenhouse, 1988). Studies on two closely related species of salmonids, but occupying different habitats, revealed one species to have a clock controlled rhythm in pineal melatonin production while in the other melatonin production remained level in constant darkness (Iigo et al, 1997c). Thus, it was proposed that ecological niches as well as phylogenic differences are important in the circadian regulation of pineal melatonin.

In non-mammals, the pineal gland contains its own photoreceptors and is situated near the surface of the brain where light can reach it after passing through the skull and skin (Falcõn, 1999). Some fish contain a semi-transparent window to allow the passage of even more light (Meissl and Brandstätter, 1992). There is strong evidence that the pineal photoreceptors are responsible for the production of melatonin (see Falcon, 1999 for review). Pineal studies using mammals (Klein

and Weller, 1970), bird (Binkley et al, 1977; 1978) and fish (Falcon et al, 1987) have demonstrated that the rhythm in melatonin biosynthesis is due to the activity of AA-NAT, whereas the activity of HIOMT remains constant throughout a light/dark cycle in fish (Falcon et al, 1987) although slightly different activity levels between night and daytime have been seen in birds (Binkley et al, 1975).

Individual pineal photoreceptors show a diurnal and circadian rhythm of melatonin production in both the lizard pineal (*Anolis carolinensis*) (Pickard and Tang, 1993; 1994) and the pike (*Esox lucius*) (Boillet et al, 1997) indicating that individual pineal photoreceptors contain their own clock. In constant darkness, the rhythm of melatonin production in isolated pineal glands eventually dampens and the time of damping is species dependant (Bolliet et al, 1994; Murakami et al, 1994) and this in turn reflects the strength of the oscillator (Murakami et al, 1994). Damping is thought to be due to desynchronization between individual pineal photoreceptor cells and melatonin may act as a coupling device among the population of photoreceptor cells (Bolliet et al, 1997).

Melatonin is thought to be the signal of darkness and plays a role in time-keeping in all vertebrates studied (Arendt and Deacon, 1997; Armstrong, 1989; Cassone et al, 1993). In nearly all species, melatonin levels are high during the night and low during the day and this rhythm of secretion is entrained primarily by light (Arendt, 2003). The duration of night-time melatonin release is also thought to play a role in circadian adjustments to seasonal daylength changes (Arendt, 2003) (see chapter 5).

Several studies have shown the effects of melatonin on circadian rhythms. Destruction of the pineal gland and removal of the eyes in pigeons destroys circadian rhythmicity which is restored with daily doses of melatonin (Chabot and Menaker, 1992). In mammals, the SCN contain the majority of melatonin receptors (Reppert and Weaver, 1994; Weaver and Reppert, 1996) and thus the driven rhythm of pineal melatonin secretion feeds back on the oscillator. Melatonin injections entrain circadian rhythms in rats (Redman and Armstrong, 1988) and this effect can be eliminated by lesions to the SCN (Cassone et al, 1986). Similarly, melatonin can phase shift circadian rhythms in humans (Lewy et al, 1980), sheep (Wood et al, 1989), birds (Heigl and Gwinner, 1995) and lizards (Underwood and Harless, 1985). Phase shifts caused by melatonin occur in a "dark-pulse" pattern, that is, opposite to that produced by a light pulse (Dawson and Armstrong, 1996). Thus, rats that have had their pineal gland removed phase shift more rapidly to new light cycles than do animals with intact pineals (Armstrong and Redman, 1985). Melatonin adminstered in constant darkness has less effect than when administered to rats during a normal light/dark schedule or in constant light (Redman and Armstrong, 1988).

1.11 Retinal and peripheral clocks

As well as the 'master clocks' in the SCN of mammals and the pineal gland in non-mammalian vertebrates, clocks are also distributed throughout vertebrate bodies (Whitmore et al, 1998; Yamazaki et al, 2000). These clocks are termed 'peripheral' clocks and their functions are poorly understood. After the discovery of clock genes, they were found in locations other than the SCN or pineal gland

with their corresponding mRNA products showing circadian rhymicity in a variety of tissues including liver, muscle, kidney and lung (Reppert and Weaver, 2001 for review). In the zebrafish, *Clock* oscillation was found in the pineal gland as well as the eye, kidney, heart and other peripheral tissue (Whitmore et al, 1998). Peripheral clocks are dependent on the central clock, although they can be decoupled from the central clock to operate independently for a short period of time under certain conditions such as restricted feeding (Damiola et al, 2000). Unlike the SCN or pineal organ, peripheral clocks show damping very rapidly when placed in isolation (Whitmore et al, 1998; Yamazaki et al, 2000).

The vertebrate retina is an example of such a peripheral clock. However, unlike other peripheral tissues, retinal rhythms do not dampen rapidly when isolated. In vertebrate retinae, several rhythms can be distinguished at the physiological, cellular and molecular levels that are regulated by local circadian clocks. Circadian control of these rhythms is thought to allow the retina to anticipate the large difference in illumination between day and night (more than 10 log units).

Using both electrophysiological and psychophysical methods, it has been shown that visual sensitivity in a number of species is under circadian control. Behaviourly measured visual sensitivity in fish showed peak values at dusk (Bassi and Powers, 1987), while for humans kept in constant dark the absolute visual threshold decreases by about 20% during the subjective night (Bassi and Powers, 1986). In rats the ability to detect flashes of light also changes with the time of day and this sensistivity variation to flashes continues in constant darkness (Rosenwasser et al, 1979). Circadian rhythms have also been described for the b-

wave component of the electroretinogram (ERG) in humans with peak values occurring at noon (Nozaki et al, 1983; Hankins et al, 1998) and fish (Dearry and Barlow, 1987) where values peaked during the night.

Photoreceptor disc shedding is also under control of a circadian clock. The outer segments of photoreceptors are renewed continuously by the addition of new membrane discs at the base and shedding of old discs at the tips in discrete packets (see section 1.2.3). Rod disc shedding usually occurs in the morning soon after light onset while in some species cone shedding also occurs in the morning and in other species it occurs after dusk (Besharse, 1982 for review). In goldfish, two peaks of disc shedding were found; one at the beginning of the dark phase due to the cones and a second peak at the beginning of the light phase attributed to rods (O'Day and Young, 1978). Rod, but not cone, disc shedding has been shown to be under clock control as rat rod photoreceptor disc shedding persists in constant darkness (La Vail, 1976; 1980).

One of the most obvious cellular/structural change that undergoes circadian rhythmicity is the retinomotor movements of cones, rods, and melanosomes within the RPE cells. Control of these will be described in greater detail below. Horizontal cell spinules, which are small finger-like projections on horizontal cell dendrites invaginating cone pedicles also show circadian rhythmicity (see chapter 7).

Neurochemicals in the retina have also been found to be under clock control. As in the pineal, melatonin in the retina is produced rhythmically using the same

enzymes and biosynthetic pathway. Retinal melatonin, or the enzymes that are responsible for its synthesis, has been found to peak at night in various species of rodents (Pang et al, 1980; Yu et al, 1981; Lucas and Foster, 1997; Pozdeyev and Lavrikova, 2000; Fukuhara et al, 2001), bird (Binkley et al, 1979; 1980; Hamm and Menaker, 1980; Pang et al, 1983; Reppert and Sagar, 1983; Zawilska et al, 2003), frog (Pang et al, 1985; Wiechman et al, 1986), and rabbit (Dubocovich ,1983). Further, persistent rhythms of melatonin production in constant darkness indicating clock control of retinal melatonin production has been demonstrated in several species including bird (Hamm and Menaker, 1980), amphibia (Iuvone and Besharse, 1983; Cahill and Besharse, 1990) as well as mammals (Tosini and Menaker, 1996). In hamster retina, *in vitro* release of melatonin in cultured retinae showed circadian rhythmicity where the melatonin levels were high at night and low in the day during a normal light/dark cycle. This pattern continued in constant darkness, free-running with a period close to 24 hours, thus suggestive of a retinal clock (Tosini and Menaker, 1996). The circadian ryhthm of melatonin was also temperature compensated (Tosini and Menaker, 1998b) further evidence of a retinal clock. Circadian rhythms of melatonin release in cultured retinae have also been reported for both the mouse and rat (Tosini and Menaker, 1998a; Tosini et al, 1998).

In fish, both the rhythm of retinal melatonin production and its control is species dependent. Retinal melatonin levels show peaks during the night in most species (Cahill, 1996; Iigo et al, 1997a, c, d) but peaks in melatonin were also noted during the day in salmonids (Gern et al, 1978; Zachman et al, 1992a and b; Zaunreiter et al, 1998b). Similarly, the rhythm of retinal melatonin production

continued in constant darkness in the goldfish (*Carassius auratus*) (Iigo et al, 1997d), zebrafish (*Danio rerio*) (Cahill, 1996), and rainbow trout (*Oncorhynchus mykiss*) (Zaunreiter et al, 1998a). However, retinal melatonin production showed no rhythmicity in constant darkness in the cyprinid oikawa *Zacco platypus* while in another cyprinid, the ugui *Tribolodon hakonensis*, a rhythm of retinal melatonin production could be seen in constant darkness (Iigo et al, 1997a). It has been suggested that as in the pineal organ, retinal melatonin production is dependent on ecological niches as well as phylogeny (Iigo et al, 1997a).

Melatonin receptor subtypes have been localized in the retina. In *Xenopus* retina, Mel1A, Mel1B, and Mel1C receptor subtype mRNA are expressed in both the pigmented epithelium and neural retina (Wiechmann et al, 1999; Wiechmann and Smith, 2001) while Mel1B and Mel1C are also expressed in both the inner segments of photoreceptor cells within the inner nuclear layer and in ganglion cells (Wiechmann and Smith, 2001). In the chick retina, Mel1A was shown in the inner nuclear layer and less so in the ganglion cells (Wiechmann and Smith, 2001) while Mel1C was expressed in the inner nuclear layer (Reppert et al, 1995). For the rat retina, Mel1A receptor mRNA is expressed in amacrine and horizontal cells in the inner nuclear layer as well as in ganglion cells (Fujieda et al, 1999).

There is substantial evidence from numerous species that the site of retinal melatonin production is the photoreceptors. In many vertebrates, including fish, NAT activity (Vivien-Roels et al, 1981; Gern and Karn, 1983; Falcon and Collin, 1991) and HIOMT-like (Wiechmann et al, 1985; Wiechmann and Holyfield, 1989) immunoreactivity have been localized within the outer retina. Similarly, in

other vertebrates, HIOMT- and AA-NAT-encoding mRNA (Weichmann and Craft, 1993; Bernard et al, 1997; Niki et al, 1998; Tosini et al, 1998) as well as melatonin binding sites (Weichmann et al, 1986; Blazynski and Dubocovich, 1991) have also been localized mainly to the outer retina. AA-NAT activity in cultured chicken retinae is increased by treatment that increases the number of photoreceptor like cells (Iuvone et al, 1990). Both in the *Xenopus* (Cahill and Besharse, 1992) and chicken (Zawilska and Iuvone, 1992; Thomas et al, 1993) eyecup preparations lacking the inner retinal layers, retinal melatonin continued to show circadian rhythmicity which in *Xenopus* was influenced both by light and dopaminergic drugs (Cahill and Besharse, 1993). Taken together, this suggests that photoreceptors are responsible for rhythmic melatonin synthesis. Further, it has been shown using different strains of mice, that rods are not required for the synthesis of melatonin but are needed for the expression of its circadian rhythmic synthesis, suggesting that cones are the sites of synthesis and that either rods or both rods and cones are required to generate rhythmic synthesis (Tosini and Menaker, 1998a).

Unlike its role in the pineal organ, there is less evidence of the function of retinal melatonin although it is also thought to act as a dark adaptive signal. In rats, melatonin has been shown to be involved in dark adaptive outer segment disc shedding (Besharse and Dunis, 1983; White and Fisher, 1989), while in amphibia, melatonin causes RPE pigment aggregation (*Ranasps*; Kraus-Rupert and Lembert, 1965; Kemali et al, 1986) and cone elongation (*Xenopus laevis*; Pierce and Besharse, 1985). Dark adaptive pigment aggregation was also seen in the RPE of guinea pigs (Pang and Yew, 1979) and hamster (Krasovich and Benson, 1983). In

fish, the role of melatonin is less clear. In the carp (*Cyprinus carpio*) and also trout (*Salmo irideus*), melatonin causes dark adaptive RPE aggregation (Chèze and Ali, 1976) while in the Crucian carp (*Carassius auratus auratus*) retina, melatonin was shown to be involved in the dark withdrawal of horizontal cell spinules (Behrens et al, 2000). However, no dark adaptive effects were seen in teleost cone movement in the midas cichlid (*Cichlasoma citrinellum*) (McCormack and Burnside, 1992) or the green sunfish (*Lepomis cyanellus*) (Dearry and Burnside, 1986).

One way in which melatonin is thought to be involved in altering retinal function is in its interactions with the neurochemical dopamine, which has been shown to have a light adaptive role (see section 1.15). Dopamine, also found in the retina, shows a reverse rhythm of production in comparison to melatonin. Dopamine is synthesized in the retina from the amino acid tyrosine by the action of two enzymes; the rate-limiting enzyme tyrosine hydroxlase and the aromatic L-amino acid decarboxylase (Dubocovich, 1988). The general pattern of retinal dopamine synthesis shows peak levels during the day, which continues in constant darkness revealing clock control in mammals (Wirz-Justice et al, 1984; Pozdeyev and Lavrikova, 2000), birds (Adachi et al, 1998; Manglaplus et al, 1999;Zawilska et al, 2003a and b), and fish (Kirsch and Wagner, 1989; Kolbinger et al, 1990; McCormack and Burnside, 1993; Zaunreiter et al, 1998a; Ribelayga et al, 2002; Ribelayga et al, 2004). Recent evidence suggests that the site of the clock controlling retinal dopamine production is within the retina itself as *in vitro* studies of isolated fish retinae showed a rhythm of dopamine production for at least 56 hours in constant darkness (Ribelayga et al, 2004).

The retinal actions of dopamine are better known than those of melatonin. In the retina, dopamine can modulate a variety of functions via G-protein coupled receptors. There are at least five types of dopamine receptors; D_1, D_2, D_3, D_4, and D_5 which are further classified into two family subgroups denoted the D_1-like (consisting of D_1 and D_5 subtypes) and the D_2-like (the D_2, D_3 and D_4 subtypes) receptors (Strange, 2000). D_1-like and D_2-like receptors are characterized according to their effects on adenosine 3', 5'-cyclic monophosphate (cAMP) production (Kebabian and Calne, 1979). Activation of D_1-like receptors causes cAMP production, whereas activation of D_2-like receptors decreases cAMP production. Further, D_1-like and D_2-like receptors interact differently with selective pharmacological agonists and antagonists, and D_2-like receptors are 2-3 orders of magnitude more sensitive to dopamine than D_1-like receptors (Kebabian and Calne, 1979). D_2-like receptors are located on photoreceptors (Dearry and Burnside, 1986; Harsanyi and Mangel, 1992; Yazulla and Lin, 1995) while D_1 receptors occur on cone horizontal cells (Mangel and Dowling, 1985, 1987).

In many vertebrates, retinal dopaminergic cells are primarily amacrine cells but in teleost fish and New World Monkeys, they are interplexiform cells (Dowling et al, 1980; Yazulla and Zucker, 1988). Many studies show dopamine to be the major light-adaptive signal within the retina (Djamgoz and Wagner, 1992 for review). In fish, for example, application of dopamine to dark adapted retinae causes light adapted cone contraction, rod elongation, RPE melanosome dispersion (Pierce and Besharse, 1985; Bruenner and Burnside, 1986; Dearry and Burnside, 1989) via D_2 receptors and spinule formation (see chapter 7) (Weiler et al, 1988a;

Kohler and Weiler, 1990; Kirsch et al, 1991) as well as decoupling of horizontal cells (Baldridge et al, 1987, 1989) via D_1 receptors.

Not only are the rhythms of melatonin and dopamine synthesis opposite, but there is also an interaction between the two. Melatonin receptors have been localized to various parts of the retina (see above) including the inner nuclear layer where their activation inhibits the release dopamine (Dubocovich, 1983; Boatright et al, 1994; Fujieda et al, 2000). At the same time dopamine can inhibit melatonin synthesis and/or release (Nguyen-Legros et al, 1996; Tosini and Dirden, 2000). It has been proposed that melatonin drives dopamine rhythmicity and dopamine modulates the melatonin rhythm but is not necessary for the generation of the rhymicity (Cahill and Besharse, 1995). Recently, using goldfish retinae, it has been shown that the melatonin rhythm is not produced by the rhythm in dopamine and that the rhythm in dopamine, in particular the decrease in dopamine release at night, is achieved by the retinal clock activating melatonin receptors (Ribelayga et al, 2004). Thus, the melatonin rhythm drives the dopamine rhythm. Similarly, in mice which cannot synthesize pineal or retinal meltonin (BALB/c), no rhythm in dopamine production is seen in constant darkness, further suggesting that melatonin is responsible for the rhythm in dopamine (Nir et al, 2000). However, in the Royal College of Surgeons rat lacking photoreceptors, a rhythm in dopamine metabolism could still be detected, indicating that melatonin does not drive this rhythm and suggesting at least two independent oscillators in the retina (Doyle et al, 2002).

Other retinal circadian rhythms include; the levels of mRNA encoding rod and cone photopigments (von Schantz et al, 1999), GABA turnover rate and release (Jaliffa et al, 2000), protein kinase C immunoreactivity (Gabriel et al, 2001) and the levels of several amino acids (Pozdeyev et al, 2000).

Studies using the *tau* mutant hamster have suggested that retinal clocks operate in a similar manner to the SCN at a molecular level. Melatonin retinal rhythms as well as disc shedding rhythms in *tau* mutant hamsters showed shorter free-running periods, indicating that they use the same basic molecular mechanism as the SCN clock (Grace et al, 1996; Tosini and Menaker, 1996). Several studies have detected genes in the retina that are part of the core clockwork in the SCN (Tosini and Fukuhara, 2002 for review). In *Xenopus*, *Clock* (Zhu et al, 2000) and *3 Cry* (Zhu and Green, 2001) homologues are expressed predominantly in photoreceptors, while *Per2* is expressed predominantly in the RPE (Zhu et al, 2000). *Cry 1* (Haque et al, 2002) and *Cry2* (Bailey et al, 2002) have also been localized to the photoreceptors of chicken retinae. *In situ* studies show the clock genes to be more widely expressed with *Clock, Bmal1,Cry, Per1* and *Per2* detected in the inner nuclear layer and ganglion cells of the *Xenopus* (Zhuang et al, 2000) and *Cry 1* and *Cry 2* are also found in the ganglion cells of chicken (Bailey et al, 2002; Haque et al, 2002). In mammals, *Per1*, *Clock* and *Bmal1* are expressed mainly in the inner retina of mouse although some expression is also seen in photoreceptors (Gekakis et al, 1998). Mouse and human *Cry1* and *Cry 2* are expressed in ganglion cells as well as cells in the inner nuclear layer (Miyamoto and Sancar, 1998; Thompson et al, 2003).

In *Xenopus*, *Per2* is thought to play a role in phase setting the retinal clock as it exhibits a light driven diurnal rhythm peaking out of phase with *Per1* and is induced by both light and dopamine (Steenhard and Besharse, 2000). Changes in PER2 protein may then interact with other components of the molecular clock to alter the circadian phase. The entrainment mechanisms and pathways involved in mammalian retinae are not known (Tosini and Menaker, 1996).

The mechanism by which the retinal clock controls the production of melatonin is not well understood. However, studies in chicken and frogs have shown both Trp-H and AA-NAT to be rhythmic in their mRNA and activity levels both *in vitro* and *in vivo* (Anderson and Green, 2000). Both retinal *Aa-nat* mRNA and AA-NAT activity show circadian changes in both the rat and mouse (Sakamoto and Ishida, 1998a and b).

1.12 Circadian regulation of retinomotor movements

Of particular importance in the context of this thesis is that retinomotor movements are under endogenous circadian regulation. In fact, retinomotor movements were the first retinal process discovered to be under circadian control. Welsh and Osborne (1937) first placed catfish in constant darkness and found that the cones continued their cyclic movements. At what would have been subjective day the cones were constricted and during what would have been night they were elongated. Since then, there have been many studies showing the presence of circadian controlled retinomotor movements (see chapter 3 for review).

The behaviour of the retinomotor movements in continual darkness, that is the degree to which they are under endogenous control, has been used to characterise fish (Wagner et al, 1992a). In retinomotor literature, strongly rhythmic fish are considered to be those that preserve their rhythm of retinomotor movements in continual darkness for at least 2 days and there may be continuation of movements in continual light conditions. Weakly rhythmic fish show a dampened continuation of the retinomotor movements in continual darkness and no movements in continual light. Arrhythmic fish show no retinomotor movements in continual darkness (Wagner et al, 1992a).

1.13 Regulation of retinomotor movements

Several studies have addressed the question whether retinomotor movements are under local control within the eye or if their control lies outside the eye (see Besharse, 1982 for review). For photoreceptors, evidence suggests that the control centre governing their movements is located within the eye. However, the situation is less clear for migratory RPE melanosomes. By projecting a small spot of light onto the dark-adapted retinae of intact Jack Dempseys (*Cichlasoma biocellatum*) it has been shown that light-adaptive reactions (cones and RPE melanosomes) are sharply restricted to the area of the retina being illuminated (Easter and Macy, 1978). This effect was also seen in Jack Dempsey (Easter and Macy) and goldfish (*Carasius auratus*) (Kirsch and Wagner, 1986) retinae after optic nerve section indicating that induction of retinomotor movements by light is

mediated locally by those retinal cells directly exposed to light and it is unlikely there is influence from a more global humoral or neural component.

In *Xenopus* eyecups, retinomotor movements persist in longterm culture (Besharse and Iuvonne, 1983), also indicating their control centre was located within the eye. Light adapted retinomotor movements were also seen in cultured fish retinae (Burnside and Basinger, 1983), again indicating that neural or humoral agents emanating from outside the eye are not required for light adaptive movements. In both the green sunfish (*Lepomis cyanellus*) (Dearry and Barlow, 1987), and the Midas cichlid (*Cichlasoma citrinellum*) (McCormack and Burnside, 1992), retinomotor movements were recorded following optic nerve section. However, both these results do not exclude the possibility of humoral agents. Whole retinae from Midas cichlids have been cultured and cone movements recorded after 21 days in culture (McCormack and McDonnell, 1995) giving the most conclusive evidence to date that cone movements are governed from within the eye while the same cannot yet be said for RPE melanosomes.

1.14 Intracellular regulation of retinomotor movements

Several studies have investigated the intracellular regulation of retinomotor movements (Burnside, 2001 for review). An increase in intracellular cyclic AMP causes dark adaptive retinomotor movements in teleost rods, cones, and RPE melanosomes (Burnside et al., 1982; Burnside and Basinger, 1983; Burnside and Ackland, 1984). An increase in cyclic AMP is seen during both a normal

light/dark cycle and during constant darkness and thus cyclic AMP is thought to couple retinomotor movements to both the diurnal and circadian cycles. Cyclic AMP acts by mobilising the photoreceptor cytoskeleton via protein phosphorylation (Liepe and Burnside, 1993; Rey and Burnside, 1999). In RPE cells, cyclic AMP acts via phosphorylation of protein kinase (Garcia and Burnside, 1994). For light adaptive retinomotor movements (see section 1.3.1), a decrease in intracellular cyclic AMP, and an increase in intracellular calcium as well as ATP is required (Burnside et al, 1982, O'Connor and Burnside, 1982). In cones, calcium acts as in other muscle and non-muscle cells by stimulating actin-activation of the myosin ATPase responsible for force production and thus contraction. In rods, the force required for elongation results from the assembly of rod myoid actin filaments (O'Connor and Burnside, 1981). An increase in calcium levels activates actin-myosin interaction and creates force production required for light induced melanosome dispersion in RPE cells (Burnside et al, 1982).

1.15 Extracellular regulation of retinomotor movements

Extracellular regulation by dopamine causes light adaptive cone and RPE melanosome movement (Dearry and Burnside, 1988, 1989; Burnside et al., 1993) (see section 1.11). In teleosts, dopamine is localised within a population of interplexiform cells (Dowling and Ehinger, 1978) (see section 1.11) which form a variety of contacts in the inner plexiform layer (Yazulla and Zucker, 1988). In the outer plexiform layer they synapse with horizontal and bipolar cells and in some species, make contact with photoreceptors (Wagner and Wulle, 1990, 1992). Dopamine has also been shown to play a role in the circadian regulation of cone

movement acting via D_2 receptors (Kolbinger et al, 1990; Douglas et al., 1991; McCormack and Burnside, 1992; Wagner et al, 1992a and b). However, dopamine is unlikely to be the only contributor to light induced and circadian cone movements as these persist, although with reduced amplitude, following lesion of interplexiform cells with 6-hydroxydopamine (Douglas et al, 1992; Wagner et al, 1992a and b). Dopamine is released from interplexiform cells into the outer plexiform layer and is thought to diffuse in a paracrine fashion to cause cone contraction via D_2-like receptors during normal light adaptation. (Behrens et al, 2000; Burnside, 2001). However, as cone contraction is not compromised in dopamine depleted retinae (Douglas et al, 1992), the above pathway may not be the only one involved.

The extracellular regulation of dark adaptive retinomotor movements is less clear. One prime candidate to signal the onset of darkness in the retina, and thus trigger dark adaptive retinomotor movements, is melatonin (see section 1.11). Both melatonin and the enzymes involved in its synthesis have been localised in the teleost retina (Iigo et al., 1994, 1997a, c, d; Sánchez-Vázquez et al., 1997; Zaunreiter et al., 1998a and b). Melatonin binding sites have also been characterised in the goldfish retina (Iigo et al, 1997b) and cone melatonin autoreceptors have been shown in the mouse (Blazynski and Dubocovich, 1991). Although patterns of melatonin levels in fish have produced ambiguous results (some fish species show peak levels during the day while others show peak levels at night, see 1.11), melatonin is still considered the likely dark adaptive candidate in fish retina for several reasons;

a/ the overwhelming evidence that it is the dark adaptive signal in other animals

b/ it has been shown to be involved in the dark withdrawal of horizontal spinules in fish retina (Behrens et al, 2000)

c/ the melatonin/dopamine antagonistic interaction has been shown in the fish retina (Behrens et al, 2000).

1.16 Basic fish circadian organization

In fish, locomotor activity is believed to be regulated by two or more groups of loosely coupled oscillators synchronized to external timing cues (Tabata, 1992). As previously described circadian oscillators have been demonstrated in the retina and pineal gland of a large number of species (Bolliet et al, 1996; Cahill et al, 1995). In some teleosts there is also another non-pineal circadian oscillator located in the brain (although its exact location is not known) as well as deep brain photoreceptors (Tabata, 1992). Pinealectomised catfish without both lateral eyes could still be entrained to a light/dark cycle indicating another oscillator and photoreceptor controlling locomotor activity. However, when the brain of these catfish was covered in foil, the fish become arrhythmic indicating photoreceptors located within the brain (Tabata et al, 1988).

In different species of fish, the coupling and hierarchal order of these oscillators may vary. In the lamprey (*Lampetra japonica*) (Tabeta, 1986; Morita et al, 1992) and a catfish (*Heteropneustes fossilis*) (Garg and Sundararaj, 1986), pinealectomy causes breakdown of locomotor activity, whereas in another catfish (*Silurus asotus*) pinealectomy, removal of both eyes, and shielding the brain from light is required to destroy locomotor activity (Tabata, 1992). Further, in the catfish,

(*Silurus asotus*) the degree to which the pineal gland and the lateral eyes contribute to the circadian rhythmicity varies according to the entraining light conditions. In dim light, the pineal gland is responsible for most of the fish's locomotor activity while in bright lights the lateral eyes play a bigger role (Tabeta, 1992).

In each cell of the pineal, retinal and the deep-brain oscillators of fish, clock genes would be expected to form biochemical loops to generate the intrinsic rhythm to the oscillators. Although there have been no previous circadian experiments on the glowlight tetra, it is likely to conform to the general findings in other studies; thus it probably has a pineal, retinal, and perhaps a non-pineal deep brain oscillator together with pineal, retinal, and deep brain photoreceptors.

Most of this thesis deals with the study of retinomotor movements in the glowlight tetra. Previously, nearly all studies have found that in constant darkness, retinomotor movements show signs of damping usually within 0-48 hours. In comparison, early experiments on the glowlight tetra showed that even after 14 days in constant darkness, the rhythm of cone movements matched that found during a normal light/dark cycle with no signs of damping. This relatively long persistence of the cone rhythm in constant darkness represented a unique opportunity to conduct classic circadian rhythm studies. This work concludes by considering whether this robust cone rhythm could also indicate a strong ocular oscillator capable of maintaining glowlight rhythmicity such as locomotor activity following pinealoctomy.

Chapter 2-General Methods

In this chapter, the general methods used throughout the bulk of this thesis will be outlined. Other experimental procedures will be described in the appropriate chapter.

2.1 Fish

All fish were obtained from a local dealer, who imported them from fish farms in Singapore. Glowlight tetras (*Hemigrammus erythrozonus*), which are the bulk of the fish used in this thesis, as well as zebrafish (*Danio rerio*), were maintained at a temperature of 25°C and a pH of 7.0. They ranged in standard length from 2.0-3.0cm for glowlight tetras and 2.7-3.2cm for the zebrafish. All fish were fed a diet of Tetramin flakes five days a week (fish were generally not fed on the weekend). Feeding always occurred during the light phase of the light/dark cycle to minimise any unwanted light during the dark phase. Feeding times were varied in order to eliminate any possible entrainment effect.

2.1.1 Fish Tanks

For both maintenance of fish and experimental purposes, fish were kept in five different tanks of similar dimensions (36cm x 92cm x 38 cm high). Each tank was painted white externally covered by a layer of black paint. Thus, the internal tank

surface reflected light back into the tank and the external tank surface was black to prevent the entry of light.

A wooden light box housing a fluorescent bulb covered the tank. The internal surface of this box was also painted white and the external surface painted black. A clear sheet of Perspex, resting on a 7cm wooden rim, separated the light box from the water surface. This rim ensured minimal entry of stray light. Finally, as an added precaution, the light box and tank were covered with black sheets to prevent the possibility of any stray light entering the tanks (Figure 2.1).

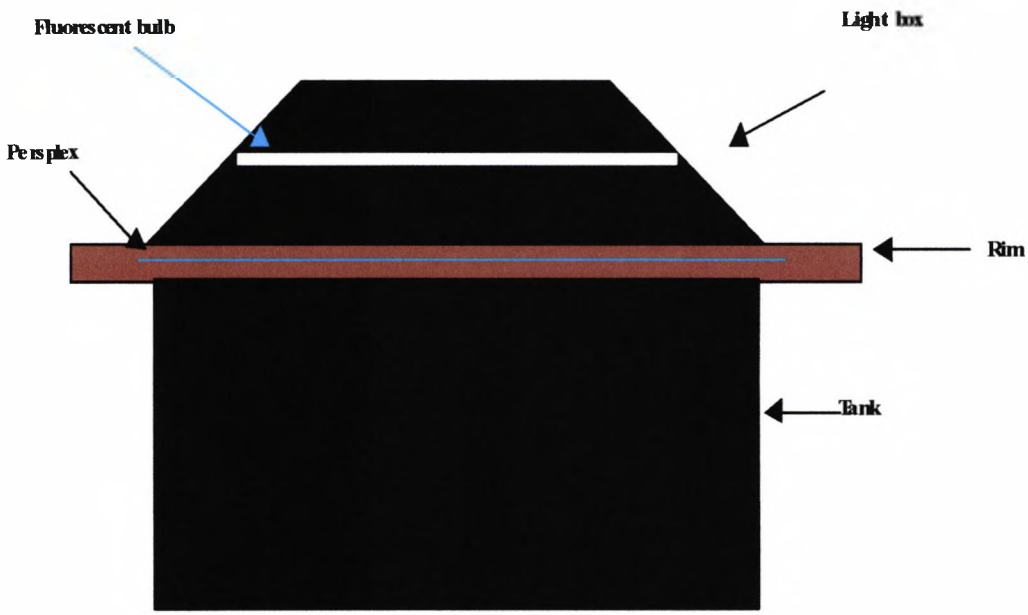

Fig 2-1 Fish tank set-up
Diagram showing set up of light box and fish tank. The above set-up was covered by black sheets to ensure that no stray light entered or left the tank.

The water inside each tank was both aerated with air pumps and heaters were used to keep the water at a constant temperature, which was monitored, on an almost daily basis, using floating thermometers.

The tanks were kept in a dark room with only red filtered light (see below) used to allow manoeuvring in the room during feeding hours. When tanks with different light schedules were used within the same room, these were placed away from each other, although the above precautions ensured that there was no stray light escaping from any tank. The temperature of the room was maintained at approximately 20-21°C.

2.2 Illumination

The fish tank lighting was provided by a fluorescent bulb (Phillips, TLD 18W/ 35D). The illumination in each tank was measured using a light meter (Macam PM203), in 9 different locations covering the entire surface of the water to yield an average illumination ranging from 0.38mW/cm^2 to 0.47mW/cm^2.

Comparison with light levels used in other studies is difficult as most of these used photometric units such as lux, rather than the radiometric units employed here. Since photometric units are adjusted for human sensitivity they have little relevance to animal studies. However, lights used in this and previous studies are likely to be comparable as most used fluorescent tubes similar to those employed here, although precise details are rarely given (see for example John and Haut, 1968; Olla and Marchioni, 1968; Levinson and Burnside, 1981; Douglas 1982a and b; McCormack and Burnside, 1991; McCormack and McDonnell, 1995;

Kolbinger et al, 1996). Natural daylight will usually be much brighter than illumination produced in the laboratory. Thus, the light levels used here and in most other studies are only a small percentage of those experienced in the wild. Some studies that have sampled fish in their natural environment (John et al, 1967; John and Gring, 1968) or outdoors in fish farms (Douglas, 1982a).

2.3 Light schedules

The time the lights came on and went off was controlled by external timers connected to the light source. In all experiments, fish were entrained to a particular light schedule for a minimum of 30 days. Previously, most other retinomotor experiments have entrained fish for a minimum of 30 days and in chapter 4 it is shown that this is more than sufficient time for good entrainment to the light/dark cycle.

The light within tanks came on and off abruptly which is different to natural dawn/dusk transitions which last for much longer (see chapter 1 for duration of twilight and latitude position). Sharp cut off points were used rather than attempting to simulate the gradual natural light transitions for better comparison with other studies (all retinomotor experiments to date using artificial lighting have used sharp cut offs to simulate dawn and dusk). Interestingly, an experiment which compared retinomotor movements in fish entrained outdoors to the natural light/dark cycle and those entrained to an artificial light regime in the laboratory found no difference in the retinomotor movements either during the light/dark cycle or subsequently in constant darkness (Douglas, 1982a).

2.4 Experimental procedures

2.4.1 In darkness

Any experiment carried out in 'darkness' was performed using only dim red light (Figure 2.2). Action spectra (Kirsch et al, 1989)(see section 1.3) indicate that such long wavelengths are ineffective in causing cone contraction, which is triggered through light absorption by rods. Further, animals sampled in their dark phase were always morphologically dark adapted with no sign of unexpected light adaptation being caused by the dim red light.

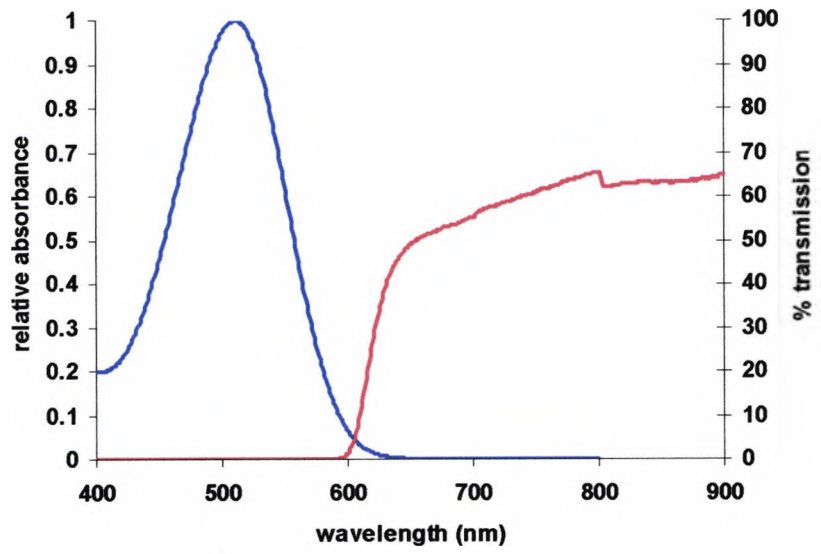

Figure 2.2 Transmission spectrum of red filter and relative absorbance of rod
The blue curve above gives the relative absorbance for glowlight tetra rods (λmax=511, see below for determination) fitted using an A_1 template from Govardovskii et al, 2000. The red curve shows the transmission spectrum of the red filter covering the torch used to access fish in 'darkness' measured using a scanning spectrophotometer (Shimadzu UV-2101PC). Clearly, the wavelength of the red light is too long to stimulate glowlight tetra rods to any significant degree.

2.4.2 Sampling fish

On the evening before the experiment was due to begin, approximately 1 hour before dusk (lights off), fish were divided into groups of three and placed into netted jars before being returned to the home tank. This procedure usually took less than 30 minutes so that fish were back in the home tank well before the lights went off. Groups of three fish were placed into jars for several reasons;

A/ three fish were sampled per time point

B/ stress, such as that experienced during capture, can potentially lead to light adaptation in both the fish being captured and in other fish within the same tank through the release of pheromones (Kirsch et al, 1989). Since fish were confined to jars they could be removed from their home tanks within a few seconds thus minimising the chance of their tank mates, which would be sampled at a later time, being affected by pheremones. Since the sampled fish themselves were immersed in fixation solution within 40 seconds of leaving the home tank, it is unlikely that significant stress induced light adaptive retinal changes would be caused within the sampling group as light retinomotor movement light adaptation takes longer than 15 minutes.

C/ fish could be sampled at exactly the required time without having to search for them!

It is unlikely that placing groups of 3 fish into small jars caused undue stress for several reasons;

A/ in all experiments, retinomotor elements were markedly different during the dark phase in comparison to the light phase. If fish were stressed, it would have

been expected that cones and RPE melanosomes remained in their light adapted positions throughout the light/dark cycle.

B/ Sampling fish during a normal light/dark cycle (LD 12:12, see chapter 3) was first done without the use of jars. 3 fish were taken straight from the tank using a small net at the appropriate sampling time. The results of this experiment were compared to sampling under the same light/dark cycle but after groups of 3 fish were placed into netted jars as described above. At each time pointed tested, there was no difference in the cone index (see below) between the two groups of fish ($p>0.2$, unpaired student t test).

C/ Groups of 3 fish left in the jar during the middle of the light phase would commence eating Tetramin flakes after only 1 hour of being placed in the jar indicating that they are not under undue stress. Previous experimenters have used the time taken to recommence feeding following experimental procedures as an indicator of the stress levels experienced by the fish (for example, Muntz et al, 1996).

2.4.3 Preparation for light microscopy

After removing fish from the experimental tanks, cervical transection and pithing was used to kill all fish. If this needed to be done in the 'dark', then a dim red-light was used. After killing the fish, the central cornea was pierced and the whole fish placed into a fixation solution consisting of 1% paraformaldehyde and 2.5% glutataldehyde (see appendix). After 12 hours or overnight in the fixative solution, the eyes were enucleated, hemisected and prepared for embedding into resin using the stepwise procedures outlined below.

A/ Fixed posterior eyecups were washed with water three times, each wash lasting for 5 minutes, before undergoing a series of graded ethanol dehydrations.

B/ The dehydration commenced with immersion in a solution containing 50:50, ethanol:water mixture for 5 minutes. This was followed by;

C/ Immersion in 70:30 solution of ethanol:water for 5 minutes.

D/ Immersion in 80:20 solution of ethanol:water for 20 minutes while rotating.

E/ Immersion in 90:10 solution of ethanol:water for 20 minutes while rotating.

F/ Immersion in 95:5 solution of ethanol:water for 20 minutes while rotating.

G/ Immersion in 100% ethanol three times, each time for 20 minutes and rotating the eyecups.

The dehydration was now complete and the next step involved removing the ethanol and preparing the eyecups for embedding into resin. This was achieved by;

H/ Immersion into a solution of Histoclear for 30 minutes again while rotating.

I/ Immersion into a 50:50 solution of Histoclear: resin for 30 minutes while rotating.

J/ Immersion into 100% resin and rotating the eyecups overnight at room temperature.

K/ Each eyecup was placed into an individual baking mould and filled with fresh resin. The eyecups were orientated to allow good radial sections of the retina to be cut.

L/ Moulds were placed into an oven at 60°C for 48 hours to allow polymerisation and hardening of the resin.

For sectioning, resin blocks containing individual eyecups were trimmed manually using a razor blade. Following this, blocks were placed into the Reichert-Jung Ultracut E microtome for sectioning. Glass knifes were made using a standard knifemaker (LKB Knifemaker, Type 7801B) and prepared for sectioning by attaching small 'boats' near the cutting edge. These 'boats' were made from water-resistant tape and could be filled with distilled water so that as the tissue/resin complex was cut, it would float into the boat to allow collection of the section from the water.

1μm thick radial sections were cut, collected in the distilled water of the boat, and removed using a small syringe. From the syringe, sections were transferred to a droplet of water resting on a clean glass slide. Up to ten sections were placed on individual glass slides to maximise the chance of good quality sections (and minimise tissue loss during the staining process). Glass slides were placed onto a hot plate set to approximately 60°C which allowed the tissue to dry and stretch slightly without folding, as it adhered to the glass slide. When all the water had evaporated, the glass slide was held over a small flame for a few seconds, to ensure that the section was stuck to the slide.

Initially, during all experiments, the resin was taken off before staining the tissue, as follows;

A/ The glass slide was immersed in sodium methoxide for 60-90 seconds, which removed the resin.

B/ The slide was rinsed with diluent (see appendix) to eliminate any sodium methoxide.

C/ It was rinsed with acetone in order to remove the diluent.

D/ Distilled water was used to rinse off the acetone.

The tissue was now ready for staining. Before all the distilled water had evaporated, droplets of 1% toluidine blue (see appendix) were placed on the tissue for approximately 20-30 seconds before being rinsed off with distilled water. The slide was placed in an oven at 60°C for drying.

Later it was decided not to remove the resin before staining to minimise exposure to toxic chemicals. In chapter 3 alone, over 700 slides (one slide for each retina examined) were prepared using this method and so a continuation of resin removal would add up to a significant time spent handling these chemicals. A simpler and more common method to stain the tissue was thus employed;

A/ After heating the slide with the flame (see above), it was returned to the hotplate. Droplets of 1% toluidine blue were added to the resin/tissue complexes and allowed to stand for between 10-20 seconds.

B/ The stain was then rinsed off with distilled water and the slide was placed in an oven at 60°C to dry.

Once the slides were completely dry, coverslips were placed over the tissue using DPX. The slides and coverslips were allowed to dry, again in the oven at 60°C, for 48 hours. By this time, the coverslips were secured strongly to the slide for oil immersion microscopy.

2.5 Determination of cone and RPE indexes

To accurately describe the position of both the cones and the RPE melanosomes, cone and RPE indices were determined. The cone index is the distance from the outer limiting membrane (OLM) to the junction of the cone inner and outer segments divided by the distance from the OLM to Bruch's membrane (Figure 2.2). Similarly, the RPE index, commonly known as the pigment index, is the distance between the RPE melanosomes nearest to the OLM and Bruch's membrane divided by the distance between the OLM and Bruch's membrane (Figure 2.2). Rather than simply using cone/pigment positions, these indices compensate for individual differences in retinal thickness and for oblique sectioning. Rods were not analysed as it is difficult to determine accurately their positions due to both their large numbers and their slender width.

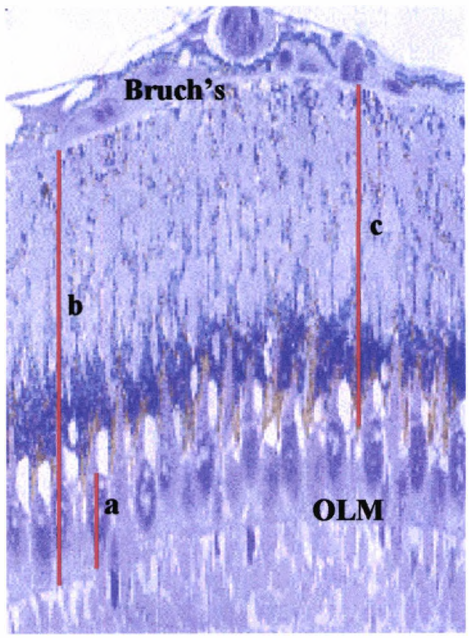

Fig 2-3 Determination of cone and pigment indices
Transverse section of glowlight tetra retina labelled to show how both the cone and pigment indices were determined. The cone index is given by 'a/b' where 'a' is the distance from the junction between the cone ellipsoid and myoid to the OLM and 'b' is the distance from the OLM to Bruch's membrane. The pigment index is given by 'c/b' where 'c' is the distance from the RPE melanosomes nearest to the OLM to Bruch's membrane.

A common problem in the histological preparation of fish retina is the tendency for the retina to detach from Bruch's membrane. It has been suggested that the addition of 0.5% cetylpyridium chloride to the fixation solution minimises this problem (McCormack and Burnside, 1991). However, this made no difference in

glowlight tetra preparations. As a consequence, in determining the cone and pigment indices in many retinas, instead of measuring from Bruch's membrane, measurements were taken from the sclerad edge of the photoreceptor layer where it is normally attached to Bruch's membrane.

Both light and electron microscopy revealed three types of cones; small and large single cones and double cones. The absorption spectra of photoreceptors in the glowlight tetra retina were determined by microspectrophotometry (MSP) of single photoreceptor cells, by Professor J.K. Bowmaker (Institute of Ophthalmology, London). Visual pigments with maximum sensitivity (λmax) at 454±3 nm, 566±5 nm and 606±7 nm corresponding to the 3 cone types were recorded, while a pigment with λmax 511±2 nm was attributed to rods (Figure 2.3).

To determine whether there were retinal regional differences in cone indices at different times during the light/dark cycle, two separate eyecups were sectioned in 20μm steps, one along the dorsal-ventral and the nasal-caudal axis, at three different times; during the middle of the light phase, the middle of the dark phase, and 1 hour before dawn, when the cones were moving to light adapted positions. Preliminary examination of each section during the middle of the light phase revealed all cones and RPE melanosomes to be in fully light adapted positions. Similarly, all slides collected from eyecups sectioned during the middle of the dark phase showed both cones and RPE melanosomes to be fully dark adapted. Cones and RPE melanosomes appeared to be in intermediate positions in all sections collected from fish sampled 1 hour before dawn.

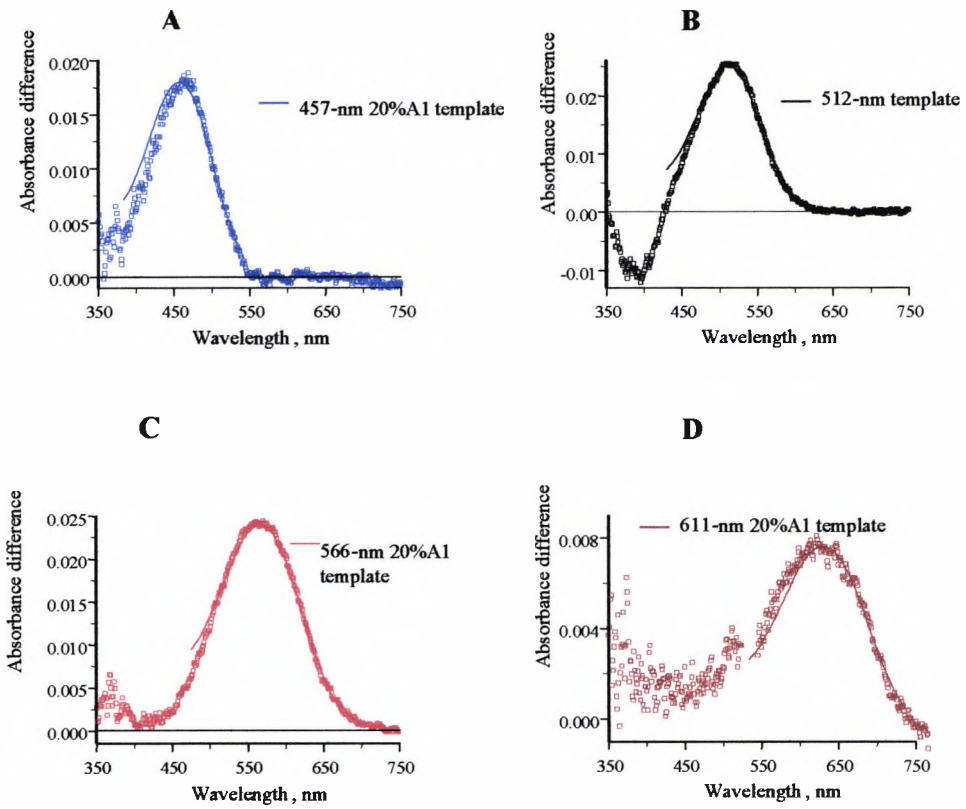

Fig 2-4 Aborption spectra of glowlight tetra photoreceptors
Absorption spectra of glowlight tetra photoreceptors determined by MSP. Curve 'A' gives a visual pigment with a maximum sensitivity at 454±3 nm (n=11) found in small single cones. 'B' indicates a visual pigment with a maximum sensitivity to 511±2 nm (n=15) located within the rods. 'C' is a visual pigment with maximum sensitivity at 566±5 nm (n=19) attributed to the long single cones while 'D' has a maximum sensitivity at 606±7 nm (n=9) and is attributed to the double cones. Rods were fitted with a pure A_1 template and cones were fitted with an 80/20 A_1/A_2 template based on the templates of Govardovskii et al, 2000.

To quantify the preliminary finding that both the cone and the pigment indices did not vary throughout the retina, they were determined at various locations throughout the retina. For the light adapted eyecup (middle of the light phase), the dorsal-ventral axis measured 2,450μm and sections cut at 300, 600, 900, 1060, 1200, 1360, 1500, 1800 and 2100μm from the dorsal edge were analysed to determine the average cone and RPE indices of 50 cones and melanosomes per section. Similarly, sections were taken along the nasal-caudal axis at 300, 600, 900, 1060, 1200, 1360, 1500 and 1800μm from the nasal edge in an eyecup with a total nasal-caudal retinal diameter of 2,100μm and average cone and pigment indices were determined. For each section, sampling of cones and melanosomes was spread along the entire section. Clearly, the spread of cones and melanosomes (50 of each per section) measured was less in sections taken nearer to the edge of the retina, which are smaller, than longer sections taken near the centre. Thus, counting 50 cones in the central sections might be less representative of the average cone index in comparison to counting 50 cones in sections taken near the edge. To offset this effect, more sections were taken in the central retina (more sections were taken in the central area also because it is in this region that most experimental sampling was done as explained below).The average cone and pigment indices were compared in all 17 locations sampled and were found to be similar using one-way ANOVA (For cones, $F=0.403$, $p=0.980$ and for pigment $F<0.001$, $p=1.000$).

The same procedure was used to determine the cone and pigment indices at 17 different locations for both the dark adapted and the intermediate retina. For the

dark adapted retina, there was no variation in either cone or pigment indices throughout the retina (one way ANOVA; for cones $F=0.658$, $p=0.836$ while for the pigment index $F<0.001$, $p=1.000$). However, for the intermediate retina there was a variation in both the cone and pigment indices (for cones; $F=2.183$, $p=0.005$ and for pigment; $F=48.540$, $p<0.001$).

As both the cone and pigment indices varied with retinal location during transitional states (while cones and melanosomes were in intermediate positions), sampling of the retina usually used sections from close to or that bisected either the dorsal-ventral axis or the nasal-caudal axis. Sections from lines close to bisecting the eyecup give the longest strip of retina and thus more cones and melanosomes can be analysed. Previously, most other studies neglected the possibility of variation in cone and pigment indices throughout the retina. A popular method in choosing retinal location to be analysed was to cut a predetermined area of retina (usually next to the optic nerve) after the retina had been fixed for several hours. This piece of retina would then be prepared for histological examination and the remaining retina discarded (see for example Levinson and Burnside, 1981; Douglas and Wagner, 1983; McCormack and Burnside, 1991).

To determine the average cone and pigment indices at each time point, at least three fish were sampled and six retinae were analysed. For each retina, the cone and pigment indices of 50 cones and 50 melanosomes were determined so that the average cone and pigment index at each time point represents the average of at least 300 cone and pigment indices (for each time point, 'n' was at least 6). Note

that 'n' was taken as the number of retinae rather than the number of fish because there is good evidence that cones are under local control within each eye (see section 1.13). Thus, each eye can be thought of as containing its own oscillator governing cone and RPE melanosome movement. In contrast, when horizontal cell spinules (see chapter 7) are analysed, 'n' is taken to be the number of fish as the formation of horizontal cell spinules in the 2 eyes of an individual is not independent (DeJuan et al, 1996).

It should be noted that the RPE of glowlight tetra contains two types of melanosomes; rod shaped migratory melanosomes, which undergo retinomotor movements, and non-migratory spherical shaped melanosomes, which remain stationary and are positioned near Bruch's membrane throughout the light/dark cycle. This is similar to the neon tetra (*Paracheirodon innesi*) (Lythgoe and Shand, 1983). In this thesis, the pigment index only refers to the migratory rod shaped melanosomes as only these undergo retinomotor movements.

2.6 Statistical analysis

Unpaired student t tests and one-way ANOVA including post-hoc tests were used to analyse data (SPSS statistical software). The level of significance was taken at 95% for all statistics performed.

Chapter 3-The basic rhythm of retinomotor movements in the glowlight tetra (*Hemigrammus erythrozonus)*

3.1 Abstract

In this chapter the basic rhythm of retinomotor movements of cones and retinal pigment epithelium (RPE) melanosomes in the retina of the glowlight tetra (*Hemmigrammus erythrozonus*) are outlined. During a normal light/dark cycle, the cones and unusually the RPE melanosomes, commenced their light adaptive movements before the onset of light, indicating an endogenous component to their control. By dawn (light onset), both cones and RPE melanosomes had reached their maximum light adaptive level where they remained until dusk (light offset). After dusk, it took 2.5 hours for maximum dark adaptive levels to be reached which were maintained throughout the dark phase until predawn light adaptation commenced.

In constant darkness, the glowlight tetra rhythm of cone contraction and elongation continued without damping for a minimum of 14 days. Meanwhile, the RPE melanosomes continued their cyclic movements for at least 7 days but only a damped rhythm persisted during the 14th day of continual darkness. After 28 days of continual darkness, the rhythms of both the cones and RPE melanosomes were abolished and both remained in their fully light adapted positions. When kept in constant light for 24 hours, the rhythm of both the cones and RPE melanosomes was extinguished as both remained in their light adapted positions throughout light exposure.

The ocular rhythm of melatonin during a light/dark cycle and in constant darkness was determined. It differed from other species and did not relate in any simple way to the observed retinomotor movements.

3.2 Introduction

Retinomotor, (also known as photomechanical movements) were shown to be under both endogenous/circadian and direct light control as early as 1937 (Welsh and Osborne, 1937). In constant darkness, catfish cones constricted at what would have been dawn and elongated when dusk was due. Subsequent experiments have established that endogenous/circadian control of retinomotor movements is widespread amongst fish and amphibia (for reviews see Burnside and Nagle, 1983; Wagner et al, 1992a; Cahill and Besharse, 1995).

While many species of fish have been found to have some circadian control to their cone movements, endogenous control of rod and RPE melanosome movement has been shown in far fewer species. For rods this is because the accurate determination of their position within the retina is difficult due to their great numbers and slender width. This was especially a problem for early workers who used paraffin embedding. Although image resolution has improved with the advent of plastic embedding, most interest has still centred on cones and RPE melanosomes as they are easier to quantify (Kolbinger et al, 1996). Still, many studies examining retinomotor movements have made unquantified remarks about the persistence of rod movements in constant dark (for example, Burnside and Ackland, 1984). In contrast to rods, RPE melanosome movement has been examined in many species and circadian control was absent in some species even when cones were subject to circadian control (for reviews see Burnside and Nagle, 1983; Wagner et al, 1992a; Cahill and Besharse, 1995).

In nearly all fish studied to date, circadian control of retinomotor movement has been shown by placing fish in continual darkness and monitoring the positions of cones and RPE melanosomes throughout the subjective day and night. In all but one study (Douglas and Wagner, 1982), in comparison to the retinomotor movements during the light/dark cycle, cone movements in constant darkness were of decreased amplitude, whilst the rhythm of RPE melanosome movement showed such damping in all studies. In isolated pineal glands placed in constant darkness, the onset of damping of the melatonin rhythm is species dependent and is thought to be an indicator to the strength of the oscillator (Bolliet et al, 1994; Murakami et al, 1994; see introduction). In the same way, when fish are placed in constant darkness, the onset of damping of the rhythm of cone and RPE melanosome movement may give an indication to the strength of circadian drive controlling their movements.

Although there is a long history to the study of retinomotor movements, there are surprisingly few studies on equatorial fish. As already described (see chapter one), the glowlight tetra (*Hemmigrammus erythrozonus*) is an equatorial animal and in its natural habitat in Guyana experiences similar daylight hours throughout the year. Therefore, circadian control of their retinomotor movements would position retinomotor elements in their light or dark adapted positions at approximately the same time each day throughout the year. In comparison, temperate species need clocks that not only can put the retinomotor elements in their light or dark adapted state at the appropriate times each day, but the appropriate times vary throughout the year. That is, temperate species' clocks need to be more sensitive to photoperiod.

In this chapter, glowlight tetras are placed in constant darkness and the duration of the rhythm of retinomotor movements (cones and RPE melanosomes) without damping is determined. The results are compared with previous studies on both equatorial and temperate species using the duration of retinomotor movements in constant darkness as an indicator of the strength of the circadian drive.

There is much evidence showing melatonin is involved in the circadian control of rhythmic retinal processes in a number of species (see chapter 1). To determine if melatonin plays a role in the circadian control of both the cones and RPE melanosomes in the glowlight tetra, ocular melatonin content was determined during a normal light/dark cycle followed by 24 hours of constant darkness.

3.3 Materials and Methods

3.3.1 Light schedule

In all experiments, fish were entrained to a 12 hour light/12 hour dark (LD 12:12) light schedule for a minimum of 30 days.

As actual experimental times differed in some experiments, for example one experiment entrained fish to a light schedule where lights come on from 09.00-21.00 and another entrained fish with lights on at 15.00-03.00, experimental times were standardised. Thus, all times have been converted to a 24 hour clock where

time t=0 denotes the time the lights come on (dawn) and t=12 the time at which the lights go off (dusk).

3.3.2 Experimental procedures

3.3.2.1 light/dark cycle, first 40 hours in constant darkness, and in constant light for 24 hours

At t=11 on the evening before the experiment was due to commence, fish were divided into groups of three and placed into netted glass jars before being returned to the home tank as described in chapter 2 (see section 2.4.2).

On the day of the experiment, fish were sampled at appropriate intervals. Small sampling intervals of 15 minutes were used around both dawn and dusk; the times that cone and melanosomes are expected to be in transitory positions.

For constant darkness, the above procedure was repeated but with the lights turned permanently off at t=12 (dusk) on the evening before the experiment was due to begin, while for constant light the lights remained on. Both the light/dark cycle and constant dark experiments were repeated on several occasions and the results pooled.

3.3.2.2 7 days of continual darkness

The same procedure as outlined above was employed with one small modification. Pellets of fish flakes were placed into the individual jars so that

fish could eat at some time during their week in continual darkness. In this way, more than 95% of fish survived the course of the experiment.

3.3.2.3 14 and 28 days of continual darkness

For these experiments, a different approach was employed. One hour before the lights were due to be turned off permanently, fish were divided into four groups and placed into four separate small tanks. Thus, during the course of these experiments fish could be fed as they were not confined in jars. Four separate tanks were used to minimise disturbance to fish during sampling. This was ensured by;

A/ using small tanks so that fish could be found and collected quickly

B/ using a sampling order where sampling from the same tank occurred only every fourth time interval.

Fish were fed in the dark every second day. Feeding times were staggered to ensure fish could not entrain to feeding.

3.3.2.4 Melatonin assays

The same sampling procedures used during the determination of retinomotor movement position were repeated except that groups of 4 fish were placed into netted jars at t=11 during the evening before the experiment. On the day of the experiment, fish were sampled at times; t=0, t=2, t=6, t=10, t=12, t=14, t=18, and t=22. This was repeated for the following 24 hours in constant darkness.

Immediately after killing each fish, they were placed in foil capsules and surrounded by embedding compound (Bright, Cyro-M-Bed) and sprayed with freezing aerosol (Cryo-Jet, Lamb's) until frozen. This procedure took less than 1 minute. Frozen specimens were transferred to a -80°C freezer (Sanyo, Ultra Low) until their transfer on dry ice to Tübingen, Germany for melatonin assay of whole eyecups. The melatonin content of eyecups was determined using a melatonin – ELISA-kit (IBL Hamburg) standardised according to ISO and CE protocols. All procedures were carried out by a team led by Prof. H.-J. Wagner of the Graduate School of Neural and Behavioural Sciences and Max Planck Research School, Tübingen.

3.4 Results

3.4.1 light/dark cycle

In the light phase of the cycle, both cones and RPE melanosomes were in light adapted positions. The cone myoid was constricted and the ellipsoid lay close to the outer limiting membrane. RPE melanosomes had dispersed into the long apical projections of the RPE cell and could be seen lying adjacent to the cone ellipsoids (Figure 3.1a).

During the dark phase of the cycle, the cone myoids were elongated and the ellipsoids positioned nearer Bruch's membrane. The RPE melanosomes had withdrawn and accumulated at the base of the RPE cells (Figure 3.1b).

While in transitory states, that is during either movements to light or dark adapted positions, the cones lay in intermediate positions with the myoids partially constricted and the melanosomes in corresponding partial dispersion (Figure 3.1c).

Glowlight tetra cones and RPE melanosomes anticipated 'dawn,' commencing their light adaptive movements approximately 2.25 hours before lights on and were almost fully light adapted by dawn (Figure 3.3). They remained in fully light adapted positions until 0.5 hours after dusk. Thus, neither the cones nor RPE melanosomes predict dusk. Maximum dark adaptive positions for both cone and RPE melanosomes were not reached until 2.0 – 2.5 hours after dusk (Figure 3.1a-c and 3.3).

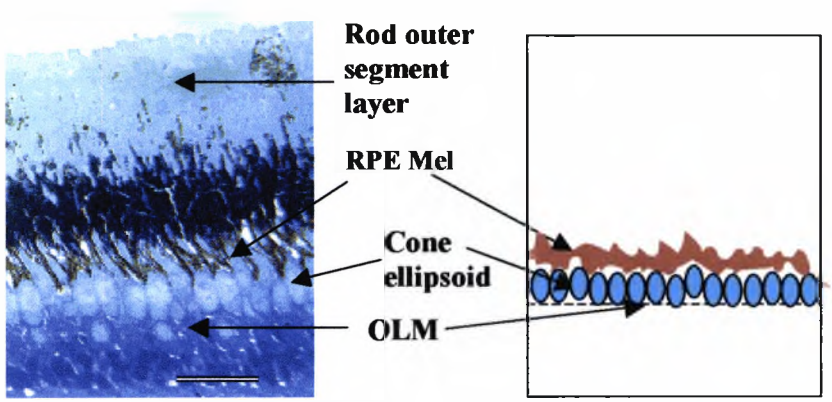

Fig 3.1(a) Light adapted glowlight tetra retina
Transverse section (left) and corresponding schematic diagram (right) of glowlight tetra at t=6 (middle of light phase) during a normal light/dark cycle. Here, and in all subsequent micrographs: OLM outer limiting membrane, RPE Mel. RPE melanosomes. Bar = 25µm.

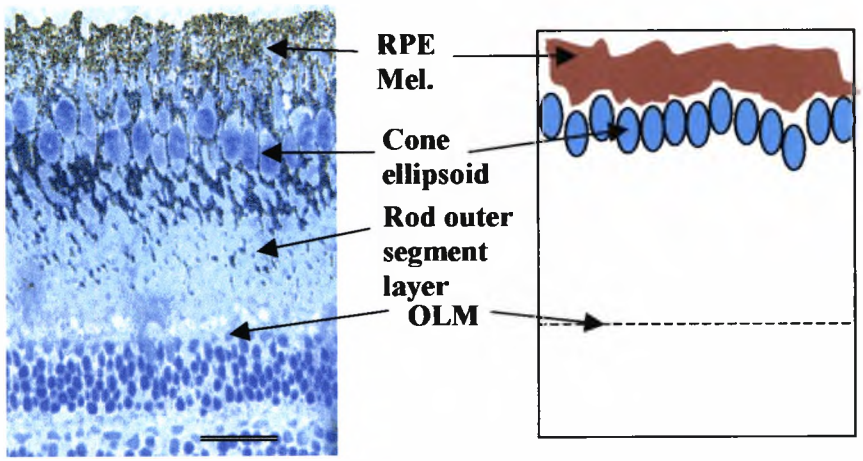

Fig 3.1(b) Dark adapted glowlight tetra retina
Transverse section (left) and corresponding schematic diagram (right) of glowlight tetra at t=18 (middle of dark phase) during a normal light/dark cycle. Bar = 25μm.

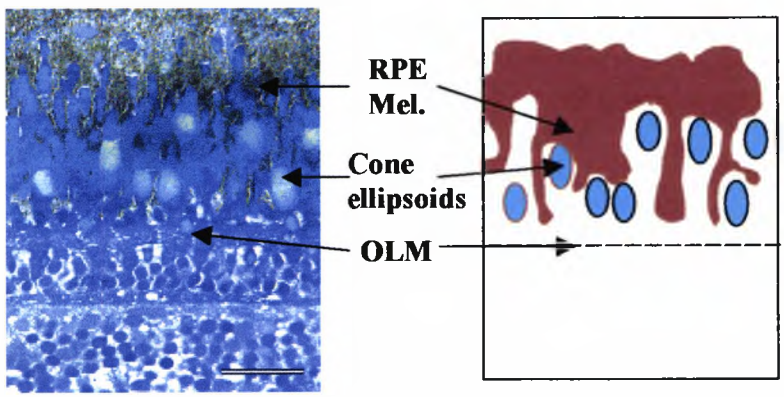

Fig 3.1(c) Intermediate glowlight tetra retina
Transverse section (left) and corresponding schematic diagram (right) of glowlight tetra at t=23 (1 hour before dawn when both cones and melanosomes are moving to light adapted positions) during a normal light/dark cycle. Bar = 25μm.

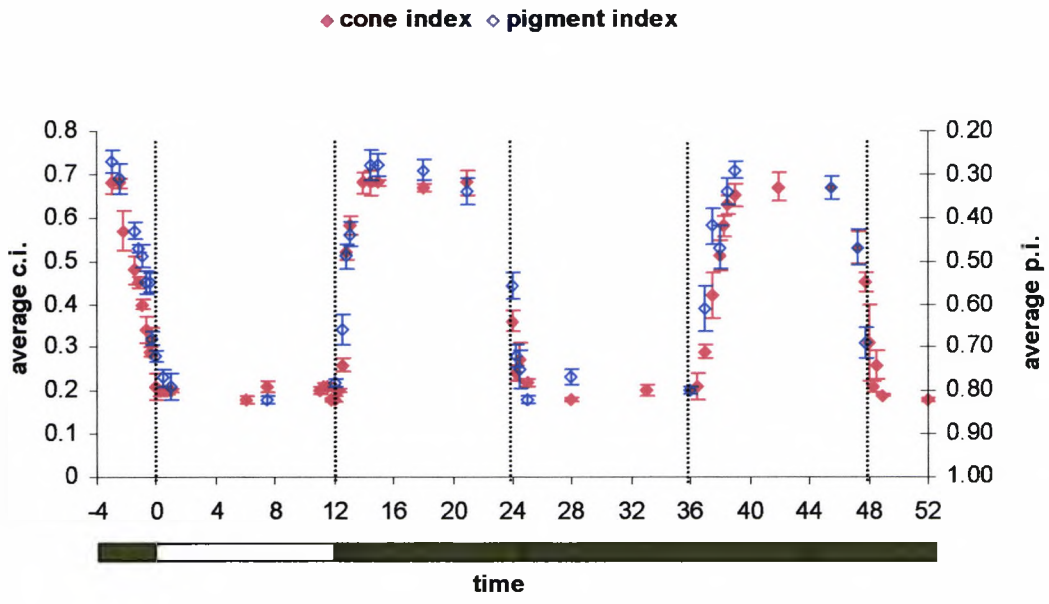

Fig 3-2 Cone and pigment indices during a normal light/dark cycle and in continual darkness

Cone (c.i) and pigment indices (p.i) during a normal light/dark cycle (LD 12:12) followed by 40 hours of continual darkness. Light onset is at t= 0 and the lights are permanently turned off at t=12. Each time point represents the mean ± the standard error (SE) for a minimum of 6 retinas. More retinae were counted at several time points. For the cone index, between 8-17 retinae were counted at the following times; t=-0.50, -0.75 0.00, 0.50, 11.50, 12.00, 12.25, 12.50, 12.75, 13.00, 37.00, and 38.00. Extra retinae for the pigment index were counted (between 9 and 11) at t= -0.75, 0.00, 12.50, 38.00. The dotted lines show the normal lights on and off times and also when they would be expected to come on and off in continual darkness (on at t=0, off at t=12, expected on at t=24 and 48, expected off at t=36) while the light/dark bar shows the light schedule during experimentation.

For statistical analysis, the average cone and pigment indices when both cones and melanosomes were in fully light and dark adapted positions were calculated. For the average light adapted value, the indices during the light/dark cycle from t=0 to t=12 when both cones and melanosomes were fully light adapted were

utilised. The average dark adapted indices were determined during the dark phase of the light cycle from t=14.5 to t=21 when both cones and melanosomes were fully dark adapted. Thus, the average fully light adapted values for the cone and pigment indices are given in table 3.1.

Table 3.1. Average light and dark adapted indices for glowlight tetra during light/dark cycle

	Cone index	**Pigment index**
Average light adapted value	0.20±0.02, n=90	0.79±0.02, n=27
Average dark adapted value	0.68±0.02, n=34	0.29±0.02, n=24

(Note here that average indices are given together with ± standard deviation. Also, note that 'n' is higher for determining the average cone than pigment indices because the average cone index was determined in more retinae than the pigment index (see figure 3.2).

3.4.2 First 40 hours in constant darkness

Both the cones and RPE melanosomes continued their cyclic movements for the first 40 hours in continual darkness closely following the pattern seen during the normal light/dark cycle (Figure 3.2). Thus, light adaptive movements commenced approximately 2.25 hours before dawn, light adapted positions were maintained until 0.5 hours after what would have been dusk, and between 2.0 and 2.5 hours later full dark adapted positions were reached and maintained until predawn migrations commenced. During the subjective day, from t=24-36, the cone and pigments indices were statistically similar to the full light adaptive values at all sampled time points ($p>0.2$ at all points, unpaired Student t test) while from t=38.5-45.5 the cone and pigment indices were similar to the maximum dark value at all points examined ($p>0.2$ at all points, unpaired Student t test). Thus the

amplitude of both rhythms showed no signs of damping as both full light and dark adaptive positions of cones and melanosomes were reached. Cones were first similar to the light adaptive values at t=24 ($p<0.05$, unpaired Student t test) and then again following elongation at t=48($p<0.05$, unpaired Student t test). Therefore, the free-running period is 24 hours (Figure 3.2 and 3.3a-c).

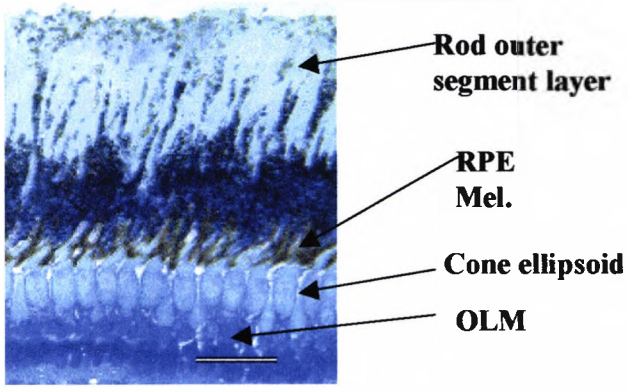

Fig 3-3 (a) Glowlight tetra retina at t =24 during 40 hours of constant darkness

Transverse section of glowlight tetra retina at t=24 (subjective 'dawn' during a normal light/dark cycle). Bar = 25µm.

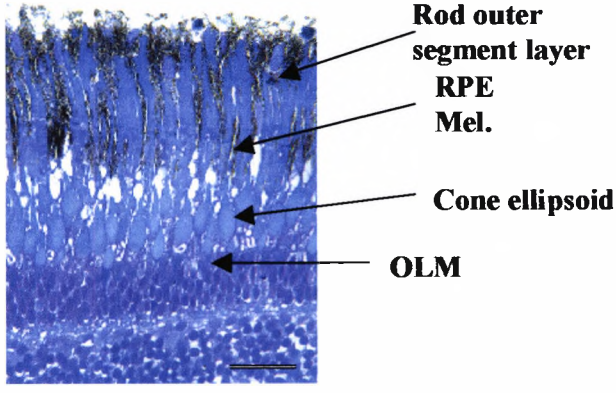

Fig 3-3 (b) Glowlight tetra retina at t =30 during 40 hours of constant darkness

Transverse section of glowlight tetra retina at t=30 (subjective mid-day during a normal light/dark cycle). Bar = 25µm.

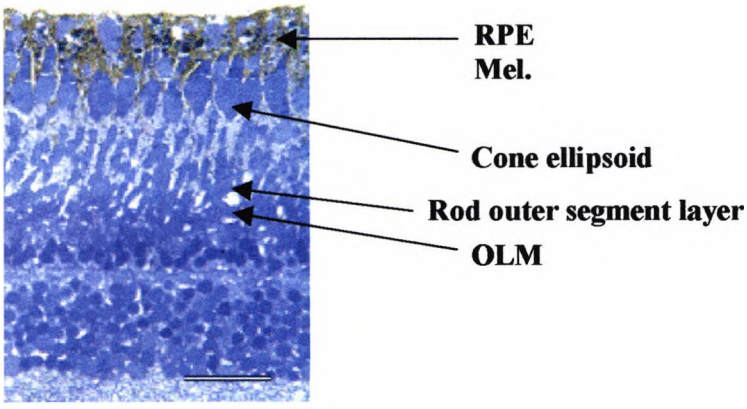

Fig 3-3 (c) Glowlight tetra retina at t =42 during 40 hours of constant darkness

Transverse section of glowlight tetra retina at t=42 (subjective mid-night during a normal light/dark cycle). Bar = 25μm.

3.4.3 7 days of constant darkness

On the 7th day of continual darkness, cones had a similar pattern of movement to that displayed during the light/dark cycle (Figure 3.4). Only at t=0 was there a statistically significant difference in the cone index between fish kept in constant darkness and during the light/dark cycle ($p<.001$, unpaired Student t test). In constant darkness, RPE melanosomes mostly followed the pattern of movements seen during the light/dark cycle except at t=0 (subjective dawn) where the melanosomes were significantly less light adapted ($p<.001$, unpaired Student t test) and at t=18(subjective midnight) where the melanosomes were significantly less dark adapted than during the light/dark cycle ($p<.001$, unpaired Student t test) (Figures 3.5 and 3.6). The inability to reach maximum dark adapted values at t=18 indicates that the amplitude of the RPE melanosome rhythm may be beginning to

dampen and it would have been interesting if further time points were sampled over the next 24 hours (day 8 in constant darkness) to see if this indeed was the case.

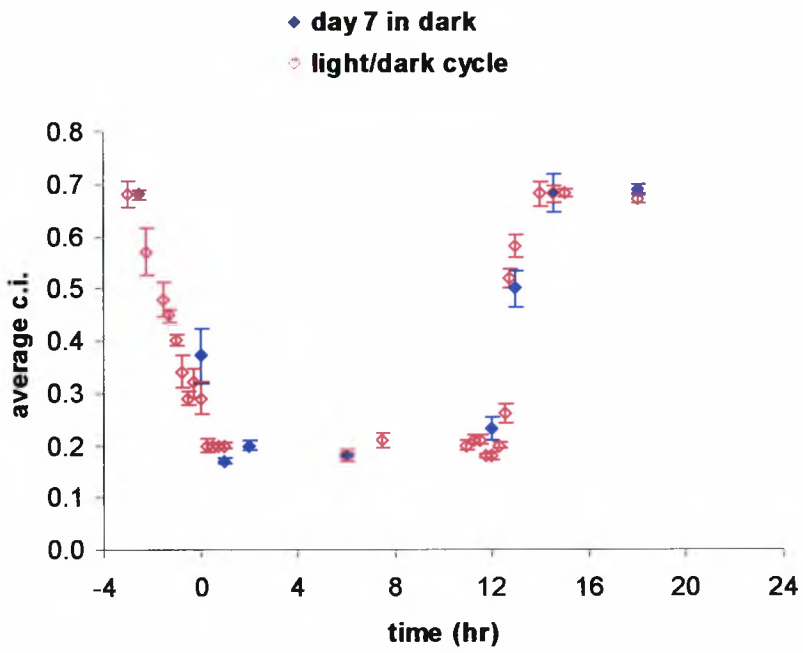

Fig 3-4 Cone indices during the 7th day in constant darkness
Cone indices (c.i.) during the 7th day in continual darkness in comparison to during the normal LD 12:12 schedule. t=0 is the time the lights would normally go on and t=12 the time the lights normally go off during the light/dark cycle. Each point during the dark experiment represents the mean and ± SE for 6 retinae.

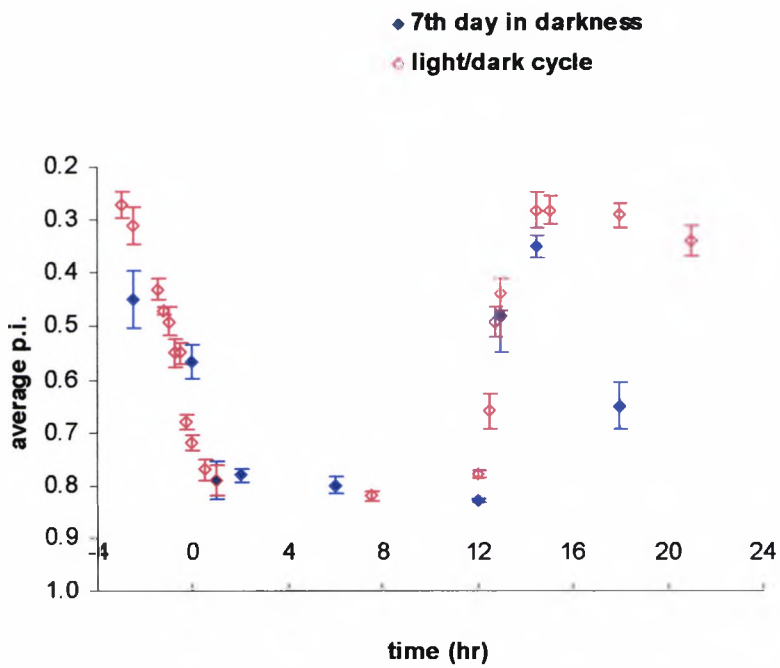

Fig 3-5 Pigment indices during the 7th day in constant darkness
Pigment indices (p.i) during the 7th day in continual darkness in comparison to during the normal LD 12:12 schedule. t=0 is the time the lights would normally go and t=12 the time the lights normally go off during the light/dark cycle. Each point during the dark experiment represents the mean and ± SE for 6 retinae.

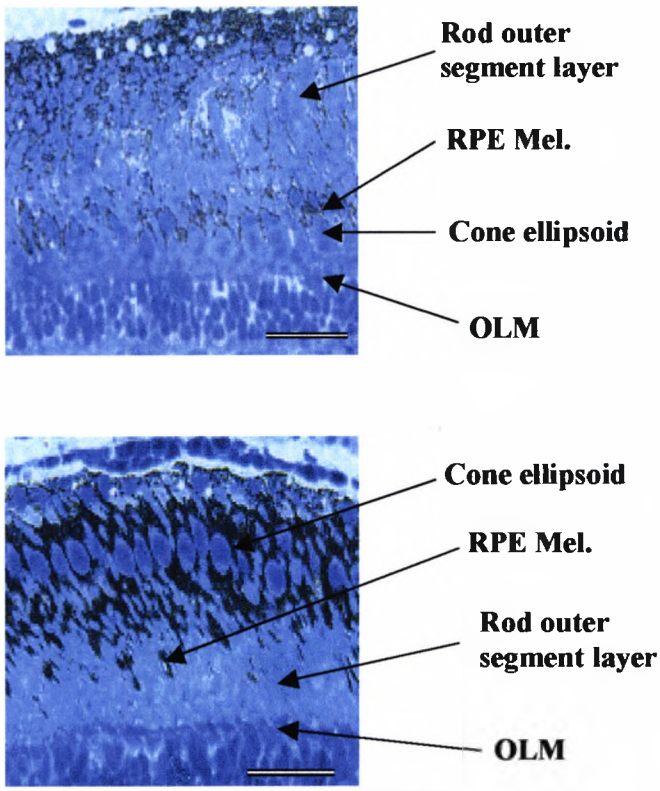

Fig 3-6 Glowlight tetra retinas during 7th day in constant darkness
Transverse sections of glowlight tetra retinae at various times throughout the 7th day in constant darkness. The top micrograph is the retina at t=6 (normally the middle of the light phase), and micrograph on the bottom is at t= 18 (the usual middle of the dark phase of the light/dark cycle). Bar=25μm.

3.4.4 14 days of constant darkness

Cones maintained their basic cyclic rhythm matching that seen during a normal light/dark cycle even after 14 days in constant darkness. The cone index at nearly all times measured was statistically similar to that during the light/dark cycle ($p>0.1$ for t=-2.5 and 23.5 and $p>0.2$ for the others, unpaired Student t test) except for at t=0 where the cones were significantly less light adapted ($p<.05$, unpaired

Student t test). The RPE melanosome rhythm, on the other hand, showed signs of amplitude damping as full dark adaptation was never reached. During the subjective dark phase at t=-2.5 (2.5 hours before subjective dawn), t=13 (1hour after subjective dusk), and t= 18 (subjective midnight), the pigment index was significantly more light adapted than the corresponding times in the light/dark cycle ($p<.001$, unpaired Student t test). At t=14.5, the pigment index was also significantly less than that found during a light/dark cycle ($p<.05$, unpaired Student t test) (Figures 3.7-3.9).

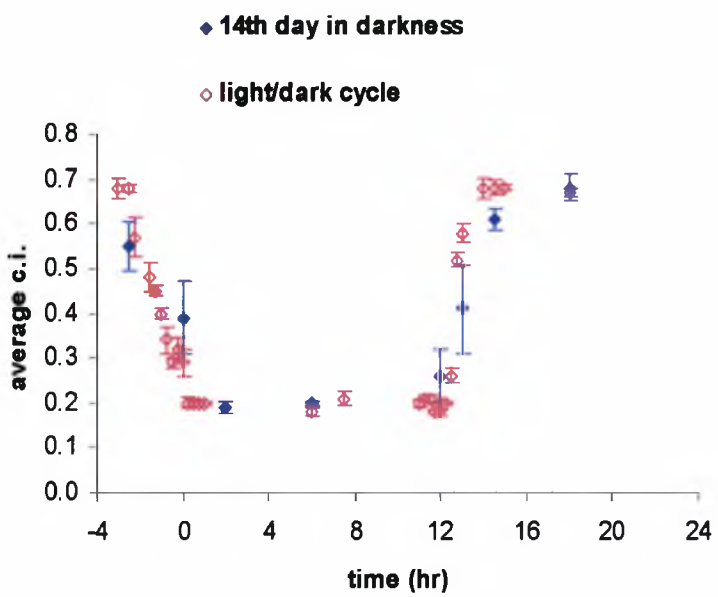

Fig 3-7 Cone indices during the 14th day in constant darkness
Cone indices (c.i.) during the 14th day in continual darkness in comparison to during the normal LD 12:12 schedule. t=0 is the time the lights would normally go on and t=12 the time the lights normally go off during the light/dark cycle. Each point during the dark experiment represents the mean ± SE for 6 retinae.

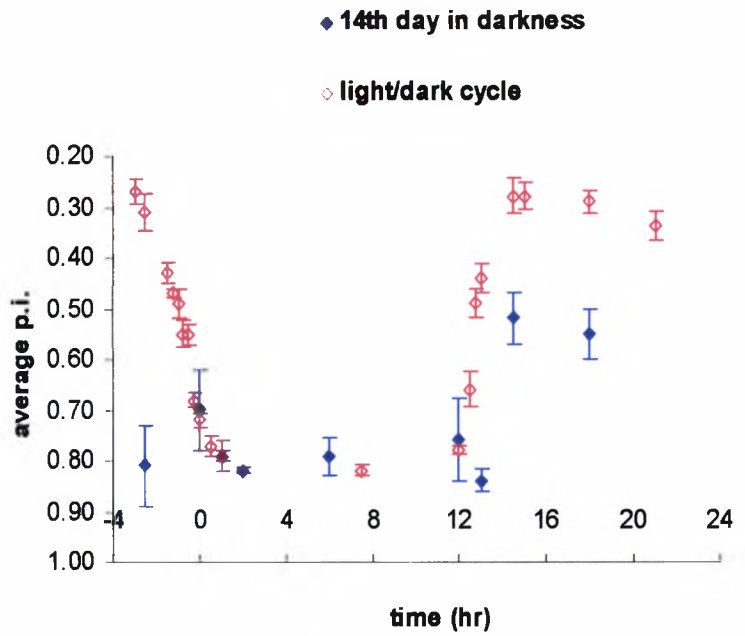

Fig 3-8 Pigment indices during the 14th day in continual darkness
Pigment indices (p.i.) during the 14th day in continual darkness in comparison to during the normal LD 12:12 schedule. t=0 is the time the lights would normally go on and t=12 the time the lights normally go off during the light/dark cycle. Each point during the dark experiment represents the mean ± SE for 6 retinae.

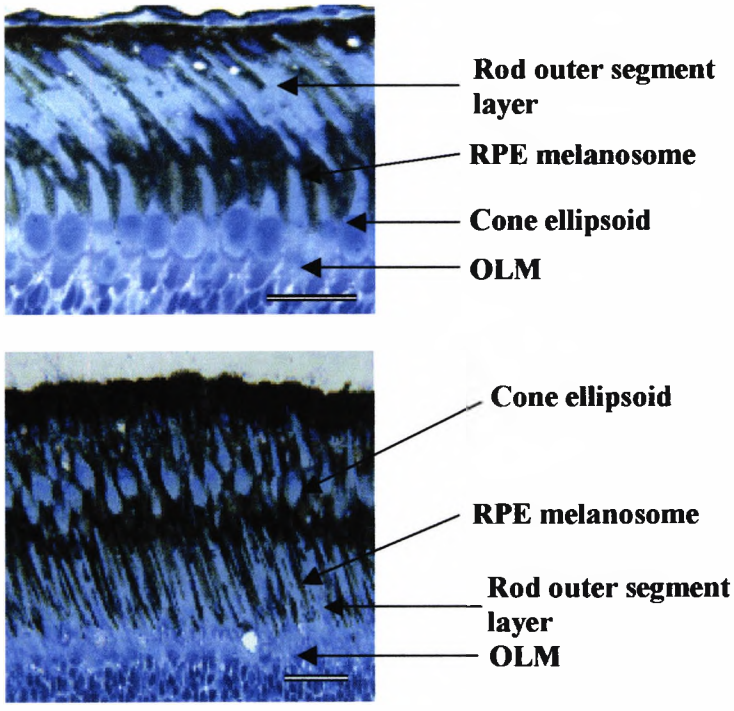

Fig 3-9 Glowlight tetra retinas during the 14th day in constant darkness
Transverse sections of glowlight tetra retinae at various times throughout the 14th day in constant darkness. The top micrograph is the retina at t=6 (normally the middle of the light phase), and the micrograph on the bottom is at t= 18 (the usual middle of the dark phase of the light/dark cycle). Bar=25μm.

3.4.5 28 days of constant darkness

Both the cone and RPE melanosome remained in their fully light adapted positions during both the subjective day and night after 28 days of constant darkness (Figures 3.10-3.12).

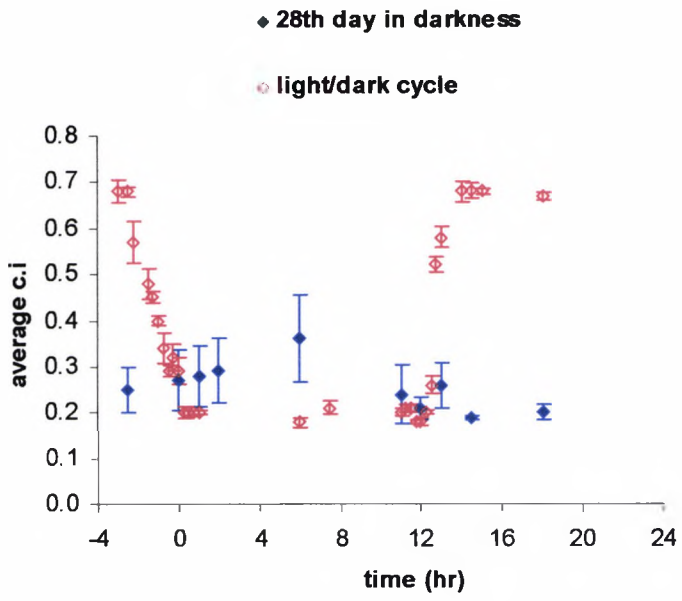

Fig 3-10 Cone indices during the 28th day in continual darkness
Cone indices (c.i.) during the 28th day in continual darkness in comparison to during the normal LD 12:12 schedule. t=0 is the time the lights would normally go on and t=12 the time the lights normally go off during the light/dark cycle. Each point during the dark experiment represents the mean ± SE for 6 retinae.

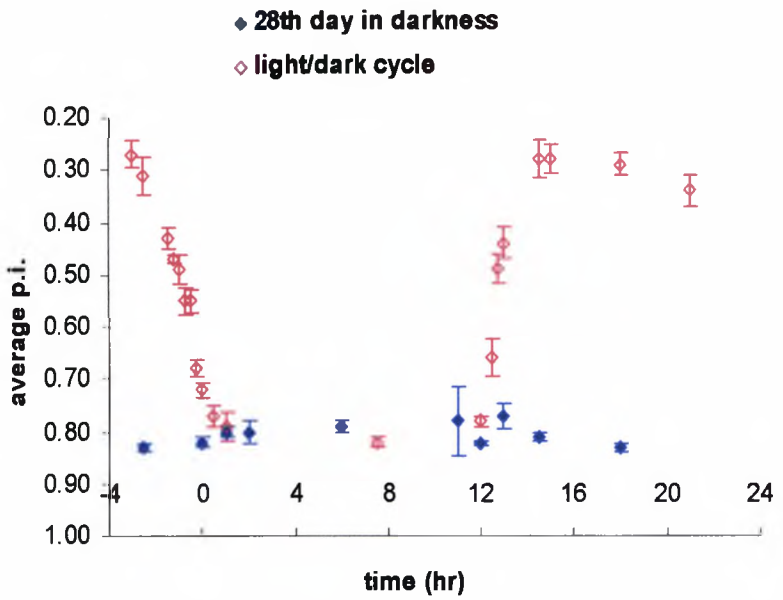

Fig 3-11 Pigment indices during the 28th day in continual darkness
Pigment indices (p.i.) during the 28th day in continual darkness in comparison to during the normal LD 12:12 schedule. t=0 is the time the lights would normally go on and t=12 the time the lights normally go off during the light/dark cycle. Each point during the dark experiment represents the mean ± SE for 6 retinae.

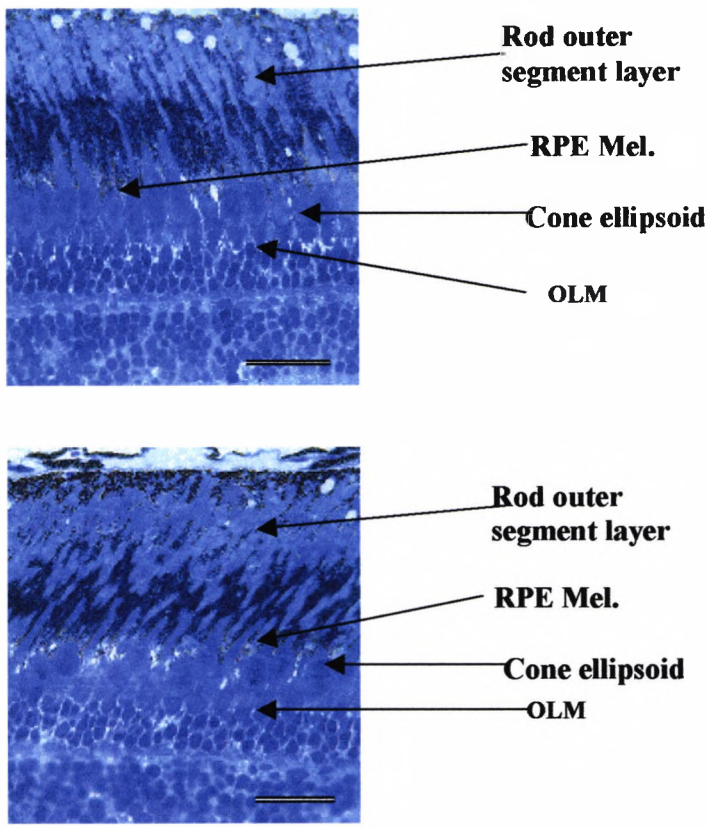

Fig 3-12 Glowlight tetra retinas during the 28th day in continual darkness Transverse sections of glowlight tetra retinae at various times throughout the 28th day in constant darkness. The top micrograph is the retina at t=6 (normally the middle of the light phase), and the bottom is at t= 18 (the usual middle of the dark phase of the light/dark cycle). Bar=25μm.

3.4.6 Constant light experiment

Constant light abolished the rhythms of both the cones and RPE melanosome movements. Both remained fully light adapted for the duration of the experiment (Figures 3.13).

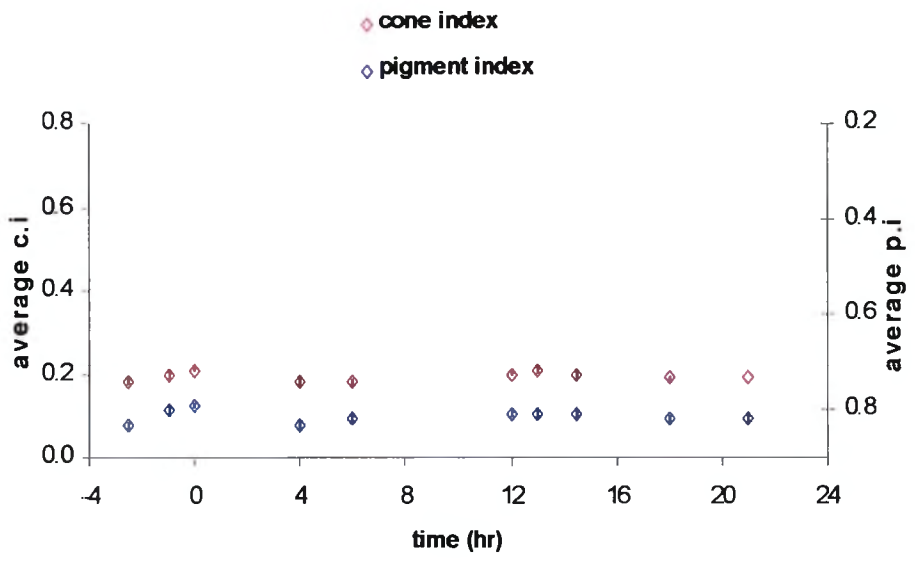

Fig 3-13 Cone and pigment indices during 24 hours of constant light
Cone (c.i.) and pigment (p.i.) indices during 24 hours of constant light. Each point during this experiment represents the mean ± SE for 6 retinae.

3.4.7 Ocular melatonin

Ocular melatonin content remained constant throughout a normal light/dark cycle (one way ANOVA) with no difference between the light and dark phase content (Figure 3.14). In comparison, most teleosts show peak levels of ocular melatonin during the dark phase of the cycle (Cahill, 1996; Iigo et al, 1997a, c, and d) (Figure 3.15).

In constant darkness, one way ANOVA showed differences in the ocular melatonin content and as such a post-hoc Bonferroni test was applied (pairs where differences were found are given in the appendix, table A2). Again, there is no clear difference between the subjective light and dark phase melatonin levels.

However, at t42 (subjective midnight), the ocular melatonin levels are significantly higher than all times except for t38 (Figure 3.14). In other teleosts, such as the goldfish (Iigo et al, 1997d-Figure 3.15), ocular melatonin content in constant darkness is higher during subjective night than subjective day, but the differences are less marked than during the light/dark cycle, indicative of damping.

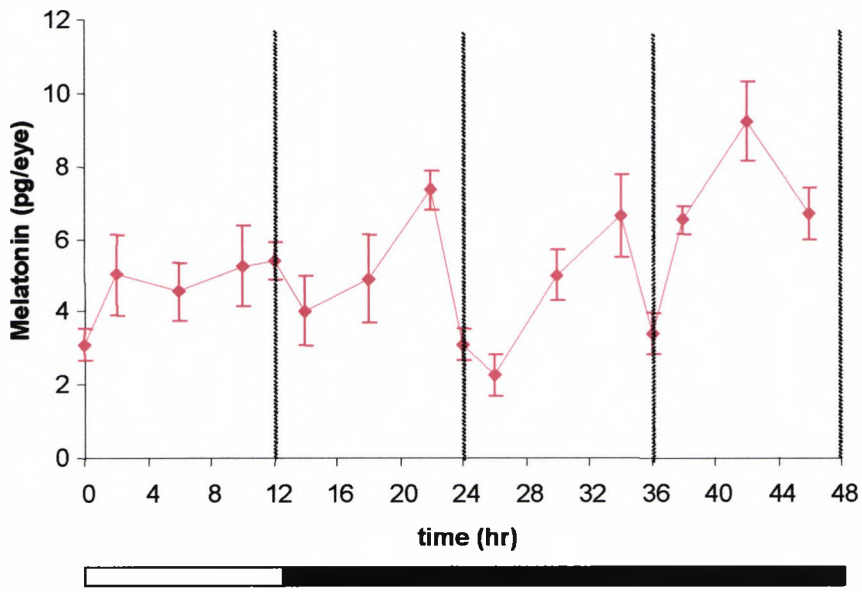

Fig 3-14 Ocular melatonin content
Ocular melatonin content during a normal LD 12:12 light cycle and in constant darkness. Here, each bar represents the mean ± SE for 8 retinae. The light/shade bar represents the light schedule given during the course of the experiment. The dotted lines give the lights-on (24 and 48) and lights-off (12 and 36) times during a light/dark cycle.

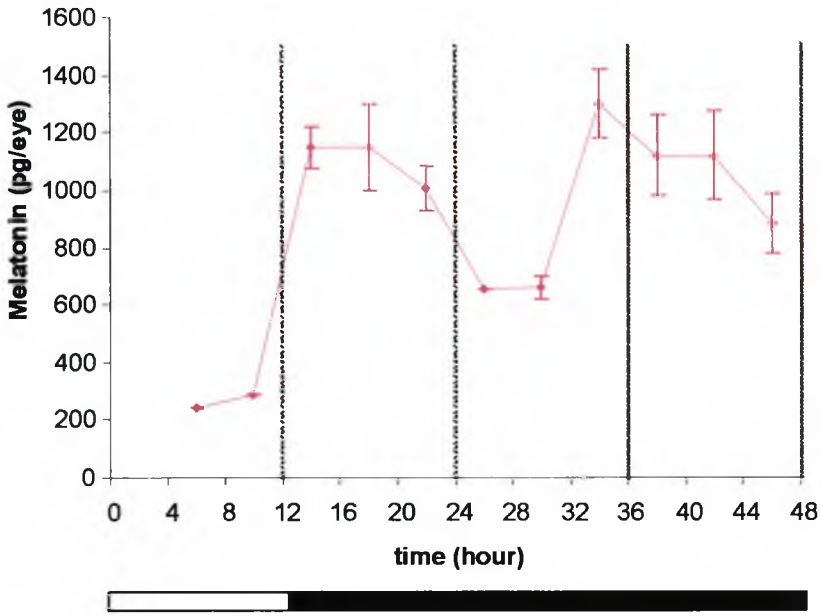

Fig 3-15 The ocular melatonin content in goldfish
The ocular melatonin content during a normal light/dark cycle and in constant darkness in the goldfish (*Carassius auratus*). Ocular melatonin values were determined by radioimmunoassay. Here, each time point represents the mean±SE for 10 retinae. The light/shade bar represents the light schedule given during the course of the experiment. The dotted lines give the lights-on (24 and 48) and lights-off (12 and 36) times during a light/dark cycle. Modified from Iigo et al, 1997d.

3.5 Discussion

Since 1937 when retinomotor movements were first found to be under endogenous control (Welsh and Osbourn, 1937), there has been a plethora of studies showing this to be prevalent amongst both fish and amphibia (for reviews see Burnside and Nagle, 1983; Wagner et al, 1992a; Cahill and Besharse, 1995).

The tables below give a summary of the major findings for the persistence of cone (Table 3.2) and RPE melanosome (Table 3.3) rhythms in constant darkness for various fish species. Their geographical distribution has been taken from Fishbase (2004). In the tables below, the duration of the rhythm gives how long the rhythm was seen to persist in constant darkness. It does not give the maximum time the rhythm persisted in constant darkness as in nearly all studies, experiments were terminated before the rhythm had extinguished. Also, the onset of damping represents when the amplitude of the rhythm in constant darkness was first seen to decrease.

Table 3.2 Duration and onset of damping of cone retinomotor movements in constant darkness.

Species	Distribution (Latitude)	Rhythm duration for at least	Onset of damping	Authors
Catfish (*Ameiurus nebulosus*)	54°N to 25°N	2 days	24 hours	Welsh and Osborne (1937)
Catsfish (*Ameiurus nebulosus*)	54°N to 25°N	4 days	24 hours	Arey and Mundt (1941)
Goldfish (*Carassius auratus*)	35°N to 23°N	3 days	24 hours	John et al (1967)
Bluefish (*Pomatomus saltatrix*)	45°N to 45°S	1 day and 15.5 hours	24 hours	Olla and Marchioni (1968)
Bluegill (*Lepomis macrochirus*)	50°N to 25°N	1day and 20 hours	0-24 hours	John and Gring (1968)
Mexican tetra (*Astyanax mexicanus*)	36°N to 24°N	6 days	24 hours	John and Kamister (1969)
Rainbow trout (*Oncorhynchus mykiss*)	63°N to 32°N	2 days	24 hours	Douglas (1982a)
Midas cichlid (*Cichlasoma citrenellum*)	15°N to 8°N	3 days	0-24 hours	Levinson and Burnside (1981); McCormack and Burnside (1991)
Goldeneye cichlid (*Nannacara anomala*)	8°N to 5°N	3 days	No damping	Douglas and Wagner (1982)
Neon tetra (*Paracheirodon innesi*)	17°S to 3°S	2 days	24 hours	Lythgoe and Shand (1983)
Green sunfish (*Lepomis cyanellus*)	50°N to 26°N	1 day	0-24 hours	Burnside and Ackland (1984); Dearry and Barlow (1987)
Blue acara (*Aequidens pulcher*)	12°N to 5°N	2 days	0-24 hours	Douglas et al, (1992)

Table 3.3 Duration and onset of damping of RPE melanosome movement in constant darkness.

Species	Distribution (Latitude)	Rhythm duration for at least	Onset of damping	Authors
Catsfish (*Ameiurus nebulosus*)	54°N to 25°N	0 days	0	Arey and Mundt (1941)
Bluefish (*Pomatomus saltatrix*)	45°N to 45°S	0	0	Olla and Marchioni (1968)
Bluegill (*Lepomis macrochirus*)	50°N to 25°N	1day and 20 hours	0-24 hours	John and Gring (1968)
Mexican tetra (*Astyanax mexicanus*)	36°N to 24°N	6 days	24 hours	John and Kamister (1969)
Rainbow trout (*Oncorhynchus mykiss*)	63°N to 32°N	2 days	24 hours	Douglas (1982a)
Goldeneye cichlid (*Nannacara anomala*)	8°N to 5°N	3 days	24 hours	Douglas and Wagner (1982)
Neon tetra (*Paracheirodon innesi*)	17°S to 3°S	2 days	0	Lythgoe and Shand (1983)
Green sunfish (*Lepomis cyanellus*)	50°N to 26°N	0 day	0	Burnside and Ackland (1984); Dearry and Barlow (1987)
Blue acara (*Aequidens pulcher*)	12°N to 5°N	2 days	0-24 hours	Douglas et al, (1992)

Thus, in constant darkness, the persistence of the rhythms of retinomotor movements without damping is longest for cones in both the glowlight tetra and the goldeneye cichlid, and for melanosome movement in the glowlight tetra. The glowlight tetra (1°N-5°N) and the goldeneye cichlid (5°N-8°N) both originate in Guyana and are the most equatorial species studied to date and therefore experience the most constant daylengths in comparison to other species. One previous study has suggested that the persistence of retinomotor movements in constant darkness is related to the consistency of the light environment within an animal's habitat (Douglas and Wagner, 1982). Thus, retinomotor movements in

constant darkness may persist for longer periods in equatorial species in comparison to more temperate ones.

In the introduction, it was suggested that the duration of retinomotor movements in constant darkness could give an indication to the strength of the circadian drive governing them (section 3.2). This suggests that equatorial species have stronger circadian drive controlling their retinomotor movements than do temperate species. One reason for this may be found by examining the duration of dawn which depends on latitude. The further from the equator, the longer the duration of dawn. Also, there is a greater variability in the duration of dawn further from the equator. For example, in the northern hemisphere, the longest periods of dawn occur in December and June whilst the shortest occur in March and September (see table 3.4 for an example of the duration of dawn at the equator (0°), and 60° from the equator).

Table 3.4 Astronomical dawn duration at the equator and 60° from the equator*

Date	**0°**	**60°**
March 21	69 mins	146 mins
June 21	75 mins	(The sun is never 18° below the horizon)
September 23	69 mins	146 mins
December 21	75 mins	166 mins

(* values taken from U.S. Naval Observatory; Astronomical dawn commences in the dark when the sun is 18° below the horizon).

As table 3.4 shows, the closer to the equator, the shorter the duration of dawn. Thus, equatorial species have less time to prepare for daylight in comparison to temperate species. It may therefore be more important for equatorial species to have clocks controlling their retinomotor movements to help prepare for the arrival of daylight in comparison to more temperate species where dawn lasts for

longer which allows more time to move retinomotor elements to the appropriate positions.

Circadian drive of retinomotor movements may be a reflection of the oscillator governing them or it might represent a more tightly coupled relationship between the 'hands of the clock', the retinomotor elements, and the oscillator. If the former were true, this would imply that equatorial species have stronger oscillators governing retinomotor movements than those found in temperate species. The latter suggests that equatorial and temperate species may have oscillators with the same strength but these are more tightly coupled to the retinomotor movmements in equatorial species.

Alternatively, there may not be any difference between temperate and equatorial circadian drive of retinomotor movements. Rather, different retinomotor movement circadian drive may simply be species specific. The different strength of the circadian drive of retinomotor movements may in turn be a reflection of both the hierarchy and interaction between different oscillators within a particular species, as seen in the oscillators regulating melatonin in birds. In birds, there are multiple oscillators involved in the regulation of melatonin located in the eyes, pineal gland, and an SCN-like structure. Even in closely related species, melatonin oscillators have varying importance and may interact differently. For example, in the house sparrow, pinealoctomy will abolish circadian rhythmicity (Gaston and Menaker, 1968), whereas in the quail removal of the pineal and both eyes is required to remove rhythmicity (Underwood, 1994). Thus, the ocular oscillator is capable of maintaining rhythmicity (via production of ocular

melatonin which enters the bloodstream) in quail but not in house sparrows. A similar mechanism may be acting in fish. In fish, circadian rhythmicity is believed to be regulated by two or more groups of loosely coupled oscillators synchronized to external timing cues (Tabata, 1992). As previously described (chapter 1), circadian oscillators have been demonstrated in the retina and pineal gland of a large number of species (Bolliet et al, 1996; Cahill et al, 1991) and also in the brain of some fish (Tabata et al, 1991). In different species of fish, the coupling and/or hierarchal order of these oscillators may vary. In the catfish (*Heteropneustes fossilis*) (Garg and Sundararaj, 1986) pinealectomy causes breakdown of circadian rhythms, whereas removal of the pineal organ altered the free-running period in the lake chub (*Couesius plumbeus*) (Kavaliers, 1979) and caused splitting of free-running activities in the white sucker (*Catostomus commersoni*) (Kavaliers, 1981). To destroy circadian rhythmicity in the catfish (*Silurus asotus*), pinealectomy, removal of both eyes, and shielding the brain from external light is required (Tabata et al, 1991). Further, in the catfish (*Silurus asotus*), the degree to which the pineal gland and the lateral eyes contribute to the circadian rhythmicity varies according to the entraining light conditions. In dim light, the pineal gland is responsible for most of the fish's circadian rhythmicity while in bright lights the lateral eyes play a bigger role (Tabeta et al, 1991). Therefore, strong circadian drive controlling retinomotor movement may not necessarily reflect an ecological adaptation to the photo environment but rather a different system of coupling and possibly hierarchal order of oscillators governing circadian rhythmicity.

Table 3.5 shows all species in which the rhythm of either cone or pigment

migration was found to persist in constant light. No persistence of either cone or melanosome movement rhythms were seen in the glowlight tetra. Previously, it has been suggested that the persistence of retinomotor movements in constant light indicates strong circadian drive (Wagner et al, 1992a). If this were true, then this clearly contradicts what was found for the persistence of retinomotor movements in constant darkness (in constant darkness, the glowlight tetra has the strongest circadian drive controlling both cone and RPE melanosome movement yet in constant light, even the catfish appears to have more robust circadian drive). One possible explanation could be that light masks the effect of the oscillators more in the glowlight tetra than in other species (in the next chapter, it is shown that light does mask rather than stop the oscillators controlling cone movement in glowlight tetras). A simple explanation could be the intensity of lights used in the experiments. For example, brighter lights would suppress the rhythm more than dim lights. Unfortunately, none of the previous studies which showed a persistence of retinomotor movements in constant light give any details of the light intensity used.

Table 3.5 Duration and degree of persistance of retinomotor movements in constant light.

Species	**Cone or RPE melanosome**	**Duration of rhythm for at least;**	**% Normal amplitude**	**Authors**
Catsfish (*Ameiurus nebulosus*)	cone	<24 hours	Not given	Arey and Mundt (1941)
Midas cichlid (*Cichlasoma citrenellum*)	cone	24 hours	34%	Levinson and Burnside (1981)
Goldeneye cichlid (*Nannacara anomala*)	Both	72 hours	38% cones and 48% RPE melanosomes	Douglas and Wagner (1982)

In isolated pineal glands, damping of the rhythm of melatonin production is thought to be due to desynchronization between individual pineal photoreceptors and melatonin may act as a coupling device among the photoreceptor cell population (Bolliet et al, 1996). A similar situation may apply to both the cones and RPE melanosome oscillators. Currently, there is evidence that the cones are the site of melatonin synthesis and that the rods and/or cones are required for the rhythmic synthesis of melatonin and that melatonin has a role in the circadian regulation of retinomotor movements (see section 1.11). Thus it is possible, although more evidence is required, that the cones contain an oscillator regulating melatonin synthesis. In this case, the melatonin synthesised in each cone could feed back onto the cone resulting in the movement seen and may also serve as a 'glue' to couple groups of cone oscillators which in turn would synchronize cone movements amongst the population. Thus, damping of the cone rhythm could be a result of desynchronization of individual oscillators. In this case, individual cone oscillators would continue to oscillate but would become out of phase with other cone oscillators causing a lack of synchrony in cone movements amongst the population leading to damping of the rhythm. Likewise, melatonin produced by the cones could also couple groups of RPE melanosome oscillators and damping of this rhythm may also follow desynchronization of individual oscillators. Interestingly, in some retinae examined during the 28^{th} day in constant darkness, there were groups of about 40 fully light-adapted cones situated alongside 40 or so fully dark-adapted cones suggesting desynchronization between large groups of cones (personal observation). Alternatively, damping of the rhythm of both cone and melanosomes may be due to damping of the oscillators themselves. In this

case, the molecular 'machinery' may be running down in constant darkness causing the damping of rhythms seen.

In many species, the damping of the RPE melanosome rhythm in constant darkness occurs earlier than for the corresponding rhythm of cones. Also, in most species, RPE melanosomes do not commence light adaptive movements until after dawn, whilst in the same species cones commence their movement before the onset of dawn. Both of these suggest that melanosomes and cones are controlled by different oscillators. Alternatively, both the cones and melanosomes may be under the same oscillator controlling the rhythmic production of melatonin in the cones as described above. One explanation for earlier damping in the melanosomes could be the distance from the site of rhythmic melatonin production to the site of receptor activation. If melatonin is produced in the cones and feeds back on the cones and the RPE melanosomes, then melatonin would be required to diffuse further to influence the melanosomes. Perhaps in constant darkness, the extra distance that melatonin must diffuse to influence the RPE melanosomes means that it is more likely to become obstructed by another particle/substance which is normally removed during the light phase of the cycle. For example, inside the leopard frog (*Rana pipiens*) myeloid bodies, which are widely distributed in the retinal pigment epithelium, phagosomes and oil droplets, change in size in constant darkness (Cai and Dickson, 1994). These changes in the myoid bodies in constant darkness may interfere with the diffusion of melatonin and thus cause the earlier damping of the rhythm of melanosomes in comparison to the cone rhythm.

3.5.1 Melatonin

During a normal light/dark cycle, the glowlight tetra ocular melatonin content remained constant. In comparison, many other species showed peak values during the night; the zebrafish (*Danio rerio*) (Cahill, 1996), the goldfish (*Carassius auratus*) (Iigo et al, 1997d), the ugui (*Tribolodon hakonensis*) (Iigo et al, 1997c) and the oikawa (*Zacco platypus*) (Iigo et al, 1997b). Peak values, on the other hand, were noted in the day for the rainbow trout (*Oncorhynchus mykiss*) (Gern et al, 1978), the brook trout (*Salvelinus fontinalis* Mitchell) (Zachman et al, 1992a) and the rainbow trout (*Oncorhynchus mykiss*)(Zaunreiter et al, 1998b). In constant darkness, the ocular melatonin content remained similar throughout the duration of the experiment although a significant rise was noted at subjective midnight. Other species have kept the same pattern in constant darkness as seen during the light/dark cycle; goldfish (*Carassius auratus*) (Iigo et al, 1997d), zebrafish (*Danio rerio*) (Cahill, 1996), the ugui (*Tribolodon hakonensis*) (Iigo et al, 1997c) and the rainbow trout (*Oncorhynchus mykiss*) (Zaunreiter, 1998a). The pattern of ocular melatonin content observed in the glowlight tetra is clearly very different to that observed in other species during both the light/dark cycle and in constant darkness and in no simple way relates to the observed retinomotor rhythms.

Chapter 4-The light control of cone and RPE melanosome movement in the glowlight tetra (*Hemigrammus erythrozonus*)

4.1 Abstract

The light control of both cone and RPE melanosome movements in the glowlight tetra was investigated by subjecting fish to light during the dark phase and darkness during the light phase of the light/dark cycle. 100 minutes of light exposure in the middle of the dark phase had no effect on the positions of the cones, whilst melanosomes were fully light adapted within 45 minutes. Thus, cone movement is not effected by light in the middle of the dark phase while RPE melanosome movement is. In comparison, 100 minutes of darkness in the middle of the light phase had no effect on either cone or melanosome movements as both cones and melanosomes remained stationary.

To determine if there is a circadian rhythm in the effects of light on cone movement, fish were exposed to light or placed into darkness at various times during the light/dark cycle. Cone movement was most influenced by light or dark during times which corresponded to the dawn and dusk transitions.

The effect of the light/dark schedule on cone movements was also examined. Fish were entrained to a light/dark cycle for 30 days before phase shifting the light cycle by 180°. The rhythm of cone movements was found to re-entrain to the new light schedule within 14 days.

4.2 Introduction

This chapter is divided into two parts. In the first, retinomotor, in particular cone, movements were investigated in order to determine whether they show a circadian rhythm to light exposure. Thus, glowlight tetras were either exposed to light at various times throughout the dark phase or they were placed into darkness at various times throughout the light phase of the light/dark cycle.

Previously, several studies have interpreted the procedures outlined above as an indicator to the strength of the circadian oscillator controlling retinomotor movements or spinule (see chapter 7) dynamics (for example, Levinson and Burnside, 1981; Douglas and Wagner, 1983; McCormack and Burnside, 1991). In particular, cone length was thought to be a function of both an endogenous (oscillator) and a directly light-driven component. Each of these constituents sent a signal to the retinomotor elements and the resultant combination would determine the overall length of the cones or positions of RPE melanosomes (Levinson and Burnside, 1981). For example, 20 minutes of light in the middle of the dark phase caused cones to fully constrict to their normal light adapted levels and this was taken to be indicative of a relatively small endogenous component and a larger light component controlling cone movements in the midas cichlid (*Cichlasoma citrenellum*) (McCormack and Burnside, 1991).

Although these experiments may be interpreted as demonstrating the presence of circadian control of cones, the effect of light exposure on the oscillator itself has

been ignored. A pulse of light, of either 0.25 or 1 hour duration, will phase shift activity rhythms of animals maintained in constant darkness (see section 1.7). Similarly, dark pulses on animals kept in constant light will shift their activity rhythms (Johnson et al, 1989). Although the experiments described above were conducted during a normal light/dark cycle, the pulse of light or 'dark' may have reset the underlying oscillators. Thus, rather than interpret the results as a gauge to the oscillator signal strength, another interpretation may be that these experiments are merely resetting the oscillator. The resultant movement or non-movement of retinomotor elements following the pulse of light or dark may give an indication to the sensitivity of the oscillators to light or dark at the particular phase the pulse was administered. However, as the oscillators were not isolated, the resultant retinomotor movements may be reflecting the coupling or uncoupling between the oscillator and the retinomotor elements. That is, light may be causing the retinomotor elements to uncouple from the oscillators. Thus, a better interpretation to these experiments could simply be that they are probing the light control of retinomotor movements. Therefore, for example, in the midas cichlid cones are strongly influenced by light as light in the middle of the dark phase caused maximum light adaptive levels within 20 minutes (McCormack and Burnside, 1991).

The first experiments described here involved exposing glowlight tetras to 100 minutes of light in the middle of the dark phase and placing them into darkness for 100 minutes in the middle of the light phase. The main purpose is to compare both the cone and RPE melanosome movements to similar experiments in other species carried out by other workers. As shown previously (chapter 3), both

glowlight tetra cones and RPE melanosomes have strong circadian drive controlling them in comparison to other species. Perhaps strong circadian drive also means that light has only a weak influence on the cones which would help to explain the persistent rhythms of cone and melanosome movement in constant darkness. The next set of experiments involves light exposure and dark placement at different times of the light/dark cycle to determine if there is a rhythm in the light control of cone movement. Are there particular times in the rhythm of cone movements which are more sensitive to light?

In the second part of this chapter, after 30 days of entrainment to a light/dark cycle, glowlight tetras are entrained to a new light cycle with a 180° phase shift. If light has little influence on cone movement, then adjusting to a 180° phase shift may be a slower process as this relies entirely on the oscillator as masking by light would not be expected to play a role. There are no previous experiments of this kind in fish and as such the results are compared to similar experiments in other animal systems.

4.3 Materials and Methods

4.3.1 Light exposure and dark placement

All glowlight tetras were entrained to a 12-hr light/ 12-dark (LD 12:12) regime for a minimum of 30 days. Groups of three fish were placed into netted jars within their home tank during the light phase of the cycle prior to the experiment. For experiments which required light exposure during the dark phase, the home tank

lights were turned on to give an illumination of 0.38-0.47mW/cm^2. Placement in dark during the light phase was achieved by simply turning the lights off in the home tanks. After sampling at selected intervals, fish were prepared for light microscopy as described previously.

4.3.1.1 Short term light exposure and dark placement during the middle of the dark and light phase

Fish were exposed to light for 100 minutes beginning at t=18 (the middle of the dark phase) or placed in darkness for 100 minutes beginning at t=6 (the middle of the light phase). This experiment was repeated on three occasions.

4.3.1.2 Prolonged light exposure at various times throughout the dark phase

Glowlight tetras were exposed to light at various times during the dark phase. As the movement of cones may give an indication of when the oscillator is sensitive to light, glowlight tetras were exposed to light at times when the cones were in transitory states, when they first reached full elongation following their transitory movements, and when they had been in their fully elongated positions for some time. Thus, glowlight tetras were exposed to light at the times given in table 4.1.

Table 4.1. Commencement and duration times of prolonged light exposure throughout the dark phase

Experiment	Light exposure	Position of cones at commencement of light exposure
a	From t=18 to t=22	The cones have been elongated for approximately 3.5 hours.
b	From t=12.75 to t=21	The cones are in a transitory state as they have just commenced to elongate to dark adapted levels.
c	From t=23 to t=12	Again, the cones are in a transitory state having commenced to constrict to light adapted levels 1 hour before light onset.
d	From t=14.5 to t=21.	The cones have just reached full dark adaptation levels when this experiment commences (thus, the light sensitivity of the oscillator may be different in comparison to when the cones have been elongated for 3.5 hours as in 'a').
e	From t=42 in constant darkness (the fish have been in darkness from t=0 to t=42) to t=45	The cones are fully dark adapted at what is subjective mid-night during the first day in constant darkness (the aim of this experiment is to determine the effects of prolonged darkness on the light sensitivity of the cone oscillator).

All experiments outline in table 4.1 are terminated close to the times when the cones normally commence constriction (around t=21.5) or elongation (around t=12.5) during a light/dark cycle.

4.3.1.3 Dark placement at different times during the light phase

Fish were placed in darkness at various times during the light phase. Analogous to the light placement experiments, fish were placed in dark at times when the cones had just reached maximum constriction and also when they had been constricted for some time. Table 4.2 gives the times for dark placement.

Table 4.2 Commencement and duration times of dark placement throughout the light phase

Experiment	Dark placement	Position of cones at commencement of dark placement
a	From t=6 to t=12	The cones have been constricted for 6 hours.
b	From t=0.25 to t=12	The cones have just reached maximum constriction levels (thus, the light sensitivity of the oscillator might be different in comparison to 'a' when the cones have been fully constricted for 6 hours).
c	From t=30 in constant light(the lights have been left on from t=0 to t=30) to t=36	The cones are fully constricted at what is the subjective midday in constant light (the aim of this experiment is to determine the effects of constant light on the light sensitivity of the oscillator).
d	From t=42 in constant light (the lights have been left on from t=0 to t=42) to t=45	The cones are fully constricted at what is subjective midnight in constant light (as in 'c'; the aim here is to determine the effects of constant light on the light sensitivity of the oscillator.

Note that experiments 'a', 'b' and 'c' were terminated before the time elongation normally commenced (t=12.5 and t=36.5) during a light/dark cycle.

4.3.2 15 minute pulse of light in the middle of the dark phase

In order to determine whether the duration of experiments rather than the duration of light exposure was important in determining the positions of cones and melanosomes, fish were exposed to 15 minutes of light in the middle of the dark phase. Subsequent cone and melanosome positions were determined at 45, 120, and 180 minutes following the pulse of light.

4.3.3 Change of light cycle experiment

After entraining glowlight tetras to a light/dark schedule with onset at 09.00 and offset at 21.00, for 30 days, the illumination cycle was phase shifted by 12 hours (lights on at 21.00 and off at 09.00). After 7 and 14 days of this new light regime, groups of 3 glowlight tetras were sampled at the appropriate intervals. Also on day 14 of the new light schedule, light and dark exposure for 100 minutes during the middle of the opposite phase was performed.

In the previous chapter, the rhythm of the cones during entrainment to a LD 12:12 schedule was outlined after sampling at many points. However, knowing the detailed form of the rhythm, its basic shape can be outlined using far fewer data points. The time points required to outline the rhythm are given in Figure 4.1. Such 'skeleton' sampling greatly reduced the number of fish utilized.

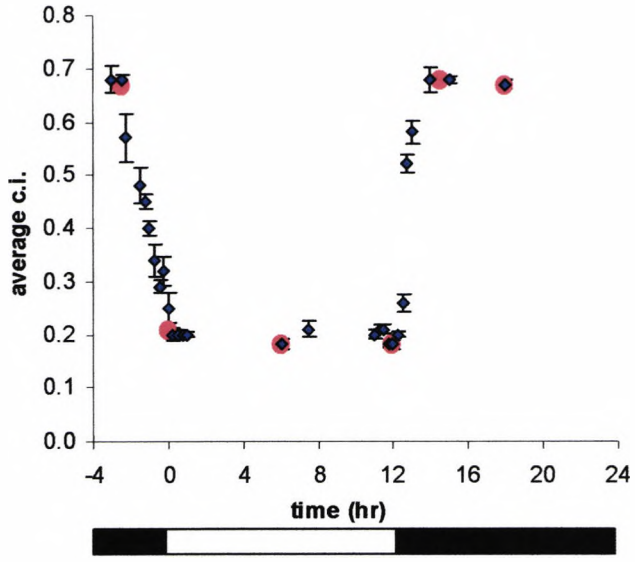

Fig 4-1. The salient points outlining the rhythm of glowlight tetra cone movements during a normal light/dark cycle.

The filled red circles indicate the minimal points required to outline the basic shape of the rhythm of cones during entrainment to a LD 12:12 cycle in comparison to that found using more extensive sampling (black data).

4.4 Results

For statistical analysis, the average light and dark adapted indices given in table 3.1 were used.

4.4.1 Part 1; light exposure and dark placement during the opposite phase of the cycle

4.4.1.1 100 minutes of light exposure during the middle of the dark phase (commencing at t=18)

For the duration of this experiment, the cones remained in their fully elongated dark adapted positions, while the RPE melanosomes commenced light adaptive movements immediately, and reached full light adaptation within 45 minutes as the pigment indices were now statistically similar to the maximum light adapted value ($p>0.2$, unpaired Student t test) (Figure 4.2a-c and 4.3).

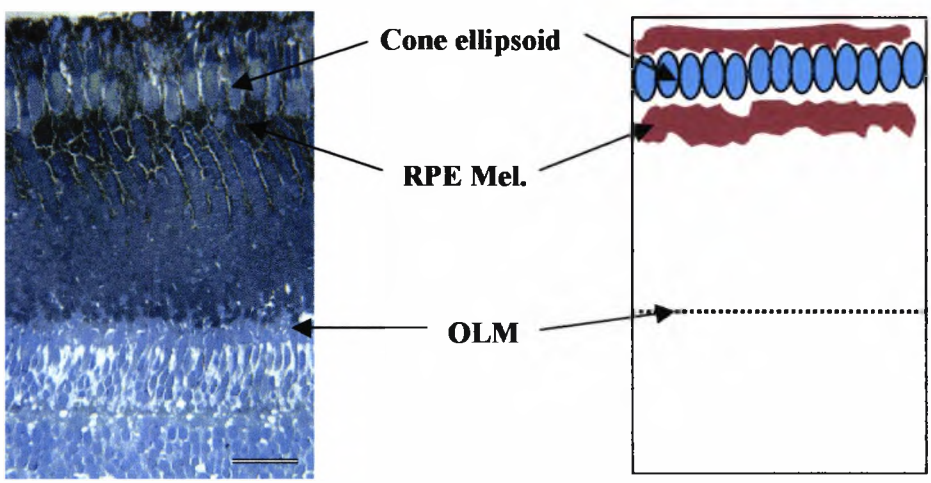

Fig 4-2a Glowlight tetra retina at t=18 (middle of dark phase)

Transverse section (left) and corresponding schematic diagram (right) of glowlight tetra at t=18 (middle of dark phase) before light exposure. Note that both the cones and RPE melanosomes are in their dark adapted positions well away from the OLM. Here, and in all subsequent micrographs: OLM outer limiting membrane, RPE Mel. RPE melanosomes. Bar = 25µm.

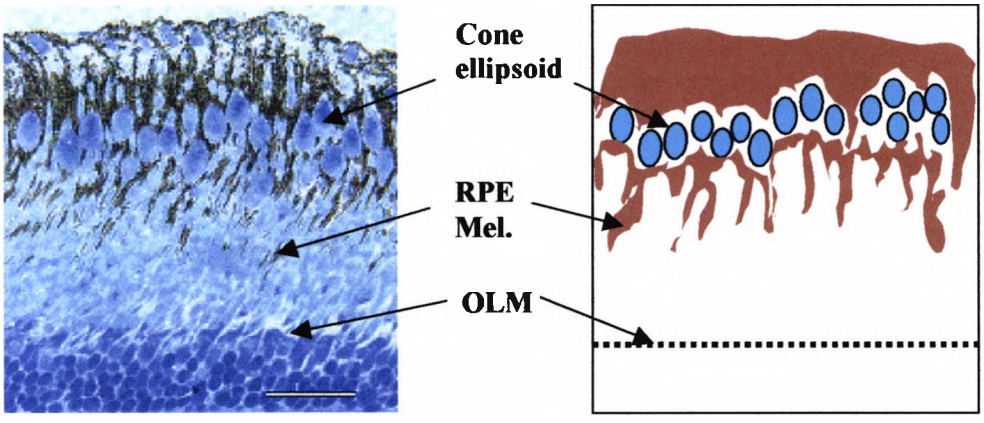

Fig 4-2b Glowlight tetra retina following 15 minutes of light exposure

Transverse/slightly oblique section (left) and corresponding schematic diagram (right) of glowlight tetra retina following 15 minutes of light exposure in the middle of the dark phase. Note that the RPE melanosomes have already commenced light adaptive movements as they are no longer withdrawn behind the cones but have dispersed vitreally towards the OLM and are covering the stationary cones. Bar = 25μm.

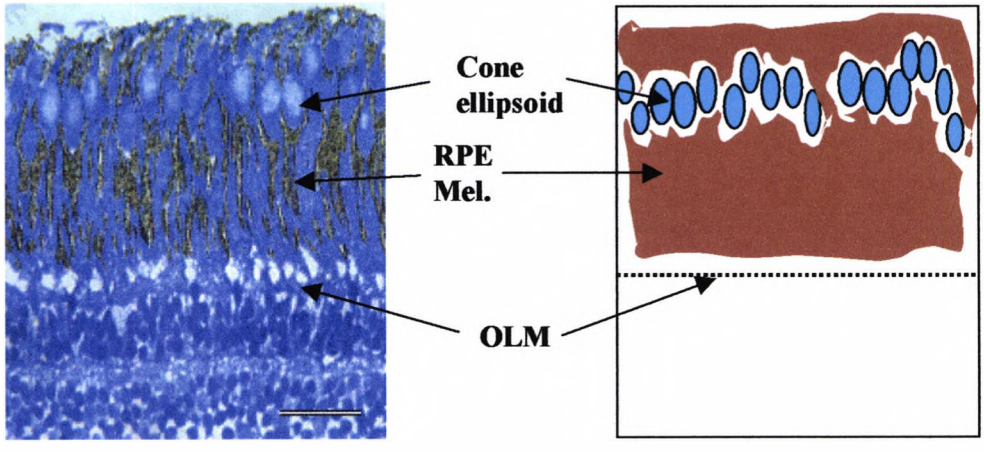

Fig 4-2c Glowlight tetra retina following 30 minutes of light exposure

Transverse section (left) and corresponding schematic diagram (right) of glowlight tetra retina following 30 minutes of light exposure in the middle of the dark phase. The RPE melanosomes have fully dispersed (15 minutes earlier than average, see text) and their innermost projection can be seen near OLM while the cones remain stationary. Bar = 25μm.

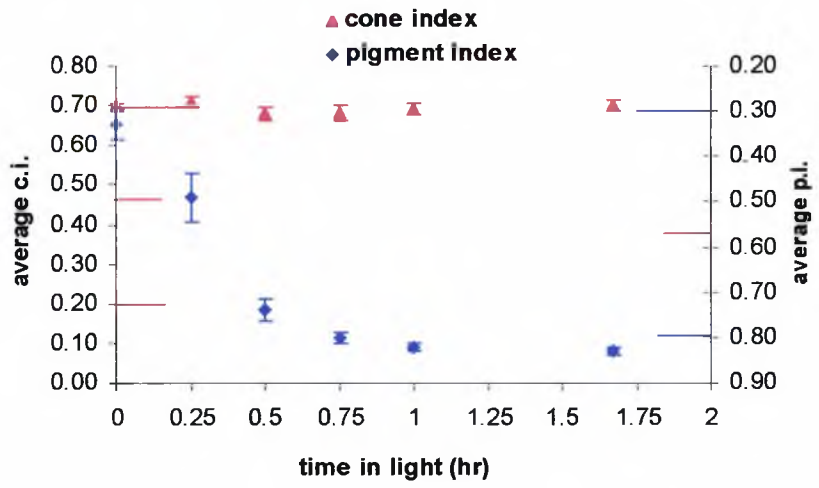

Fig 4-3 Cone and pigment indices during 100 minutes of light exposure in the middle of the dark phase

Cone (c.i.) and pigment (p.i.) indices during 100 minutes of light exposure during the middle of the dark phase. Each point represents the mean ±SE for 6 retina for the pigment index and between 9-12 retina for the cone index. The short bars represents the normal light adaptation values (red, cone index and blue, pigment index) while the longer bars give the normal dark adaptation values.

4.4.1.2 100 minutes of darkness in the middle of the light phase (commencing at t=6)

Both the cones and the RPE melanosomes remained in their fully light adapted positions near the OLM, throughout this experiment (Figures 4.4 and 4.5).

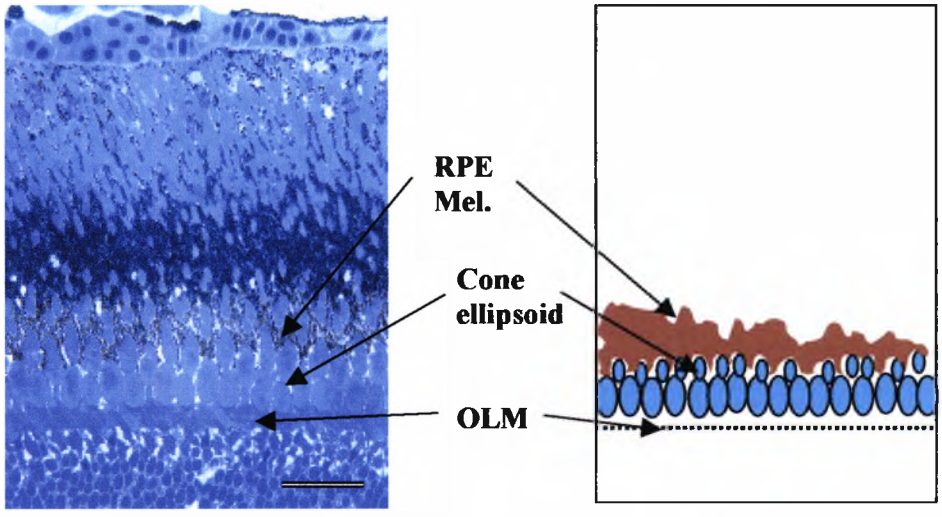

Fig 4-4 Glowlight retinae following 100 minutes of darkness in the middle of the light phase

Transverse section (left) and corresponding schematic diagram (right) of glowlight tetra retina following 100 minutes of darkness in the middle of the light phase. Bar = 25μm.

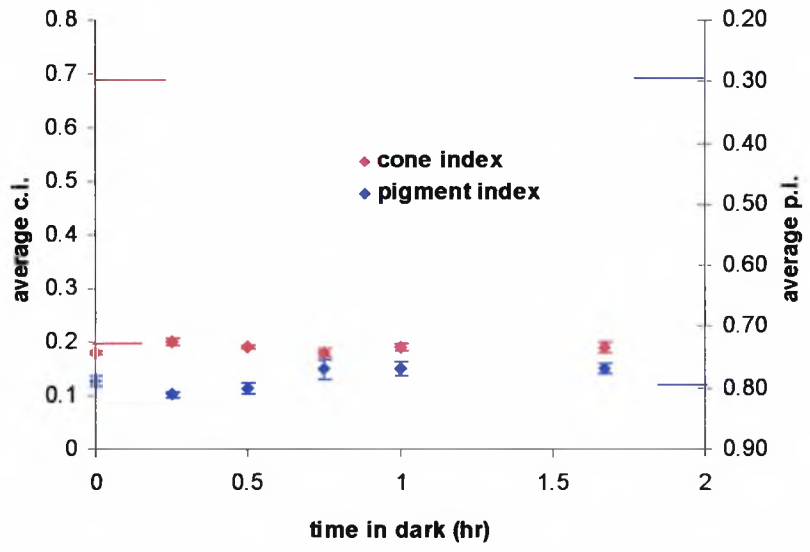

Fig 4-5 Cone and pigment indices during 100 minutes of darkness in the middle of the light phase

Cone and pigment indices during 100 minutes of darkness in the middle of the light phase. The short bars represents the normal light adaptation values (red, cone index and blue, pigment index) while the longer bars give the normal dark adaptation values. Each point represents the mean ±SE for 6 retinae for the pigment index and between 7-12 retinae for the cone index.

4.4.2 Prolonged light exposure during the dark phase

a/ Light exposure from t=18 until t=22

After 2 hours of light exposure, the cones had begun to move as the cone index is significantly different to the fully dark adapted value ($p<0.05$, unpaired Student t test). Even after 4 hours of light, however, the cones are not fully light adapted as the cone index is significantly different to the fully light adapted value ($p<0.001$, unpaired Student t test) (Figure 4.6, series 'a').

b/ Light exposure from t=12.75 until t=21

During the first hour of light exposure, the cones remain in the same intermediate positions they were during the commencement of this experiment (after 30 and 60 minutes of light exposure, the cone index is similar to that found before light exposure commences; $p>0.2$ at both time points, unpaired Student t test). Only after 240 minutes of light do the cones commence constricting (the cone index is significantly more light adapted in comparison to before light exposure commences; $p<0.001$, unpaired Student t test) (Figure 4.6, series 'b'). After 8.25 hours, the cones have reached maximum light adaptive levels (the cone index is now the same as the light adapted value; $p>0.2$, unpaired Student t test).

c/ Light exposure from t=23 until t=12

Within 0.25 hours of the lights coming on, the cones have fully constricted and remained so for the duration of the experiment (at all times, the cone index is similar to the light adaptive value; $p>0.2$, unpaired Student t test) (Figure 4.6, series 'c').

d/ Light exposure from t=14.5 until t=21

After 4 hours of light, the cones have constricted to intermediate values. The cone index is significantly different to the maximum dark value ($p<0.001$, unpaired Student t test). However, maximum constriction is not achieved until after 6.5 hours when the cone index is found to match the light adaptive value ($p >0.2$, unpaired Student t test) (Figure 4.6, series'd').

e/ Light exposure from t=42 (in constant darkness) until t=45

Even after 3 hours of light exposure, the cones remained fully elongated (Figure 4.6, series 'e').

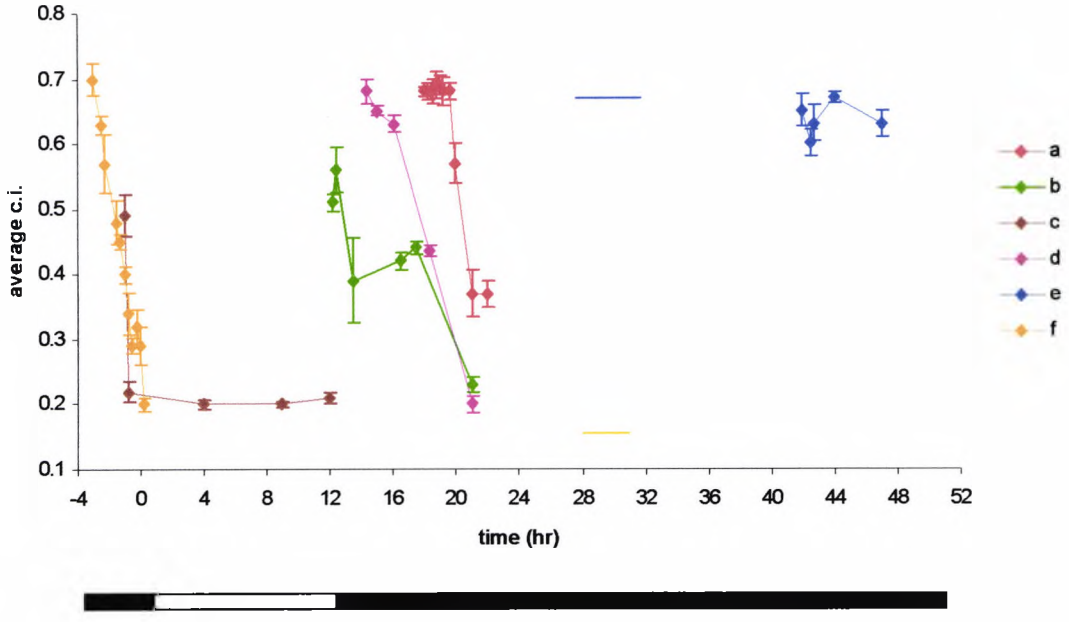

Fig 4-6 Cone indices following prolonged light exposure at various times

Cone indices (c.i) during light exposure commencing at various times throughout the light/dark cycle and also in continual darkness. Series 'a' represents light exposure during the middle of the dark phase commencing at t=18. 'b' represents light exposure commencing 0.75 hours after dusk, when the cones are in intermediate positions and becoming elongated. 'c' represents commencement 1 hour before dawn again when the cones are in intermediate positions becoming light adapted. 'd' shows commencement of light 2.5 hours after dusk when the cones are first fully elongated. 'e' demonstrates the effect of light exposure following 30 hours in constant dark and 'f' shows the cone indices for dawn during a normal light/dark schedule. Each point represents the mean ±SE for a minimum of 6 retinae. Note maximum light and adaptive indices are approximately 0.20 (orange bar) and approximately 0.68 (blue bar) repectively.

4.4.3 Prolonged placement in darkness during the light phase

a/ Dark placement commencing at t=6 until t=12

The cones remained constricted for the duration of this experiment (Figure 4.7, series 'a').

b/ Dark placement from t=0 until t=12

The cones remained fully constricted for the 12 hours of this experiment (Figure 4.7, series 'b').

c/ Dark placement from t=30 (in constant light) until t=33

The cones remained constricted during this experiment (Figure 4.7, series 'c').

d/ Dark placement from t=42 (in constant light) until t=45

The cones commenced dark adapting after 30 minutes (the cone index was significantly different to the light adapted value; $p<0.001$,unpaired Student test) and had not reached full dark adaptation levels after 2 hours (the cone index is different to the dark adapted value; $p<0.05$, unpaired Student t test) but after 3 hours were fully elongated (the cone index is similar to the dark adapted value; $p>0.2$, unpaired Student t test) (Figure 4.7, series 'd').

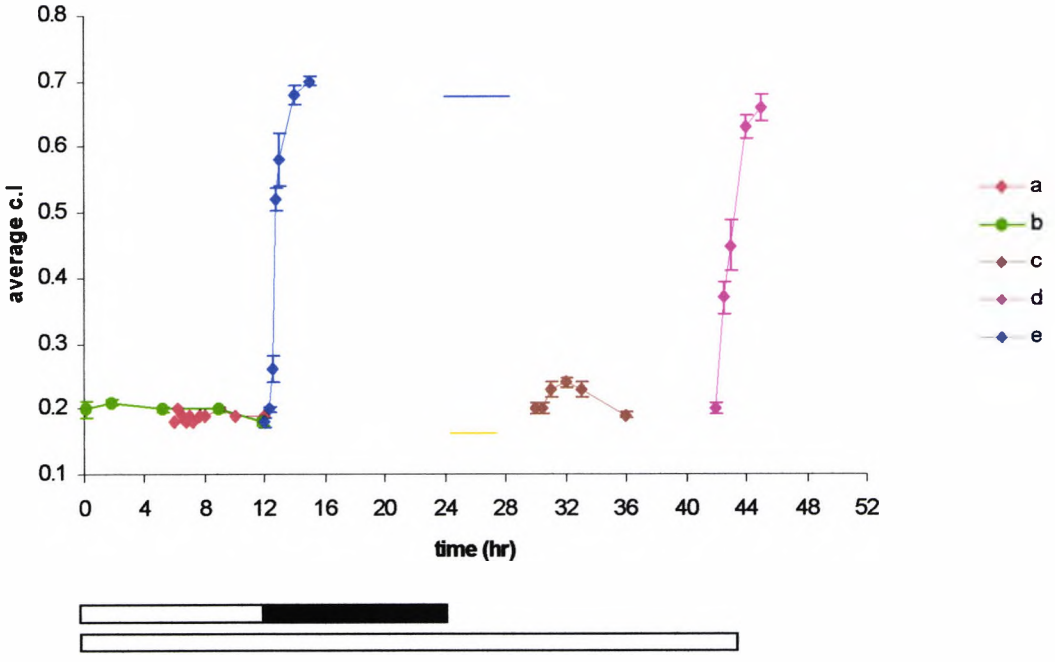

Fig 4-7 Cone indices during prolonged dark placement and at various times

Cone indices (c.i) following placement in darkness at various times throughout the light/dark cycle (lower bars show) and also in continual light (upper bar). Each point represents the mean and ±SE for a minimum of 6 retinae. Series 'a' shows the cone index during dark adaptation in the middle of a light/dark cycle; 'b', is the cone index during dark adaptation commencing at dawn; 'c', is the cone index during dark adaptation in the middle of what would have been the light phase following 30 hours of light; 'd' is the cone index during dark adaptation in what would have been the middle of the dark phase following 42 hours of light; 'e' shows the cone index during a normal dusk. Note maximum light and dark adaptive indices are approximately 0.20 (orange bar) and approximately 0.68 (blue bar) respectively.

4.4.4 15 minute pulse of light during the middle of the dark phase

Both the cones and the RPE melanosomes remained in their fully dark adapted positions for the duration of the experiment (Figure 4.8).

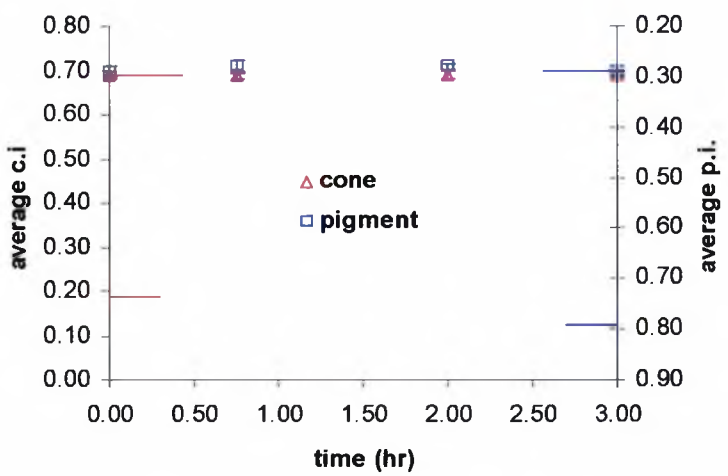

Fig 4.8 Cone and pigment indices following a brief pulse of light

Cone and pigment indices following a 15 minute pulse of light in the middle of the dark phase. The short bars represents the normal light adaptation values (red, cone index and blue, pigment index) while the longer bars give the normal dark adaptation values. Each point represents the mean ±SE for 6 retinae.

4.4.5 Part 2; change of light cycle

The important factor in these experiments is the new time the fish are being entrained to. Therefore, only these will be used in the analysis. Again, t=0 represents lights on and t=12 gives lights off.

4.4.5.1 7 days of the new cycle

After 7 days of the new cycle, the rhythm of the cone movements closely follows the new light settings. It is only at t=0 (time of lights on), that the cone positions are significantly different (less light adapted) than the basic 24- hour cycle ($p<0.001$, unpaired Student t test) (Figure 4.9).

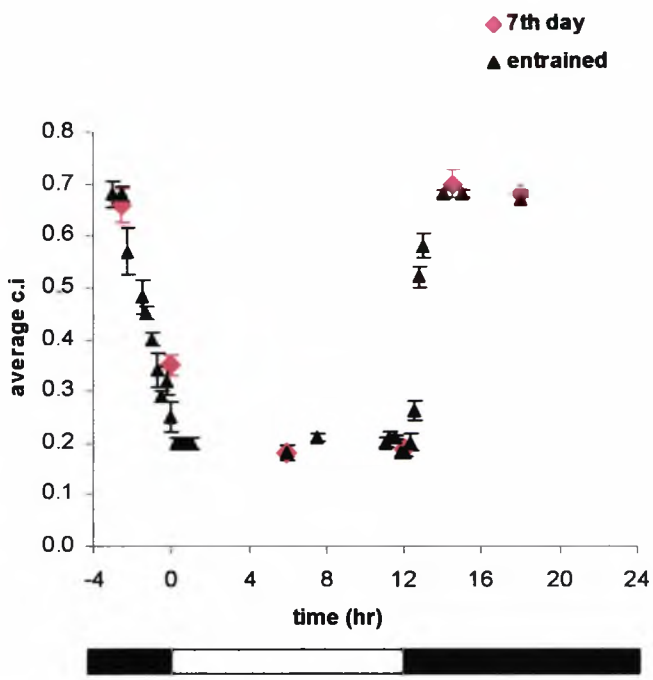

Fig 4-9 Cone indices on day 7 of phase shifted cycle

Cone indices (c.i) on the 7th day of entrainment to the 180° phase shifted light cycle in comparison to those after entrainment for 30 days. Each point on the 7th day curve represents the mean ±SE of 6 retinae during the phase shifted cycle. The data for the entrained curve comes from Figure 3.3.

4.4.5.2 14 days of the new cycle

During the 14th day of the new cycle, the cone indices match those found following 30 days of entrainment at all times indicating that the rhythm of cone movements has adapted to the new light regime (Figure 4.10).

To show further that adaptation to the new cycle has occurred, animals were exposed to light and dark out of phase with their cycle. After 100 minutes of light exposure in the middle of the dark phase and 100 minutes of darkness during the middle of the light phase (Figure 4.10), no movement of cones is seen as is the case in fish fully entrained to the light/dark cycle (see 4.3.1.1).

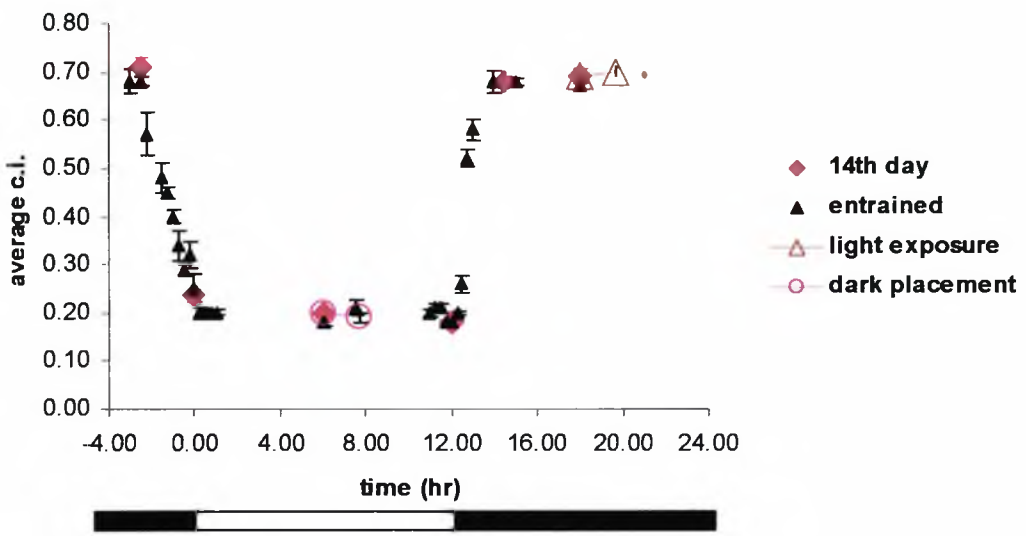

Fig 4-10 Cone indices on day 14 of phase shifted cycle

Cone indices (c.i) on the 14th day of entrainment to the 180° phase shifted light cycle in comparison to those after entrainment for 30 days. Also shown are the effects of 1.67 hours of light exposure during the middle of the dark phase and dark placement during the middle of the light phase during the same day. Each point on the 14th day, light exposure and dark placement curves represents the mean ±SE of 6 retinae. The data for the entrained curve comes from Figure 3.3.

4.5 Discussion

In the first part of this chapter, glowlight tetras were either exposed to light in the middle of the dark phase or placed in the dark in the middle of the light phase for 100 minutes. Light exposure in the middle of the dark phase had no effect on the cone positions whilst the RPE melanosomes light adapted within 45 minutes. This suggests that there is no light control of cone movements in comparison to strong light control of melanosome movement during light exposure in the middle of the dark phase. During dark placement in the middle of the light phase, no light control of either cone or melanosome movements was demonstrated as neither cone or RPE melanosome movement were seen.

Previous studies have tended to interpret such results differently. As the cones did not move while the melanosomes reached maximum light adapted levels during exposure to light in the middle of the dark phase, this could be interpreted as representing a strong circadian signal to the cones and a weaker circadian signal to the RPE melanosomes (Levinson and Burnside, 1981; McCormack and Burnside, 1991). Likewise, the lack of movement of both cones and melanosomes during dark exposure in the middle of the light phase may indicate a strong circadian signal to both the cones and melanosomes. Although this interpretation supports earlier findings that both the cones and RPE have relatively strong circadian drive controlling their movements (see chapter 3), it does not allow for the effect of light on the oscillators themselves as a pulse of light or dark will reset the

oscillator. Also, it does not consider the coupling between the oscillators controlling retinomotor movements and the retinomotor elements themselves. That is, light may simply be decoupling the retinomotor elements from the oscillators rather than overriding the oscillator itself.

In comparison to the glowlight tetra, cones in the midas cichlid (*Cichlasoma citrinellum*) were fully light adapted within 20 minutes of light exposure in the middle of the dark phase and dark adaptation in the middle of the light phase saw elongation to half the maximal levels (McCormack and Burnside, 1991). Likewise, rainbow trout (*Oncorhynchus mykiss*), carp (*Cyprinus carpio*), goldfish (*Carrius auratus*), and Jack Dempsey (*Cichlasoma biocellatum*) all showed maximal dark adapted positions of both cones and RPE melanosomes within two hours of being placed in the dark during the middle of the light phase (Easter and Macy, 1978;Munz and Richard, 1982; Wagner and Douglas, 1983). In the tench (*Tinca tinca*), whose cones do not undergo retinomotor movements, RPE melanosomes were also dark adapted within 2 hours of being placed in dark (Wagner and Douglas, 1983). Clearly, the glowlight tetra cones are influenced less by light in the middle of the dark phase while both cones and RPE melanosomes are less effected by darkness in the middle of the light phase in comparison to other species. One possible explanation could be that as the glowlight tetra is an equatorial species, retinomotor movements need not be as sensitive to light or dark exposure as daylengths are constant throughout the year. In comparison, more temperate species will experience variable daylengths which means retinomotor movements must be sensitive to the changing daylength if they are to remain entrained to the light/dark cycle (see section 3.5).

Although these experiments do not isolate the oscillators controlling retinomotor movements, there is still the possibility that the light sensitivity of these oscillators is being examined. If this were the case, then the glowlight tetra oscillators controlling cone and melanosome movement could be thought to be relatively insensitive to light in comparison to other more temperate species (temperate species oscillators must be more sensitive to the changing daylengths). Lesser sensitivity to light in glowlight tetras in comparison to other species might also be reflected in the PRC curves (for retinomotor movements). Perhaps a pulse of light or dark is simply hitting the 'dead' zone in the glowlight tetra while it is stimulating the 'advance' phase parts in other fish. The 'dead' zone in the glowlight tetra may also be longer in comparison to other species.

Alternatively, there may not be any differences in the oscillator sensitivity to light. Rather, the photoreceptor input to the oscillator may vary with different species. Sensitivity of rod mediated vision in goldfish has been shown to vary throughout the day and peaks at dusk (Bassi and Powers, 1987). It has also been shown that rods first detect the incoming light at dawn which triggers light adaptive movements in cones, melanosomes and rods probably by causing the paracrine release of dopamine (Kirsch et al, 1989; Wagner et al, 1993). Thus, rather than the cone oscillator being insensitive to light in the middle of the night, it could be that the rod mechanisms for detecting light is at its daily ebb thereby not detecting the light and not sending a signal which would reset the retinomotor oscillators in glowlight tetra. At the same time, in other fish, the sensitivity to light could be at a maximum during the middle of the night. However, it would be unlikely that the

rod mechanisms is at its daily ebb during the any part of the night in the glowlight tetra as this is when rods are expected to be functioning.

Light exposure and placing fish in darkness at various times during the light/dark cycle showed that cone movement was least affected by light or darkness when the cones were either fully contracted (from t=0 to t=12) or fully elongated (from t=14.5 to t=21.5). However, during the normal dawn (t=21.5 to t=0) and dusk (t=12.5 to t=14.5) transitions, light had more influence on cone movements. The most rapid movement of cones was seen following light exposure at t=23 when cones, not quite fully elongated, reached their maximum light adapted levels within 15 minutes of light exposure, indicating light has its most influence during normal pre-dawn cone constrictions. Light exposure at t=12.75 (during the normal dusk transitions) had some effect on the cone positions, as full dark elongation was never reached and it took 6.5 hours for maximum contraction to be reached. Thus, although cone movement appears to be insensitive to light or darkness for a great deal of the cone rhythm, there are two small 'windows' each day which show increased sensitivity.

Following 24 hours of constant light, the light control of cone movement remained unperturbed. When placed into darkness at the subjective middle of the day, the cones remained fully light adapted. However, when placed into darkness at the subjective dusk, the cones elongated to dark levels at the same rate as during a normal light/dark cycle. Previously, the rhythm of cone movement in constant light was found to be extinguished, with cones remaining in their light adapted positions throughout the cycle (see chapter 3). As the light control of cone

movement remains unchanged, this suggests that the effects of constant light are to mask the output of the cone oscillator rather than stopping it.

After 30 hours of constant darkness, even 3 hours of light in the middle of the subjective day had no effect on the position of the cones. In comparison, during the light/dark cycle, after 2 hours of light in the middle of the dark phase, cone movement had begun and by 3 hours the cones were in intermediate positions. This suggests that the light control of cone movement has decreased as a result of being placed in constant darkness. One possible explanation for this could be the photoreceptors becoming desensitized with prolonged darkness. In carp, cone horizontal cells, for example, become less responsive to light in constant darkness (Yang et al, 1994) and cone input to horizontal cells is reduced in constant darkness in perch (Mangel et al, 1994).

Although glowlight tetra cones showed no movement in response to 100 minutes of light in the middle of the dark phase and both cones and RPE melanosomes showed no movement in darkness during the middle of the light phase, there may be other explanations apart from sensitivity to light. One possible explanation could be the temporal characteristics of the mechanisms behind the movement of glowlight tetra retinomotor elements. Both dawn and dusk movements during a light/dark cycle take approximately 2.5 hours, possibly suggesting that this is the time it takes for the actin/myosin filaments and microtubules to interact appropriately for either constriction or elongation to be achieved. This implies that no movements of cones will be seen until about 2.5 hours after the onset of light or dark exposure. If this were so, then a short pulse of light (15 minutes for

example) in the middle of the dark phase should cause the cones to contract to the same degree after 3 hours as that given by 3 hours of continuous light. This was clearly not the case as even after 3 hours following a pulse of light, no movements of cones were noted. Also, during light exposure at dawn (t=23), the cones fully constricted from intermediate levels within 15 minutes when it normally takes 1 hour. Clearly, in this case, the actin/myosin mechanisms controlling cone constriction could be accelerated. Similarly for the melanosomes, the mechanisms guiding their movement could be made to go faster than at 'dawn' by light exposure in the middle of the dark phase.

In the second part of this chapter, glowlight tetras previously entrained to a LD 12:12 schedule were given a 12-hour phase shift. After 7 days of the new cycle, there was only one point (t=0) where the cones appeared not to have adjusted to the new cycle. Circadian influence was demonstrated by partial constriction of cones at this point (t=0), indicating preparation for dawn. By 14 days, the cone rhythm matched that following 30 days of entrainment at all times measured, indicating that the circadian rhythm had fully adjusted. To verify that full entrainment had been reached by day 14, the glowlights were exposed to light for 100 minutes during the middle of the night and also placed in total darkness for 100 minutes during the middle of the day. Both treatments had no effect on cone position, indicating that the rhythm of the cones had adjusted to the new cycle.

All experiments on the glowlights in this thesis were conducted after entraining for a minimum of 30 days. It can be safely assumed in light of these results that the glowlights had fully entrained to the light schedules.

The retinomotor movements of glowlight tetra adjust to a 180° phase shift between 7-14 days. However, since adaptation is almost complete by day 7 it is likely to be nearer to this than day 14. Other species and systems have also been subjected to 180° phase shifts. For locomotor activity in cockroaches and sparrows, 4 days of entrainment to the new cycle is required for full entrainment. Rats (locomotor activity) require 8 days and humans (sleep/activity) 11 days to be fully entrained to the new cycle (Aschoff et al, 1975). Therefore, cone movements entrain to light/dark schedules in about the same time found in other systems.

4.6 Summary

Cone movement is not influenced by light or dark exposure in the middle of the night and day respectively. In contrast, melanosome movement is driven by light exposure during the middle of the night. A circadian rhythm in light control of cone movement was demonstrated as there were two periods during a full cycle of cone movements where exposing fish to light effected the positions of the cones. Following a phase shift of 180°, the cone rhythm adjusted to the new light schedule within 8-14 days.

Chapter 5-Entrainment to light/dark cycles with variable photoperiods

5.1 Abstract

Glowlight tetra (*Hemigrammus erythrozonus*) retinomotor movements have already been shown to entrain well to a 12-hour light/ 12-hour dark cycle (LD 12:12) which, with the exception of the speed of the light and dark transitions, is similar to that in their natural habitat. Here, glowlights are entrained to light/dark cycles with different day lengths ranging from a LD 1:23 to LD 14:10. After a minimum of 30 days exposure to the novel light schedules, the rhythm of cone movements is examined during the light/dark cycle and in continual darkness. The main purpose of these experiments is first to determine whether the rhythm of cones can adjust to novel photoperiods and secondly whether entrainment to inappropriate light schedules is a possible explanation to earlier damping of the cone rhythm seen in other species.

Glowlight tetras are found to entrain well to all light schedules presented and no damping of rhythm is seen during constant darkness. After entrainment to each light schedule, the free-running period was determined. In most cases, the free-running period was found to be similar to that following entrainment to a LD12:12 cycle. Possible mechanisms of entrainment are discussed.

5.2 Introduction

Early experiments involving retinomotor movements in fish paid little attention to their light history. Fish were sampled during the middle of the day or the middle of the night to simply show the presence of retinomotor movements or whether these movements continued in constant darkness (eg. Welsh and Osborne, 1937). In later experiments, the number of sampling intervals increased and attention was given to the light history. Many experiments involved fish that were entrained to the natural day/night cycles. Fish were sampled in their natural environment or taken from their natural environment before being placed into constant darkness (eg. John et al, 1967; John and Gring, 1968; Olla and Marchioni, 1968). Most of the later and more detailed experiments entrained fish to artificial laboratory light schedules. However, rainbow trout entrained to the natural light/dark cycle were shown to have similar retinomotor movements to those exposed to an artificial light/dark cycle (Douglas, 1982a and b).

The choice of light schedules in previous studies sometimes appears arbitrary with 12- hour light / 12- hour dark (LD 12:12) being the most favoured. For example, Burnside and Ackland (1984) entrained the Green sunfish to a LD 12:12 cycle whilst Dearry and Barlow (1987) entrained the same fish to a LD 13:11 cycle. Green sunfish have been found to occur anywhere from 26°N to 50°N latitude (Fishbase, 2004). Thus, green sunfish originating at 50°N would experience a variation in the amount of daylight hours ranging from 8 hours (winter) to 16 hours (summer). Burnside and Ackland indicate that their fish have been raised in local fish farms and also that the experiments were performed during the summer

months. However, during the summer months in Sebastopol (38°N, California), where the experiments were performed and the fish were raised, the daylight hours were reaching 15 hours in June (US Naval Observatory). Clearly, a LD 12:12 schedule for entrainment does not coincide with the Green sunfish's natural light exposure during summer. Dearry and Barlow do not indicate the source of their fish but an attempt to match the natural light cycle is made to entrain the sunfish which is why they used a LD 13:11schedule. Later, McCormack and Burnside (1991) entrained the Midas cichlid to a LD 15:9 cycle from hatching for at least 3 months. However, the Midas cichlid is a tropical fish originating in South America. At most, it would experience daylight hours ranging from about 11 hours to just under 13 hours. Again, the 15-hour light schedule appears to show little resemblance to the Midas' natural environment. Similarly, Douglas and Wagner (1982) entrained the Goldeneye cichlid to a LD 14:10 cycle when a LD 12:12 regime would have suited this equatorial fish better.

As outlined above, in many experiments involving laboratory-entrained fish, there is an underlying assumption that the retinomotor movements of the fish under investigation would adapt to the experimental light settings. Perhaps one of the reasons that the rhythm of both cones and RPE melanosomes continued in constant darkness for a longer duration in the glowlight tetra in comparison to other species, is that the artificial light/dark schedule used to entrain glowlight tetras closely matched their natural environment. To answer this question, glowlight tetras were entrained to light schedules with different photoperiods and the rhythm of cone movements was examined during a light/dark schedule and in constant darkness to see if there is any sign of damping.

As described previously (chapter 3), the glowlight tetra is an equatorial species and as such the oscillators controlling retinomotor movements function primarily to put photoreceptors and melanosomes in the correct positions (light or dark) at the same time every day of the year. In comparison, temperate oscillators must be sensitive to the changes in the daylight hours throughout the year (up to several hours depending on the geographical location) which would mean that the times the photoreceptors need to change positions each day is variable. In chapter 3, the rhythm of cone movements following entrainment to a LD 12:12 light schedule was found to continue in constant darkness for at least 14 days which was interpreted as indicative of a strong oscillator controlling cone movement. In this chapter, attempts are made to determine whether the glowlight tetra cone movement oscillator can adapt to varying photoperiods as experienced by more temperate species or whether this strong oscillator represents a more rigid mechanism. Possible mechanisms of entrainment are discussed and a comparison is made to other animal systems.

5.3 Materials and Methods

Fish were entrained to a variety of 24 hour LD cycles, with the light period ranging from 1-14 hours (Table 5.1). So that the previous LD cycles (for example from the fish dealer or the natural light/dark cycle) did not influence the entrainment to novel photoperiods, these were run at 'unusual' times. For example, for the LD 10:14 cycle, the light phase was from 22.00-8.00. For comparison between light schedules, 'lights-on' was always taken as t=0.

Table 5.1. Table of light schedules and entrainment duration.

Light schedule	**Duration of entrainment period**	**Experiment**
LD 14:10	90 days	Cone rhythm during the normal light/dark cycle and in constant darkness
LD 10:14	31 days	Cone rhythm during the normal light/dark cycle and in constant darkness
LD 8:16	31 days	Cone rhythm during the normal light/dark cycle and in constant darkness
LD 8:16	365 days	Cone rhythm in constant darkness[1]
LD 1:23	31 days	Cone rhythm during the normal light/dark cycle

[1] Due to an unfortunate incident during the last quarter of the entrainment period, many fish were lost, so this experiment only involved analysing the cone rhythm in constant darkness.

5.4 Results

As in the previous chapters, to allow statistical analysis of data, the average light adapted cone index is 0.20±0.02 while the average dark adapted cone index is 0.68±0.02 (see section 3.4.1 for explanation).

5.4.1 14-hour light/10-hour dark cycle (LD 14:10)

5.4.1.1 Cone movements during the light/dark cycle

After 90 days of entrainment the animals closely followed their new light/dark cycle with 3 notable differences (Figure 5.1).

Dawn transition

At t = 0, animals on the 14:10 LD cycle only showed partial constriction of cones as the average cone index (0.59) was significantly different to the night time values ($p<.001$, unpaired Student t test). At the same time, the average cone index of animals on the LD 12:12 cycle was similar to the light adapted value ($p>0.2$, unpaired Student t test).

Period of cone constriction

During the LD 14:10 cycle, at each point from t=0.25 to t=14, the cone index did not differ from the maximum light adapted value ($p>0.2$ at each point, unpaired Student t test), indicating that the cones were fully constricted throughout this period. At t=14.25, the cone index was significantly different to the light adapted value ($p<.001$, unpaired Student t test). Thus, the cones were fully constricted for approximately 13.75 hours.

In contrast, during the LD 12:12 cycle, the cones were fully constricted from t =0 to t=12.5 (cone indices at all points measured are similar to the light adapted value; $p>0.2$, unpaired Student t test) [Note that at t =-0.25 and 12.75, the cone indices are significantly different to the maximum light adapted value ($p<0.001$ in both cases, unpaired Student t test)]. Therefore, the cones were fully constricted

for approximately 12.5 hours. Thus, during the LD 14:10 cycle, the cones were constricted for an extra 1.25 hours in comparison to the LD12:12 cycle.

Dusk transition

During the LD 14:10 cycle, the cones are fully constricted at t=14 and first reach full elongation at t= 15 (no significant difference from maximum dark adaptation; $p>0.2$, unpaired Student t test). Thus, it has only taken 1 hour to go from full light to full dark adaptation levels. In comparison, during the LD 12:12 cycle, the cones are fully constricted at t=12.5 and first fully elongated at t=14.5 and so take 2 hours to go from fully constricted to fully elongated levels.

5.4.1.2 Cone movements in continual darkness

Period of cone contraction

In continual darkness the animals entrained to the novel rhythm did not continue to display the prolonged cone contraction exhibited during the light/dark cycle. Cones were fully constricted for only approximately 11.5 hours from t=25.5 to t=37.0 ($p>0.2$ at each point, unpaired Student t test) during the LD 14:10 cycle. In comparison, the pattern of movements remained the same as that found during the light/dark period in the LD 12:12 cycle and the cones remained fully constricted from t=24 to t=36.5 ($p>0.2$ at all points; unpaired Student t test).

Dusk transitions

The commencement of the subjective dusk transitions (subjective dusk at t=36) has been delayed during the LD14:10 cycle in comparison to the LD 12:12. At

t=36.5, the cones are fully constricted during both cycles and by t=37, the cones have commenced elongation during the LD12:12 ($p<0.001$, unpaired Student t test), while they are still fully light adapted in the LD 14:10 ($p>0.2$, unpaired Student t test). At t=38, the cone index in the LD14:10 is significantly different to light adaptive values ($p<0.001$, unpaired Student t test) indicating the commencement of elongation. However, cones are more elongated in the LD12:12 cycle in comparison to the LD 14:10 as the cone indices are significantly more dark adapted in the LD12:12 ($p<0.05$, unpaired Student t test). The termination of the dusk transitions also appears to be delayed in the LD 14:10. Full elongation is reached at t=38.5 in the LD12:12 cycle and at t=39 during the LD14:10 cycle ($p>0.2$ for both time points, unpaired Student t test).

Dawn transitions

Both the commencement and termination of the dawn transition (subjective dawn at t=48) appears to have also been delayed in the LD14:10 cycle. At t=47.25, the cones have already commenced constriction during the LD12:12 cycle as the cone index is significantly different to the dark adapted value ($p<0.001$, unpaired Student t test) while even at t=47.5, the cone index is similar to the dark adapted value in the LD14:10 cycle ($p>0.2$, unpaired Student t test). Cones are fully light adapted at t=48 during the LD 12:12 cycle ($p>0.2$, unpaired Student t test) and at t=50 during the LD14:10 cycle ($p>0.2$, unpaired Student t test). Although the cones may have reached light adapted levels earlier, it is unlikely that this happened much sooner than 0.5 hours considering that it generally took cones 2.0-2.5 hours to undergo full light adaptive contraction from full dark elongation at subjective dawn following entrainment to different photoperiods. Thus, the dawn

transition may have been delayed by upto 2 hours in comparison to the LD 12:12 cycle.

Free-running period

During the LD 14:10 cycle in constant darkness, the cones were first light adapted at t=25.5 ($p>0.2$, unpaired Student t test), and then again following a period of full dark adaptation at t= 50 ($p>0.2$, unpaired Student t test). The free-running period is therefore 24.5 hours (Figure 5.1).

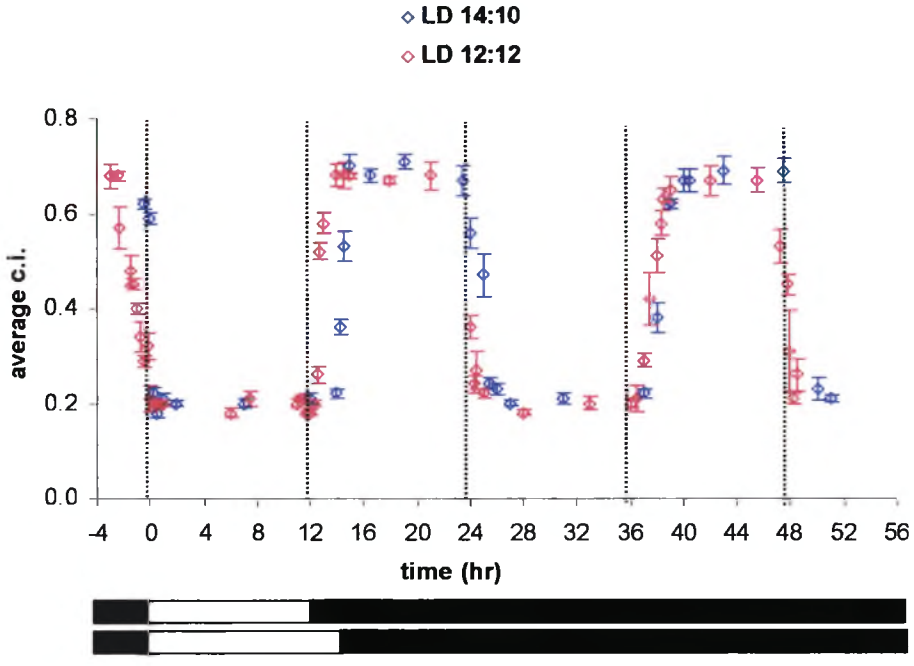

Fig 5-1 Cone indices during a LD 14:10 and LD 12:12

Cone indices (c.i) during a LD 14:10 (blue) light schedule followed by continual darkness in comparison to those found during a LD 12:12 (red) light cycle. Each time point represents a mean value ±SE for at least 6 retinae. The top light/shade bar denotes the light schedule for the LD 12:12 cycle and the bottom bar that for the LD 14:10. The dotted lines represents the lights on (0, 24, 48) and lights off (12, 36) times for a LD 12:12 cycle.

5.4.2 10-hour light/14-hour dark schedule (LD 10:14)

5.4.2.1 Cone movements during the light/cycle

Dawn transition

Animals adapted to the LD 10:14 cycle behaved in an identical manner to animals on the standard LD 12:12 cycle during the early phase of the cycle, anticipating dawn to the same degree, taking the same time for the dawn transitions and following the same pattern until dusk at t=10 (no significant difference between the cone indices measured at comparable time points for both the LD 10:14 and LD 12:12; $p>0.2$, unpaired Student t test).

Period of cone constriction

During the LD 10:14 cycle, the cones are fully constricted for 11 hours, from t=-0.5 (before dawn) until t=10.5 ($p>0.2$, unpaired Student t test). Thus, during this schedule the cones are constricted for 1.5 hours less than during the LD 12:12 cycle (12.5 hours, see above).

Dusk transitions

After dusk, the cones in the LD10:14 cycle take only 1 hour to go from being fully light adapted at t =10.5 to next fully elongated with cone indices similar to the dark adapted value, at t=11.5 ($p>0.2$, unpaired Student t test) in comparison to 2 hours during the LD 12 :12 cycle. Thus, although the dawn transition has not changed, that at dusk occurs much quicker (Figure 5.2).

5.4.2.2 Cone movements during continual darkness

Period of cone constriction

In continual darkness, in the LD 10:14 entrained animals, the cones are fully light adapted for at least 10 hours from t= 24 until t = 34 ($p>0.2$ at all points, unpaired Student t test).

Dusk transitions

Subjective dusk transitions (t=36) appear to have been advanced during the LD 10:14 cycle. At t=35 the cones have already commenced elongation as shown by the cone index which is different to the light adaptive value ($p<0.001$, unpaired Student t test) and full elongation is reached by t=37.5 ($p>0.2$, unpaired Student t test). In comparison, cones do no commence movement until t=37 and full elongation is not reached until t=38.5 during the LD 12:12 cycle. Thus, dusk has been advanced by approximately one hour during the LD10:14 cycle.

Dawn transitions

Subjective dawn transitions (t=48) in the LD10:14 group appear to closely follow those seen in the LD12:12 cycle (at t= 48, cones are fully light adapted in both cycles) although due to the lack of comparable sampling points, one cannot rule out that dawn was also slightly advanced.

Free-running period

In constant darkness, the cones in the LD10:14 fish were first seen to be fully light adapted at t=24 and following elongation, again at t=48 (at both times, the cone

index was similar to the light adapted value; $p>0.2$ at both time points, unpaired Student t test) giving a free running period of 24 hours. Due to the lack of data, the free-running period may be shorter than 24 hours but this is unlikely considering the shape of the curve during the previous 2 dawn transitions in darkness (dawn at t=0 and subjective dawn at t=24) (Figure 5.2).

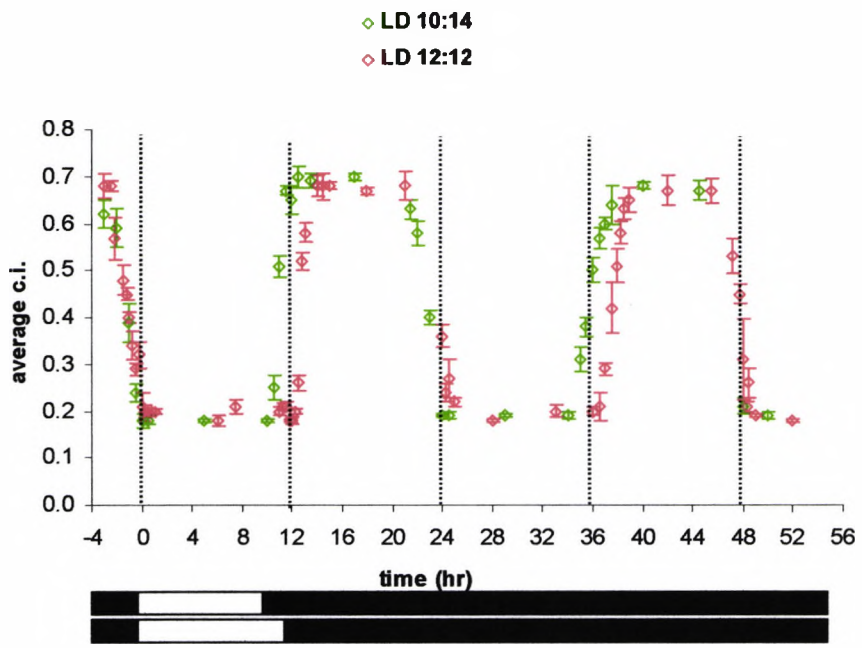

Fig 5-2 Cone indices during a LD10:14 and LD 12:12

Cone indices (c.i) during a LD 10:14 (green) schedule followed by continual darkness in comparison to those during a LD 12:12 (red) light cycle. Each time point represents a mean value ±SE for at least 6 retinae. The top light/shade bar denotes the light schedule for the LD 10:14 cycle and the bottom bar that for the LD 12:12. The dotted lines represents the lights on (0, 24, 48) and lights off (12, 36) times for a LD 12:12 cycle.

5.4.3 8-hour light/16-hour dark cycle (LD 8:16)

5.4.3.1 Cone movements during the light/cycle

Animals entrained to the LD 8:16 cycle showed differences in comparison to the standard LD 12:12 cycle (Figure 5.3);

Dawn transitions

Cones commenced their predawn constriction and reached maximum light adapted levels earlier during the LD 8:16 cycle but the dawn transition time remained at about 2.5 hours. At t=-3.5 (3.5 hours before dawn) the cones were fully elongated (the cone index was similar to the dark value; $p>0.2$, unpaired Student t test).By t=-2.5, the cones had already commenced their light adaptive movements as the cone index was significantly different to dark adapted values ($p<.001$, unpaired Student t test) during the LD 8:16 light cycle, while at the corresponding time in the LD 12:12 schedule, the cones are still fully elongated. At t=-1.0, 1 hour before the lights are due to come on, the cones have reached maximum light adapted levels in the LD 8:16 cycle ($p>0.2$, unpaired Student t test) while at the same time in the LD 12:12 cycle, the cones were at intermediate levels.

Period of cone constriction

During the LD 8:16 cycle, the cones were fully constricted for 10 hours from t=-1.0 (1 hour before dawn) to t=9.0 ($p>0.2$ at all points, unpaired Student t test) which is 2.5 hours shorter in comparison to the LD 12:12 cycle.

Dusk transitions

At t=9.0, the cones were fully light adapted in the LD 8:16 cycle and first reached maximum elongation at t=11.0 (cone indices are similar to the maximum dark value; $p>0.2$, unpaired Student t test) taking 2 hours for the dusk transition which is similar to during the LD 12:12 cycle.

5.4.3.2 Cone movements during continual darkness

Period of cone constriction

In continual darkness, the cones are fully constricted for 11 hours from t=23 (1 hour before subjective dawn) to t=34 (no difference to light adapted values at all times measured; $p>0.2$ for all times except at t=34 where $p>0.1$, unpaired Student t test).

Dusk transitions

The subjective dusk transition (t=36) has been advanced in comparison to the LD12:12 cycle. At t=35, the cones have already commenced elongation (the cone indices are different to the light adapted value; $p<0.001$, unpaired Student t test) and full elongation is reached at t=38 ($p>0.2$, unpaired Student t test). Thus,

although the dusk transition commences 2 hours before the LD 12:12 (elongation commences around t=37) cycle, full elongation is only reached 0.5hours earlier.

Dawn transitions

The dawn transitions (t=48) appear also be advanced, as light adaptive levels are reached 1 hour earlier (t=47; $p>0.2$, unpaired Student t test) than the LD 12:12 cycle.

Free-running period

At t =23, cones in the LD8:16 fish were first fully light adapted in constant darkness and following their elongation, could be seen to be light adapted again at t=47 (at both times, the cone index was similar to the light adapted value; $p>0.2$ at both times, unpaired Student t test) giving a free-running period of 24 hours. Again, the paucity of data during this '3rd dawn' means that the free- running period may have been less than 24 hours but considering the shape of the curve during the previous two 'dawns' (dawn and subjective dawn) this is unlikely (Figure 5.3).

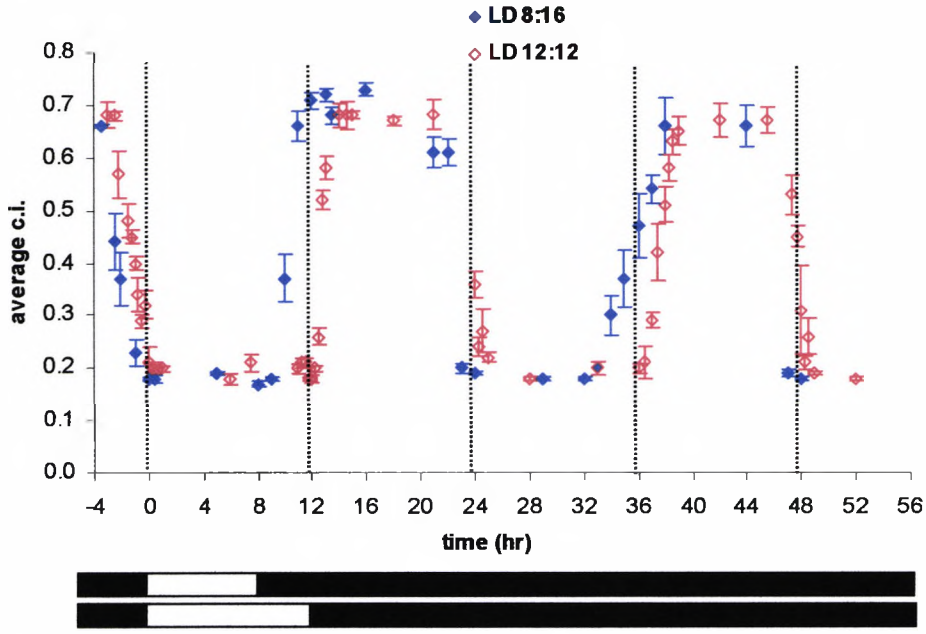

Fig 5-3 Cone indices during a LD 8:16 and LD 12:12

Cone indices (c.i) during a LD 8:16 schedule (blue) followed by continual darkness in comparison to those during a LD 12:12 (red) light cycle. Each time point represents a mean value ±SE for at least 6 retina. The top light shade bar denotes the light schedule for the LD 8:16 cycle and the bottom bar that for the LD 12:12. The dotted lines represents the lights on (0, 24, 48) and light offs (12, 36) times for a LD 12:12 cycle.

5.4.3.3 8-hour light/16-hour dark cycle (LD 8:16) after 365 days of entrainment

To determine whether the pattern of cone movements seen in continual darkness following entrainment to the above light schedules is due to an insufficient length of entrainment, fish were subjected to an LD 8:16 cycle for 365 days. Due to an unfortunate incident during the last quarter of the entrainment period, many fish were lost. Therefore, this experiment only involves looking at the endogenous rhythm in constant darkness following 1 year of entrainment to this light schedule.

5.4.3.4 Cone movements during continual darkness

After 365 days of entrainment to an LD 8:16 cycle, the endogenous rhythm of cone movement in constant darkness is consistent with that found following entrainment for 30 days. All comparable points are similar ($p>0.2$ for all times measured, unpaired Student t test) except at t=23 where the cone index is significantly more dark adapted following 365 days of entrainment ($p<.05$, unpaired Student t test) (Figure 5.4).

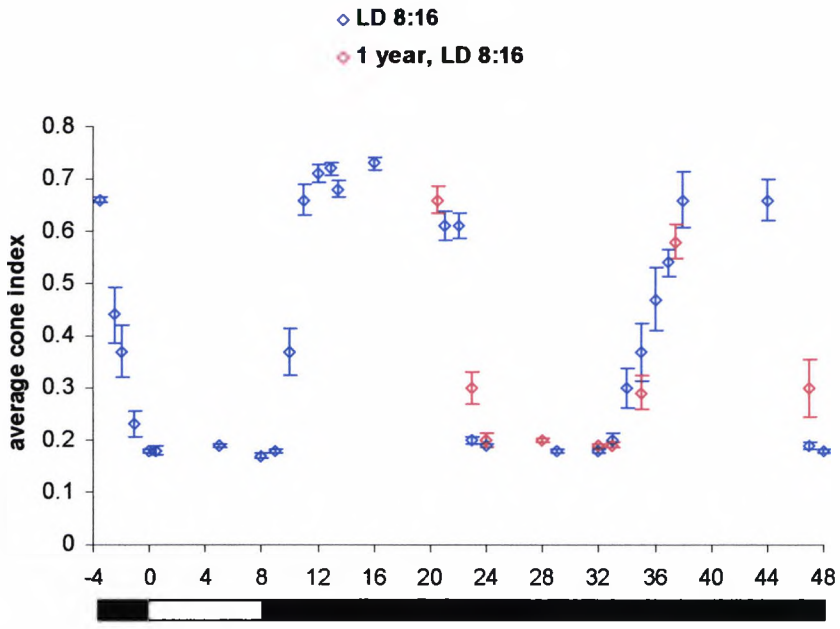

Fig 5-4 Cone indices following 1 year entrainment

Cone indices (c.i) after 1 year of entrainment to a LD 8:16 cycle (red points) in comparison to those following entrainment for 30 days (blue points). Each point represents the mean ±SE of 6 retinae. Light onset and permanent offset is given by the light/dark bar.

5.4.4 1-hour light/23-hour dark schedule (LD 1:23)

5.4.4.1 Cone movements during the light/cycle

The cone movement rhythm entrains well to this light regime and appears to have undergone an advance phase shift in comparison to the standard light schedule. Predawn constriction commences much earlier and the cones have reached light adapted levels at least 4 hours earlier than during the standard light schedule (at t=-4, there is no difference between the cone index for the LD1:23 cycle and the maximum light adapted value; $p>0.05$, unpaired Student t test). Maximum dark adaptive elongation levels are reached 7.5 hours after dusk ($p>0.2$, unpaired Student test). If this rhythm is moved forward by 4 hours, then it more closely follows that found during entrainment to the standard light condition except at t=12.5 where the cone indices are significantly different ($p<0.001$, unpaired Student t test). The period of cone constriction (12.5 hours) and the free-running period (24 hours) is similar to that found during the LD 12:12 cycle (Figures 5.10 and 5.11).

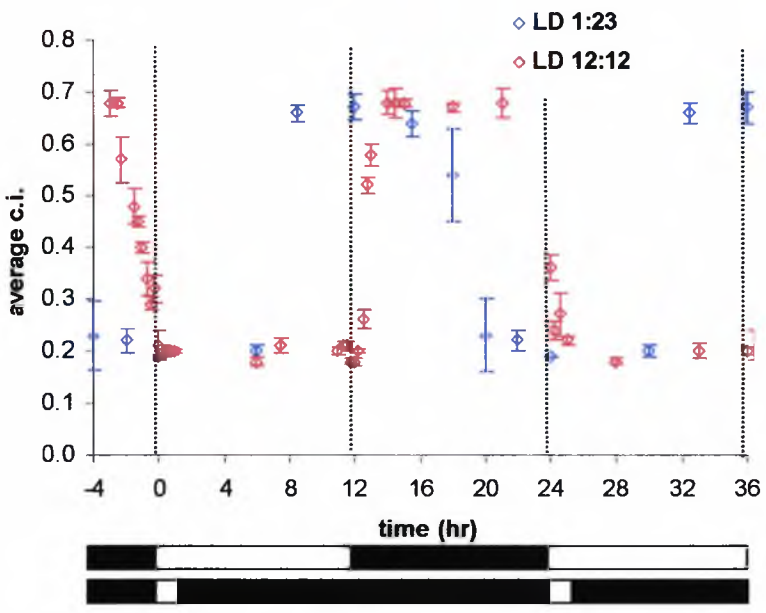

Fig 5-5 Cone indices during LD 1:23 and LD 12:12 cycles

Cone indices (c.i) during entrainment to a LD 1:23 (blue) light schedule in comparison to during a LD 12:12 (red) cycle. Here, the LD 1:23 cycle has been duplicated to give the appearance of 2 cycles to facilitate comparison between this cycle and the LD 12:12 cycle. Each point represents the mean ±SE of 6 retinae. The upper light/dark bar represents the LD12:12 light schedule and the bottom the LD 1:23. The dotted lines represents the lights on (0, 24,) and lights off (12, 36) times for a LD 12:12 cycle.

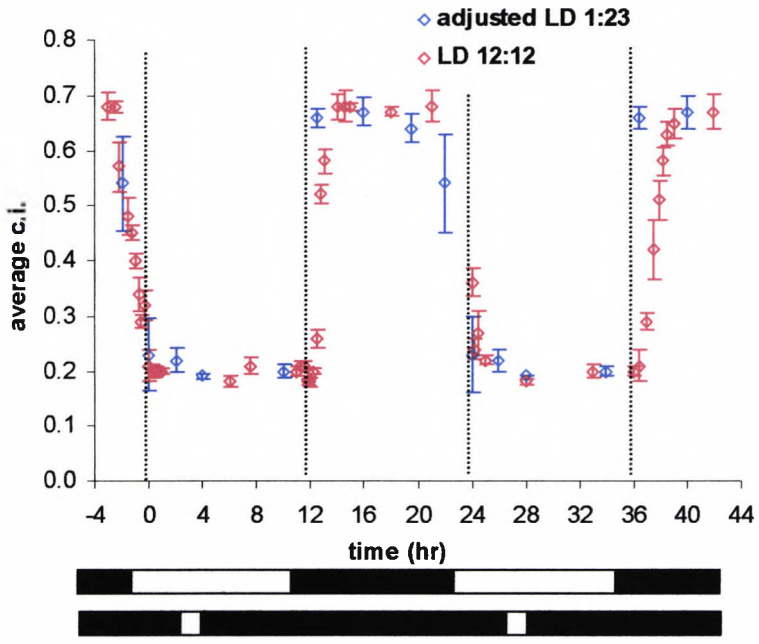

Fig 5-6 Adjusted LD 1:23 cone indices

Cone indices after the LD 1:23 cycle (blue) has been moved forward by 4 hours (adjusted) and during entrainment to the LD 12:12 (red) cycle. The adjusted LD 1:23 cycle has been duplicated to give the appearance of 2 complete cycles and thus allow better comparison with the LD 12:12. The upper bar shows the LD 12:12 light schedule and the bottom the phase shifted LD 1:23. The dotted lines represents the lights on (0, 24,) and lights off (12, 36) times for a LD 12:12 cycle.

5.4.5 Summary of results

Table 5.2 summarises the major differences in cone position between animals exposed to various LD cycles and fish experiencing a LD 12:12 regime.

Table 5.2. Summary of results

Light cycle	Period that the cones are fully contracted during L/D cycle	Relative (to LD12:12) dusk transition in constant darkness	Relative (to LD12:12) dawn transition in constant darkness	Free-running period
LD12:12	12.5 hours	_	_	24 hours
LD14:10	13.75 hours	0.5hour delay	0.5hour delay	24.5 hours
LD10:8	11 hours	1 hour advance	Matches LD12:12	24 hours
LD8:16	10 hours	2 hour advance	1 hour advance	24 hours
LD1:23	12.5 hours	4 hour delay	4 hour delay	24 hours

5.5 Discussion

5.5.1 Mechanisms of entrainment to different photoperiods

In this chapter, the rhythm of cone movements in glowlight tetras was found to entrain successfully to different light schedules. In other studies, the ability of species to entrain to a different light schedule has been predicted following determination of the phase response curve (PRC) (section 1.7). To construct a PRC curve for glowlight tetra cone movement would involve a substantial amount

of work (approximatley 1.5-2.0 year's work) and therefore the strategy employed in this chapter was used.

Other studies have demonstrated that circadian rhythms can expand and compress in response to different photoperiods. The daily duration of drinking and feeding activity in rats, for example, was progressively compressed as the duration of the dark phase of the cycle was shortened (Rosenwasser et al, 1983). Longer light periods also caused a compression of the activity profiles of nocturnal mice (Refinetti, 2002). Similarly in the diurnal chipmunk, the rhythm of locomotor activity was gradually compressed as the light phase of the light/dark schedule was shortened (DeCoursey, 1972). The drinking activity in diurnal squirrel monkeys also compressed in response to shortening of the photoperiod (Sulzman et al, 1982), as did the feeding activity in diurnal pigeons (Basco et al, 1996).

The rhythm of cone movement observed following entrainment to the standard cycle (LD 12:12) probably resembles the natural rhythm. During entrainment to different light schedules with a period of 24 hours, the rhythm of cone movements in glowlight tetras behaved in a similar manner to circadian rhythms found in other species as outlined above, showing both compression and expansion. During long photoperiods (LD 14:10) glowlight tetras stretched the period that the cones were fully constricted. Similarly, in response to entrainment to shorter photoperiods (LD 10:14, LD 8:16 and LD 1:23), they compressed the time the cones were fully light adapted.

The compression and expansion of circadian rhythms can be explained using mechanisms that rely on either a single oscillator or two oscillators ('dual oscillator' theory).

5.5.2 Single oscillator

One possible mechanism that has been suggested is a non-parametric effect of photic stimulation on the oscillator, causing a change in the rhythm expressed (Pittendrigh, 1981). In mice, it has been shown that this effect can only be slight as there is little difference between the activity rhythm of mice entrained to a 15 minute pulse of light (non-parametric stimulus) and those maintained in constant darkness (Refinetti, 2002). Similarly, this is unlikely to have a role in the expansion and contraction of the cone rhythm seen in the glowlight tetra. The LD 1:23 cycle can be considered to be a non-parametric stimulus as the duration of light is relatively small. The period of this rhythm closely matches that of the free-run rhythm, although there is a slight compression in the period that the cones are fully constricted.

Another possible mechanism could be a parametric compression or decompression of the circadian oscillator's cycle. In the rat, using light induced c-fos expression as a marker, the intrinsic rhythm of the suprachiasmatic nucleus (SCN) was found to adjust to different photoperiods (Sumová et al, 1995).The results of the LD 14:10, LD 10:14, LD 8:16 indicate a parametric effect on the circadian pacemaker. In these cases, the amount of expansion and compression seems to be directly related to the photoperiod (i.e. the period of cone constriction

is compressed more in going from the standard cycle LD 12:12 to the LD 10:14 condition and is further compressed during the LD 8:16 cycle, while it is expanded in going from the LD 12:12 to the LD 14:10 cycle). Although the LD 1:23 cycle contradicts this pattern as the period of cone constriction was greater than even the LD 10:14 cycle, this regime could be thought of as a non-parametric stimulus as the duration of daily light is relatively short.

Changes in the photoperiod may cause the photic sensitivity of the circadian oscillator to alter as prolonged exposure to constant darkness has been found to affect the oscillator's phase-shifting response to light pulses (Daymude and Refinetti, 1999; Shimomura and Menaker, 1994). There is a compression of the period the cones are constricted in going from a LD 12:12 cycle to a LD 10:14 and LD 8:16 cycle and an expansion in going from the LD 12:12 to LD 14:10 cycle, which may be a result of changes in sensitivity of the oscillator. However, the LD 1:23 cycle cannot be neglected even though it is a non-parametric stimulus. As the cones are constricted for longer periods during the LD 1:23 cycle in comparison to both the LD 10:14 and LD 8:16 cycles, this suggests that a change in photosensitivity of the oscillator plays little role in expanding and compressing the rhythm of cones.

Negative masking, the inhibition of activity caused by light in nocturnal animals, may also have a role in the mechanism of expansion and compression of circadian rhythms (Refinetti, 2002) and cannot be ruled out as a possible mechanism for causing expansion of the period that the cones are fully light adapted. The cones remain fully constricted for an extra 2 hours as dusk is delayed during the LD

14:10 cycle in comparison to the standard cycle. After dusk the cones elongated more rapidly than during the standard cycle. The extra time the cones are constricted may be due to either masking, the direct effect of light on the cones, or due to the light affecting the oscillator controlling the cones.

5.5.3 Dual oscillator theory

A popular explanation of how circadian rhythms adjust to different photoperiods was first described by Pittendrigh and Daan (1976c). After noting the splitting of activity rhythms in hamsters under constant conditions, they proposed a pacemaker (oscillator) consisting of two coupled oscillators; an evening (E) and a morning oscillator (M). E controls the evening component, is sensitive to evening light and can be thought of as tracking dusk, whilst M controls the morning component, is sensitive to morning light and tracks dawn. The periods of E and M, which may differ, are dependent on the overall light intensity, whilst the interaction between E and M, which is dependent of the phase difference between them, determines the overall period of the pacemaker.

More than one oscillator has been demonstrated by pinealectomy in hamsters which caused earlier splitting of locomotor activity (Aguilar-Robiero and Vega-Gonzalez, 1993), while melatonin coupled desynchronized activity rhythms of rats kept in constant light (Chesworth et al, 1987). In hamsters, a unilateral lesion of one of the SCNs eliminated the splitting and suggested that each SCN contained one oscillator (Pickard and Turek, 1982).

The dual oscillator model has also been used to explain the entrainment mechanisms in the circadian rhythm of N-acetyltransferase (NAT) activity in the rat pineal gland (Illnerová and Vaněček, 1982). The evening rise and the morning decline of NAT were taken as phase markers of the NAT rhythm. Pulses of light affected these phase markers differently leading to the suggestion that an evening component (E) may control the evening rise of NAT whilst a morning component (M) controls the morning decline (Illnerová and Vaněček, 1982). Similarly, entraining rats to light cycles with different photoperiods also revealed different effects on both E and M (Illnerová and Vaněček, 1983, 1985; Vaněček and Illnerová, 1985). The phase relationship between E and M is given by the period between the evening NAT rise and the morning NAT decline. Thus, the phase relationship between E and M might show when NAT activity is elevated and therefore when melatonin production is high (Illnerová and Vaněček, 1982, 1983, 1985). For example during long summer days in temperate species, the nocturnal release of melatonin is compressed possibly due to a delay in E and an advance in M oscillators controlling NAT activity. Similarly, the short winter days cause a decompression of nocturnal melatonin release which could be brought about by advancing E and delaying M (Sumová et al, 2000). The two coupled oscillator model has also been proposed in other studies (for example, Daan and Berde, 1978; Meijer et al, 1990; Strogatz and Stewart, 1993; Drijfhout et al, 1997).

The two oscillator model could also apply to the oscillator/pacemaker controlling the rhythm of cone movements in the glowlight tetra. In this case, M, the morning component can be taken as controlling the period when the cones are constricting from dark to light levels; the dawn transitions. Similarly, E, the evening

component may function when the cones are elongating from light to dark levels; the dusk transitions. When entraining glowlight tetras to different photoperiods, the positions of the transitions governed by E and M can be compared relative to those during the natural schedule, the LD12:12 cycle. Thus, during entrainment to the LD14:10 cycle, E and M have both been delayed by 0.5hour (table 5.2). Entrainment to the LD10:14 advances M by 1 hour and has no effect on E. E is advanced by 2 hours and M by 1 hour during the LD 8:16 cycle, while entrainment to the LD 1:23 cycle delays both E and M by 4 hours. Thus, entrainment to both the LD 10:14 and LD8:16 photoperiods have moved E and M by different amounts, indicating that they are independent oscillators. During entrainment to the LD14:10 and LD1:23 cycles, both E and M have moved by approximately the same amount indicating that these photoperiods might have the same effect on the two oscillators.

The same mechanisms proposed by Illnerová and Vaněček (1982, 1983, and 1985) for the control of the NAT rhythm in rat pineal glands may apply in the glowlight tetra retina. In chapter one, evidence for the possible role of melatonin in controlling circadian cone rhythms were given. If melatonin were indeed involved in the circadian control of cone rhythms in the glowlight tetra, then it may be that retinal NAT activity may also be under control of a dual oscillator as in rat pineal gland. Thus, the rhythm of cone movements, in particular the E and M components of the rhythm may be a direct reflection of the rhythm of retinal NAT activity. However, previously the evidence that melatonin has a role in the control of retinomotor movements in the glowlight tetra was inconclusive (see section 3.4.7 and 3.5.1).

5.5.4 After-effects

Following entrainment to the different light schedules, the free-running period changed only after entrainment to the LD 14:10 cycle by half an hour. Thus, although the pattern of cone movements in constant darkness differed to that found during the light/dark cycle for all light schedules (LD 14:10, LD 10:14, and LD 8:16), the free running period remained unchanged at approximately 24 hours.

Previously, entrainments to 24-hour light/dark cycles with different photoperiods have caused after-effects in hamsters (Refinetti, 2002), flies (Tomioka et al, 1997), rats (Madrid et al, 1998), mice (Pittendrigh and Daan, 1976a-c), and sparrows (Binkley and Mosher, 1986). That the cone rhythm in the glowlight tetra shows little, if any, after-effects following entrainment to different light regimes indicates a strong endogenous pacemaker with little flexibility. Alternatively, this may be due to the two oscillators, E and M, being coupled in a manner that no matter how one moves with respect to the other, the overall period of the rhythm will remain unchanged.

5.5.5 Entrainment by a pulse of light

The LD 1:23 cycle represents an example of entrainment by a pulse of light. To determine at what phase of the cone rhythm this pulse was given, the light history needs to be known. The glowlights would have to be entrained to a LD 12:12 schedule for 2 weeks and then the lights would be left permanently off at a specified time. A pulse of light can then be administered at any circadian time.

This procedure was not carried out in this experiment and therefore during entrainment to this light cycle, the circadian time the pulse of light was given is not known. However, following entrainment, the circadian time the light pulse was given can be determined. By moving the rhythm so that it matches the free-run rhythm (Figure 5.11), it can be seen that a pulse of light given at ct4 will allow stable entrainment. Therefore, it can be predicted that there will be transient rhythms that will advance and delay the free-run rhythm until the light pulse is situated at ct4.

The above assumes that both the duration and intensity of light was bright enough to entrain the rhythm. However, this is not necessarily the case. In some cases when the entraining light/dark cycle is inadequate to cause proper entrainment, the circadian rhythm may appear to be entrained for a long time before breaking away from the Zeitgeber and then later returning to the schedule to appear entrained again. This process has been called 'relative co-ordination'. Previously, 'relative co-ordination' has been found after following the circadian rhythm for up to 200 days in continual darkness (Pittendrigh and Daan, 1976 b). For obvious reasons, this has not been done in the glowlight tetra.

5.5.6 365 days of entrainment to the LD 8:16 cycle

In continual darkness following entrainment for 365 days, the rhythm of cone movement matched that found after only 30 days. Therefore, 30 days is enough to entrain glowlight tetras to the various light schedules used in these (and other) experiments. Whether raising glowlights from birth or conception under this light

regime would make a difference is difficult to say. In both mice (Davis and Menaker, 1981) and hamsters (Refinetti, 1998) no effect of the early environment was noted. Also, the presence of a light/dark cycle is not necessary for the development of a circadian clock as animals raised in either constant darkness or light since birth exhibit the same free-running rhythm as those that have experienced a 'normal' light history (Richter, 1971; Tomioka et al, 1997).

5.5.7 Implications for fish studies

The rhythm of glowlight tetra cone contraction entrained well to 24 hour light/dark cycles with light periods ranging from 8 to 14 hours, compressing and expanding the time the cones were contracted to fit the prevailing conditions. In all cases, the cones were constricted around dawn and began elongation after dusk. This range is similar to that used in previous retinomotor movement experiments and indicates that fish should be able to entrain to the arbitrarily chosen light schedules used in previous studies, especially as the most popular has been the LD 12:12 cycle.

However, the LD 14:10 cycle demonstrates how an inappropriate light schedule can mask the circadian control of the cones. Here, the cones do not appear to be predicting dawn as they have only just started to constrict at lights on. It is dawn that causes the rapid cone constriction. Similarly after dusk during the shorter photoperiod schedules of LD 8:16 and LD 10:14, there appears to be a more rapid elongation of the cones, implying that dusk (the external light) is overriding the endogenous signal. Thus, although the rhythm can entrain well to the above light

schedules, the light schedules appear to either mask or actually affect the endogenous control of the cones.

In continual darkness following entrainment to the LD 14:10, LD 10:14 and LD 8:16, the rhythm of cones altered in comparison to that seen during the LD 12:12 cycle. Thus, if a light cycle that is significantly different to the fish's natural habitat is used for entrainment, this may lead to erroneous results when cycling in continual darkness, especially if only few points are sampled. For example, during the LD 8:16 the cones are fully dark-adapted 3 hours after dusk (at t=11, the cone index is statistically similar to the dark value; $p>0.2$, unpaired Student t test). However, 24 hours later in continual darkness, the cones have only just commenced elongation (at t=35 the cone index is 0.37). If sampling were infrequent, this would indicate that the rhythm has dampened in continual darkness when clearly it has not. Thus, entraining to the natural light schedule of the fish is important if one is to determine the endogenous rhythm in constant darkness, although frequent sampling throughout the light/dark cycle can reduce this problem.

Although the rhythm altered in continual darkness following entrainment to the above light schedules, it cannot be assumed that this indicates that the endogenous rhythm is weakening. It may be that following entrainment to the various photoperiods, the rhythm of cone movements is simply returning to the shape following entrainment to the LD 12:12 cycle similar to that of the glowlight tetra's habitat. Thus, if fish were left in continual darkness following entrainment to the above light schedules, the rhythm may continue for 14 – 28 days without

damping as found previously following entrainment to the LD 12:12 schedule (chapter 3).

5.6 Summary

Glowlight tetras entrained well to all light schedules presented and unusually the free running period remained at approximately 24 hours, except following entrainment to the LD14:10 where the free running period may have increased to 24.5 hours. The LD1:23 cycle might be an example of entrainment following a pulse of light or it may represent 'relative co-ordination', that is, incomplete entrainment. Theories using models either involving one or two oscillators could explain the results for the glowlight tetra.

The results of these experiments have some implications in assessing previous retinomotor fish studies. Ideally, fish should be entrained to their natural light schedule or frequent sampling of fish around the clock should be undertaken.

Chapter-6 Retinomotor movements and the dorsal light response in two species of fish

6.1 Abstract

As outlined in chapter one, there are several possible functions of retinomotor movements including regulating visual sensitivity. In this chapter, efforts are made to determine whether retinomotor movements influence visual sensitivity, which is taken here to be the perception of relative brightness. For reasons outlined in the introduction, the angle of tilt during the dorsal light reaction (DLR) was used as an indication of visual sensitivity.

The DLRs of two species, the glowlight tetra (*Hemigrammus erythrozonus*) and zebrafish (*Danio rerio*) were measured during light adaptation at two different times;

A/ In the middle of the night.

Under these conditions the cones of the glowlight tetra remained in their elongated dark positions while RPE pigment migrated within 30 minutes. In the zebrafish, both the cones constricted, and the RPE pigment moved, to their light adapted positions within 45 minutes.

B/ In the middle of the day.

The cones and RPE pigment of both species remained in their light adapted positions throughout the course of the experiment.

Under both conditions, and in both species, there was a similar change in visual sensitivity as measured by the DLR. It is thus concluded that while sensitivity changes during light adaptation, this is not related to retinomotor movements.

6.2 Introduction

There has been much speculation as to the function of retinomotor movements (see section 1.3). However, it is still unclear what, if any, role retinomotor movements have in determining the level of relative brightness or visual sensitivity. That is, do retinomotor movements have a role in determining that light appears brighter in an eye facing unilateral illumination compared to the less illuminated eye? There is a temporal correlation between electroretinogram (ERG) sensitivity changes during both light and dark adaptation and retinomotor movements in the rainbow trout (*Salmo gairdneri*) (Douglas, 1982b), providing circumstantial evidence for a link between retinomotor movements and visual sensitivity. In the green sunfish (*Lepomis cyanellus*), retinomotor movements in constant darkness mirror endogenous ERG sensitivity during the subjective daytime but not the subjective night, indicating that the circadian rhythm of ERG sensitivity is not purely a function of retinomotor movements (Dearry and Barlow, 1987). Links between the ERG and retinomotor movements have also been demonstrated in the salmon (*Salmo salar ouaniche*) (Gramoni and Ali, 1970) as both responses were dependent on the intensity of the preadapting illumination and light adaptation proceeds quicker than dark adaptation. However, there was no difference in the sensitivity of the midas cichlid (*Cichlasoma citrenellum*) retinas, as determined by ERG, in prolonged darkness in animals whose cones were elongate and in those whose cones were held in a contracted position pharmacologically (Powers et al, 1992).

In this chapter, the DLR is used as an indicator of visual sensitivity in order to determine whether retinomotor movements are important in the determination of sensitivity. An overview of the DLR including evidence that it is an indicator of sensitivity will first be given.

Most fish determine their orientation in the water column by combining luminance information from both eyes with a gravitational signal coming from the vestibular system (von Holst, 1935; Pfeiffer, 1964). Both the luminance and the gravitational signal act to keep the fish in an upright position. In turbulent waters where the vestibular system may send erroneous signals to the fish, the ocular luminance information will maintain the fish in its upright position. Likewise, in the dark, the vestibular system keeps the fish in its normal position. Fish normally swim with their dorsum towards the incident light which allows an equal luminance to reach each eye, and when there is even illumination from above, as is usually the case, remain in an upright position. If illuminated from the side, however, fish will orient their backs toward the light source by tilting about the longitudinal axis in order to maintain equal luminance to both eyes. The amount of tilting will be countered by gravitational signals from the vestibular system acting to keep the fish upright, and the fish will tilt to an intermediate position. This is the dorsal light response (DLR) (von Holst, 1935) (Figure 6.1).

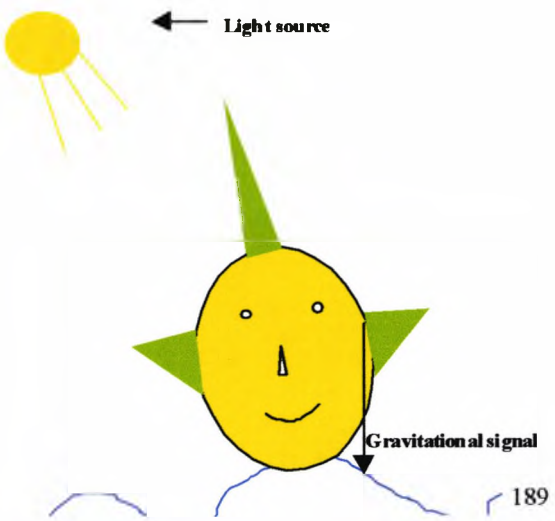

Fig 6.1 The DLR

Diagram showing DLR in fish where the amount of tilting to orient the back towards the light source is countered by the gravitational signal.

Von Holst (1935) first investigated the DLR in the wrasse, *Crenilabrus rostratus* which tilted about its longitudinal axis towards a light on one side. Further studies have revealed the DLR to be present in most species of free swimming fish. Canella (1937), for example, found the DLR in 13 species of fish but not in flat fishes (bottom dwelling with both eyes positioned on one side of the fish) or seahorses (habitually attach themselves to weeds). Thibault's (1949) study of mostly temperate freshwater fish showed the DLR in all ten species, while Von Holst (1950) found it in all eight tropical freshwater species studied and Bogenschütz (1961) noted the absence of the DLR in bottom dwelling catfish but found it to be present in four other species.

Following the description of the presence of the DLR, investigations into its properties became popular. For example, Von Holst (1935) and Lang (1967) both found that increasing the intensity of light increases the angle of tilt, while Braemer (1957) and Bogenschütz (1961) varied the angle of incident light to determine the effect on the tilt angle. Thus, the degree of tilt caused by light exposure to a particular area of the retina could be compared to the corresponding photoreceptor distribution. Similarly, at very low intensity, light that impinges the upper half of the retina where there are more rods than in the central retina of the black tetra, *Gymnocorymbus ternetzi*, produced a greater angle of tilt than light directed at the central retina (Braemer, 1957). The amount of tilt during dark adaptation was also compared to the state of photoreceptor adaptation (Braemer, 1957), showing that the tilting increased at the same time that rods began to move.

6.2.1 The DLR as a measure of visual sensitivity

Several factors suggest the DLR can be used as a measure of visual sensitivity; 1/ Increasing the brightness of light directed to one side of a swimming fish increased the angle of tilt (Von Holst, 1935; Lang, 1967).

2/ As the DLR needs no training and does not diminish with time, it has been widely used to determine the spectral sensitivities of fish (Thibault, 1947a, b and 1949; Lang 1967; Silver, 1974; Powers, 1978; Muntz et al, 1996). In all cases, the spectral sensitivity curves constructed using the DLR reflected the absorption spectra of the relevant cone pigments (determined using microspectrophotometry) indicating the DLR is a reliable measure of visual sensitivity in fish.

3/ Experiments to determine the role of dopamine during light adaptation (Lin and Yazulla, 1994; McCormack and McDonnell, 1994) further imply that the DLR can be used to assess sensitivity. When dopaminergic interplexiform cells are destroyed in one eye by intraocular injection of the neurotoxin 6-hydroxydopamine (6-OHDA), or following unilateral blocking of dopaminergic receptor sites, fish tilt towards this eye. As dopamine is the major light adaptive signal within the teleost retina (Djamgoz and Wagner, 1992; Witkovsky and Schutte, 1991 for reviews), its removal or inhibition in one eye will cause that eye to dark adapt. As a consequence, this eye will be more sensitive to light. Thus, the perceived intensity of the overhead illumination will be greater in the eye that has been injected and the fish will tilt towards it.

As the DLR probably reflects sensitivity, it was used here to determine whether retinomotor movements influence visual sensitivity. To do this, the DLR of glowlight tetras was measured during 100 minutes of unilateral light exposure in the middle of their dark phase. Previously under similar conditions, the glowlight tetra cones remained stationary while RPE melanosomes migrated to light adapted positions (see chapter four). Therefore measuring the DLR under these conditions could be interpreted as measuring visual sensitivity while cones remain stationary and RPE melanosomes migrate. The DLR of the glowlight tetra was also measured during 100 minutes of unilateral light exposure in the middle of the light phase. Under these conditions, the cones and RPE melanosomes remain in their fully light adapted positions so that sensitivity is being measured while the cones and RPE remain stationary.

Similar experiments were performed on zebrafish (*Danio rerio*). Preliminary examinations showed that under similar conditions to the glowlight tetra, 100 minutes of bright light in the middle of the dark phase caused both the cones and the RPE melanosomes to migrate to fully light adapted positions, while placing zebrafish in the dark for 100 minutes during the middle of their light phase caused both cones and RPE melanosomes to move to intermediate dark adapted positions (unpublished results). Therefore, during measurements of the DLR in the middle of the dark phase, both the cones and RPE melanosomes become light adapted within 100 minutes of unilateral illumination. Similarly, the cone and RPE melanosomes should remain stationary in their light adapted positions, during measurements of the DLR in the middle of the light phase. In summary,

measuring the DLR under these two conditions in the zebrafish, could be interpreted as measuring the sensitivity when both cones and melanosomes are migrating in comparison to when cones and RPE melanosomes are stationary.

6.3 Materials and Methods

6.3.1 Animals

As for previous experiments, glowlight tetras (sls 2.0 – 2.7cm) and zebrafish (sls 2.7 – 3.1cm) were purchased from a local supplier and maintained on a 12-hour light /12 hour dark cycle for a minimum of 30 days prior to experimentation. Two light schedules were used. In one tank (A) containing both species of fish, the lights were on from 15.00 – 03.00, while in another tank (B), also containing both species, the light phase was 04.00 – 16.00. Having different light schedules allowed two experiments to be run per day. On each day of experimentation, light adaptation in the middle of the night experiments commenced at 08.30 using specimens from tank A. On completion, a second experiment, light exposure in the middle of the day, commenced at 10.30 using fish from tank B. After each experiment, fish were returned to another tank (but not used for further experimentation).

6.3.2 Apparatus

Measurements of the DLR were made in a purpose built Perspex flume (Figure 7.2). Water (25°C) from an adjoining tank was pumped through to create a current for the fish to swim against and a removable plunger was positioned to ensure that fish swam as close as possible to the end facing the camera (Figure 6.2). The rate of flow of the water current was controlled by adjusting the water pump until the fish took up a position facing the current that it could maintain for the duration of the experiment.

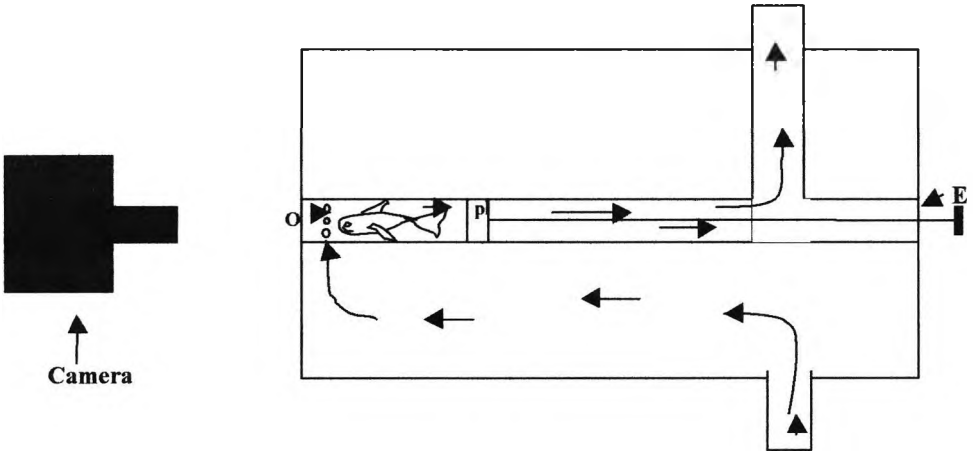

Fig 6-2 Direction of water flow and fish in flume

Side view of the DLR flume showing both fish and the direction of water flow as indicated by the arrows. Note that the fish is swimming against the water flow and facing O, the opening through which filming took place. Here; pl, plunger; E, entrance to flume.

The flume was normally blacked out except for 2 openings: The front face of the inner tube of the flume (O in Figure 6.2) was transparent to allow filming of the

animal, while the stimulus light entered a narrow rectangular slit (4.5 cm long, 1.5cm wide) above the swimming fish 60° from the vertical. The black out could be temporarily removed from the top of the flume to allow illumination with either infrared light or room lights during periods when the animals were acclimating to the flume before experimental unilateral exposure (Figure 6.3).

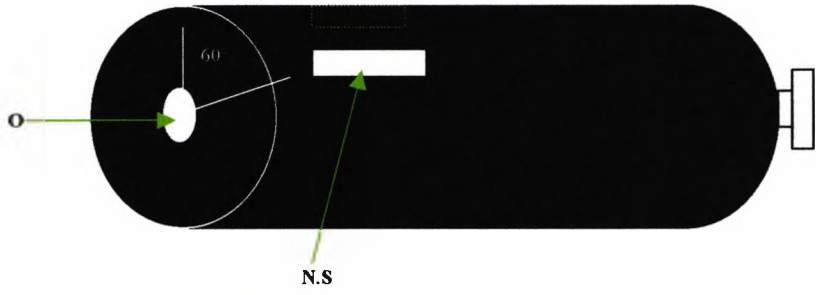

Fig 6-3 Flume narrow slit position

Side view of almost totally blacked-out flume showing O; the opening toward which the fish swam and could be filmed and N.S;the narrow rectangular slit through which the adapting light entered. The dotted green line shows the removable black cover that allowed infrared and room light to be directed through enabelling viewing of fish in the dark and light before the adapting light was turned on.

Adapting illumination was provided by a Kodak Carousel S-AV projector fitted with a 250W quartz halogen bulb positioned within a box at 60° to the flume (Figure 6.4). The output of the projector was beamed through a heat glass and adjusted by a neutral density filter to provide 1.97 mW/cm^2 incident on the flume measured using a light meter (Macam PM203).

During each experiment, fish were filmed using a Computer Zoom lens 18 – 108/2.5 attached to a Cohu CCD high performance camera, a Panasonic VHS

recorder, a For> Video Timer, and a Hitachi monochromatic monitor. Red filters were placed over the monitor to shield the flume from any stray light.

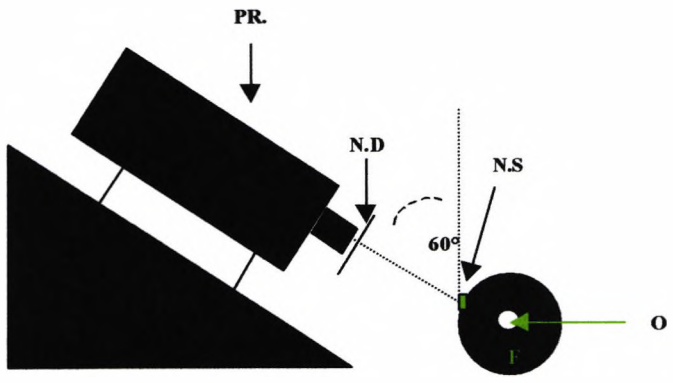

Fig 6-4 Projector light and flume set up

Diagram showing the projector light (PR) and flume (F) set up from in front. ND, neutral density filter, NS narrow slit where the projector light entered, and O the opening to allow filming of fish.

6.3.3 Procedure

10 minutes before each experiment, the pump in the adjoining tank to the flume was turned on, allowing the flume to be flushed with water of the correct temperature. For experiments in the middle of the night, fish were removed from their home tanks having been placed in netted black jars the preceding dusk. The pump was turned off, the plunger removed from the flume and fish were transferred to the flume via a funnel. The plunger was replaced carefully to position the fish at the appropriate end of the flume before turning the pump on again. While viewing fish with infrared illumination, the pump flow was adjusted so that the fish faced the current and could be filmed. Fish were then given 15 minutes to settle. Towards the end of this period, filming commenced. After 15

minutes in darkness the adapting light was turned on and fish filmed for 100 minutes. All procedures commencing with removing the fish from their tanks until the adapting light was turned on, were carried out only using a dim red light (see section 2.4.1).

For light adaptation during the middle of the day, fish were transferred from the home tanks to the flume with the room lights on and acclimated to the flume while illuminated by the room lights. After 15 minutes the room lights were turned off, the adapting illumination commenced, and fish were filmed for 100 minutes.

If a fish either failed to 'settle' before the experiment or swam around excessively during the experiment, the experiment was terminated and the fish was discounted. Either 6 or 7 fish of each species were light adapted during both the day and night.

6.3.4 Analysis of the DLR experiments

Where possible individual frames were 'grabbed' just before the adapting light went on and at exactly one minute intervals for the first 20 minutes of unilateral illumination. Thereafter frames were taken every 5 minutes. If the fish was not directly facing the camera or touched the sides of the flume, the image was rejected and another chosen as close as possible to the required interval (and always within 10 seconds of it). Images were analysed using Scion Image and a line drawn along the mouth (glowlight tetras) or joining the corners of the mouth (zebrafish) (Figure 6.5 and 6.7) gave the angle of tilt of the fish. The locations of

these lines were chosen because they were easy to see and it is for this reason that others have also chosen a line along the mouth as the reference point (Muntz et al, 1996).

6.3.5 Histological Procedure and Analysis

To determine the state of cone and RPE adaptation during light adaptation in the middle of the night and day, the above experiments were repeated without filming the fish. Instead, fish were sacrificed at specific time points and prepared for light microscopy. During the middle of the night, fish were sacrificed at 0, 15,30,45,60, and 100 minutes after the unilateral light was turned on. These points were chosen because light adaptation in the middle of the dark phase has previously been investigated at these time points, although under different lighting conditions (chapter 4) and as such, the positions of the retinomotor elements could be predicted. For light adaptation in the middle of the day, only three time points were investigated (0, 45,100 minutes after unilateral light illumination began) in order to confirm that the cones and RPE remained light adapted throughout the experiment. 3 fish were sacrificed at each time point.

During these experiments the right eye faced the adapting illumination. However, the 2 eyes of an individual were not analysed separately for retinomotor position. Ideally this should have been done. It is unlikely that the retinomotor movements of the 2 eyes of an individual differed significantly as the adapting light was a long way above the threshold needed for retinomotor movements and scatter within the flume will almost certainly have resulted in both eyes receiving enough illumination to trigger such changes. Although it is unlikely, the possibility that

retinomotor light adaptative movements took place at different rates in right and left eyes of an individual needs to be addressed (see discussion).

6.4 Results

6.4.1 Light adaptation in the middle of the night

6.4.1.1 DLR

6.4.1.1a Glowlight tetra

The tilt angle was measured in 6 different fish during 100 minutes of unilateral illumination. Because the maximum tilt angle differed in each fish (17°-23°), to determine the pattern of change of tilt angle with time, data were first normalised in each fish. Thus, the maximum tilt during the experiment was given the value of 1 and all other angles expressed relative to this. The average normalised tilt angles for all fish at each time point can then be determined (Figure 6.6).

Before light exposure, the average normalised tilt angle was slightly below zero as some fish swam with a subtle tilt away from the projector light. On light exposure, the average tilt angle increased for about 8-9 minutes at which time it began to decrease. The average tilt angle then decreased until about 15-16 minutes before it began to rise slowly before eventually levelling off (Figure 6.5 and 6.6).

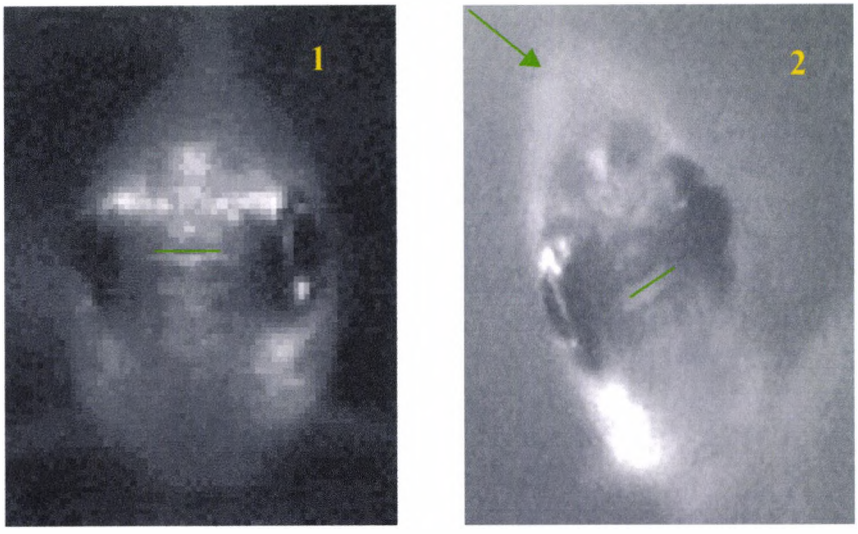

Fig 6.5 Glowlight tetra in flume (dark phase)

Photographs of glowlight tetra swimming in the flume during the middle of the dark phase **1/** in the dark before the light has been switched on and **2/** 9 minutes after the unilateral light has been switched on. Note that in **2** the direction of tilt is towards the light source indicated by the green arrow. The green dotted line drawn along the mouth gives the angle of tilt.

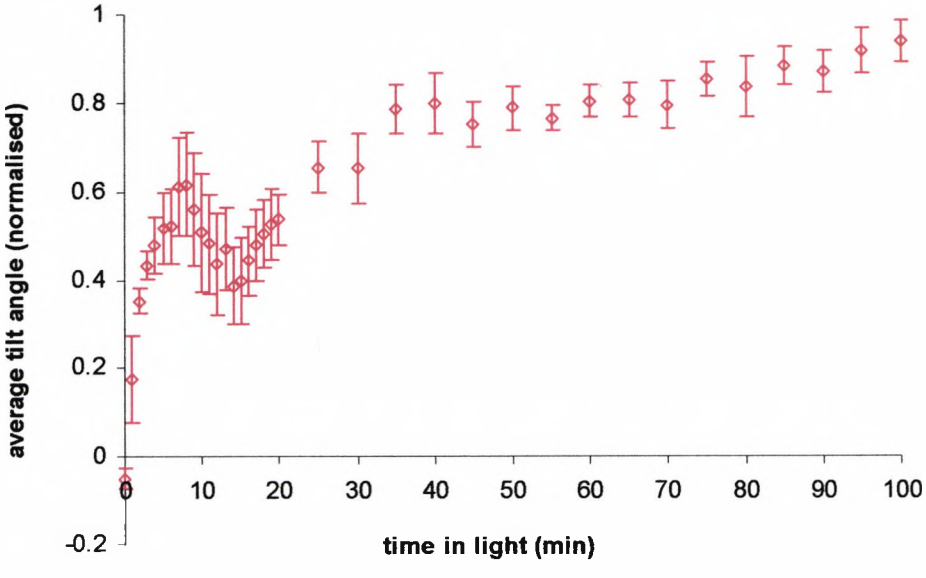

Fig 6-6 Glowlight tetra average normalised tilt angle (dark phase)

Averaged normalised tilt angle in glowlight tetras during 100 minutes of light exposure in the middle of the dark phase. Each point represents the mean and ±SE of 6 fish.

6.4.1.1b Zebrafish

The tilt angle was measured in 7 different zebrafish during 100 minutes of illumination. Again, as the maximum tilt angle varied (10°-14°), the data were normalised and averaged (Figure 6.8).

Before the lights were turned on, the average normalised tilt angle is slightly below zero indicating that some fish, with glowlight tetras, tilted slightly away from the projector light. When projector light was turned on, the angle of tilt gradually increased until it eventually levelled off. There is a suggestion of a slight decline of angle from about 11-16 minutes (Figure 6.7 and 6.8).

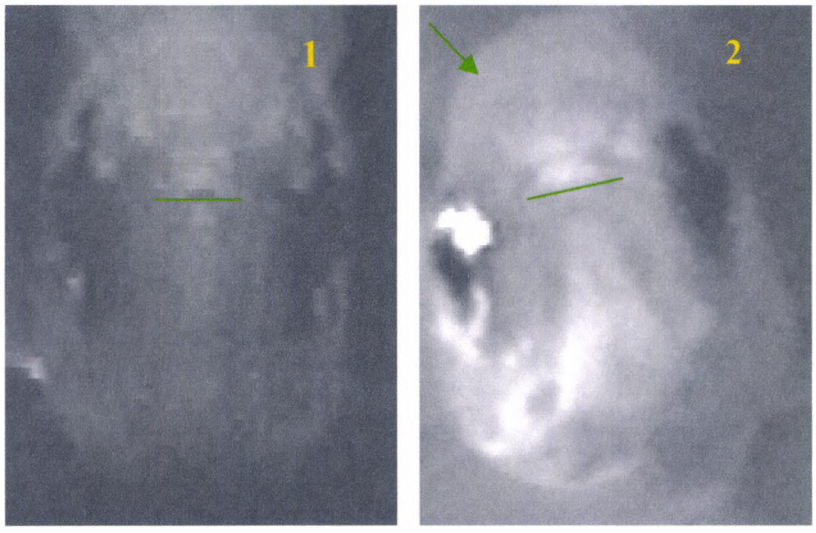

Fig 6-7 Zebrafish in flume (dark phase)

Zebrafish swimming in the flume during the middle of the dark phase **1/** before the light has come on when there is a slight tilt away from the direction of the light source and **2/** 14 minutes after unilateral light exposure when the fish now tilts towards the unilateral light source (green arrow). The green dotted line drawn joining the corners of the mouth gives the angle of tilt.

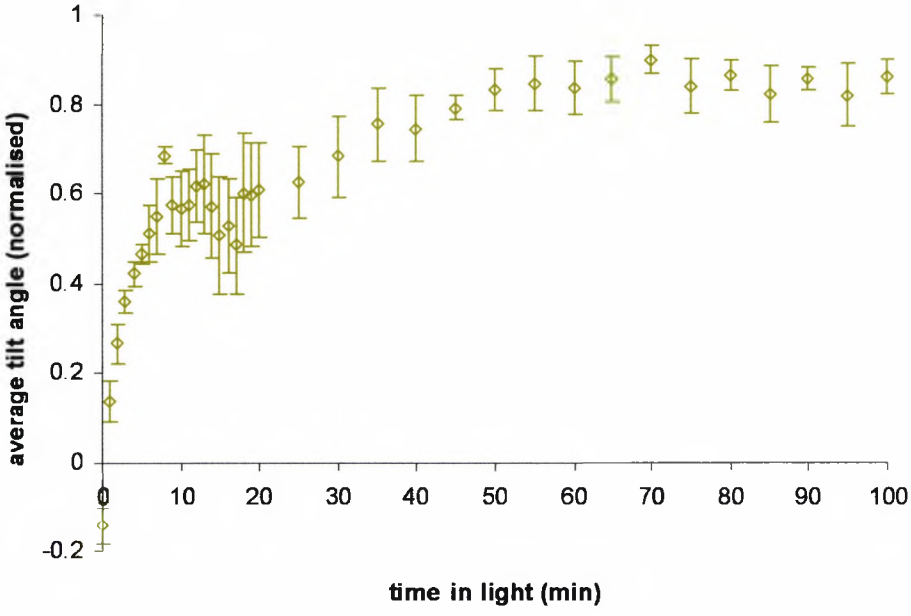

Fig 6-8 Zebrafish normalised tilt angle

Averaged normalised tilt angle in zebrtafish during 100 minutes of light exposure in the middle of the dark phase. Each point represents the mean ±SE of 7 fish.

6.4.1.2 Retinomotor responses

The cones of the glowlight tetra remained elongated and fully dark adapted throughout the course of the experiment while the RPE melanosomes migrated to reach their fully light adapted position within 30 minutes (Figures 6.9 and 6.10). Meanwhile, the cones and RPE melanosomes of the zebrafish commenced their light adaptive movements within 15 minutes of light exposure and became fully light adapted by 45 minutes (Figures 6.9 and 6.11).

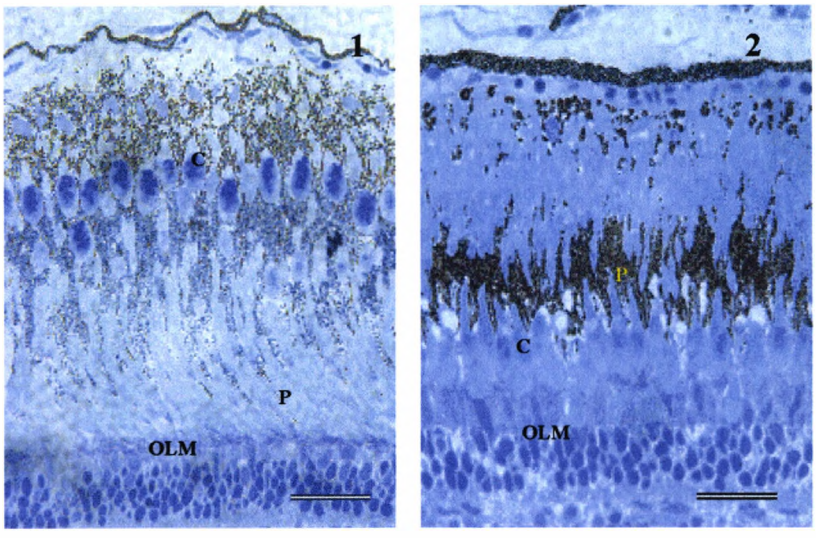

Fig 6-9 Glowlight and zebrafish retinae (dark phase and light exposure)

1/Transverse section of glowlight tetra retina at t=30 (after 30 minutes of light exposure in the middle of the dark phase). Note here that the cones remain fully elongated while the migratory RPE melanosomes (P) can be seen near the OLM. 2/ Transverse section of zebrafish retina following 45 minutes of light exposure in the middle of the dark phase (t=45). Here, both the cones and the RPE melanosomes are in light adapted positions. Bar = 25μm.

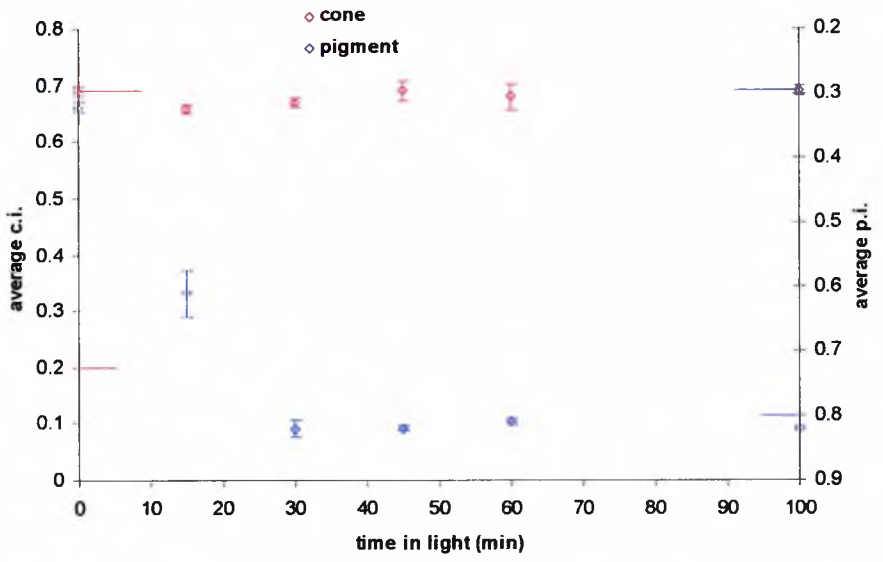

Fig 6-10 Glowlight tetra cone and pigment indices (dark phase and exposure) Glowlight tetra cone and pigment indices during 100 minutes of light exposure in the flume during the middle of the dark phase. The short bars (red, cones; blue, pigment) give the normal light adapted values and the long bars the normal dark adapted values. Each point represents the mean ±SE for 6 retina.

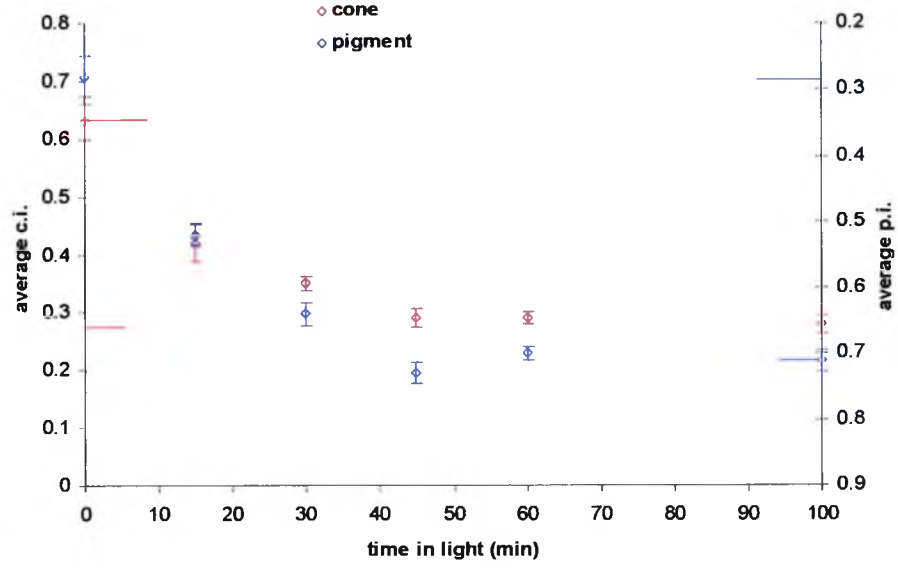

Fig 6-11 Zebrafish cone and pigment indices (dark phase and exposure)

Zebrafish cone and pigment indices during 100 minutes of light exposure in the flume during the middle of the dark phase. Here, the short bars (red, cones; blue, pigment) give the normal light adapted values and the long bars the normal dark adapted values. Each point represents the mean ±SE for 6 retina.

6.4.2 Light adaptation in the middle of the day

6.4.2.1 DLR

6.4.2.1a Glowlight tetra

The tilt angle was measured in 6 fish during illumination (maximum tilt angle range 17°-25°). Again, the average normalised tilt angle for the 6 fish was determined at each time point (Figure 6.12).

Before the projector light was turned on, the average tilt angle was negative indicating a subtle tilt away from the projector light. When the light was switched on, the average tilt angle increased for about 12 minutes before it declined for a further 2-8 minutes before increasing again until it eventually levelled off (Figure 6.12).

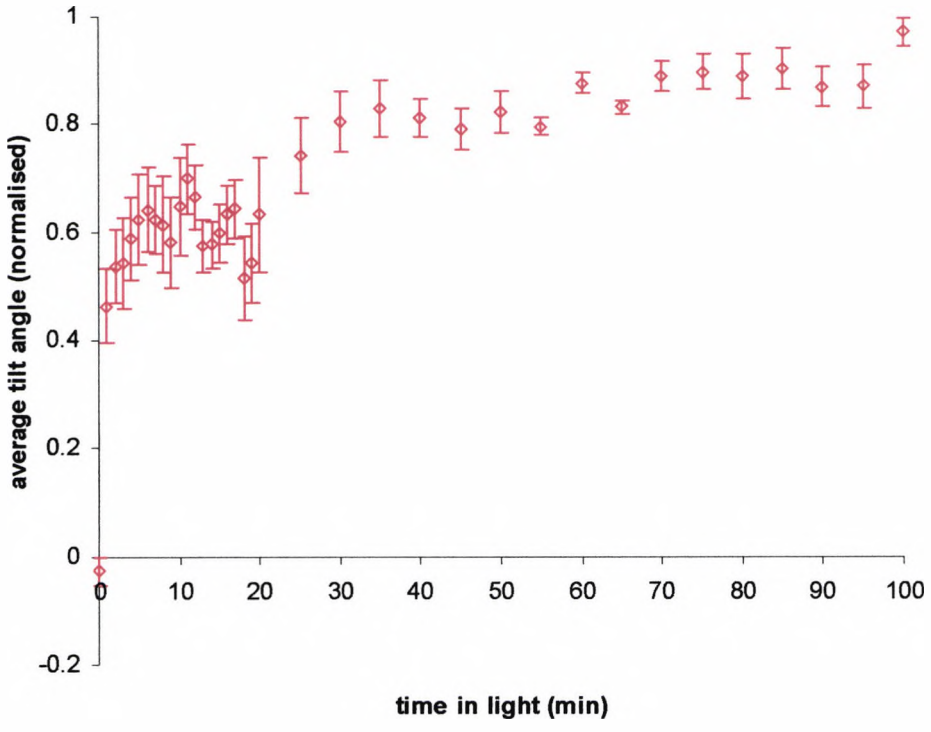

Fig 6-12 Glowlight tetra normalised tilt angle (light phase)

Averaged normalised tilt angle in glowlight tetras during 100 minutes of light exposure in the middle of the light phase. Each point represents the mean ±SE of 6 fish.

6.4.2.1b Zebrafish

6 fish were used to measure the tilt angle during unilateral illumination and the average normalised tilt was determined as above (maximum tilt angle in these fish varied from 12°-14°) (Figure 6.13).

Again, the average tilt angle is slightly negative before the projector light is turned on. When the projector light is switched on, the average tilt angle increases for the first 10-12 minutes before it declines rapidly for the next 2-5 minutes. It then increases gradually before levelling off (Figure 6.13).

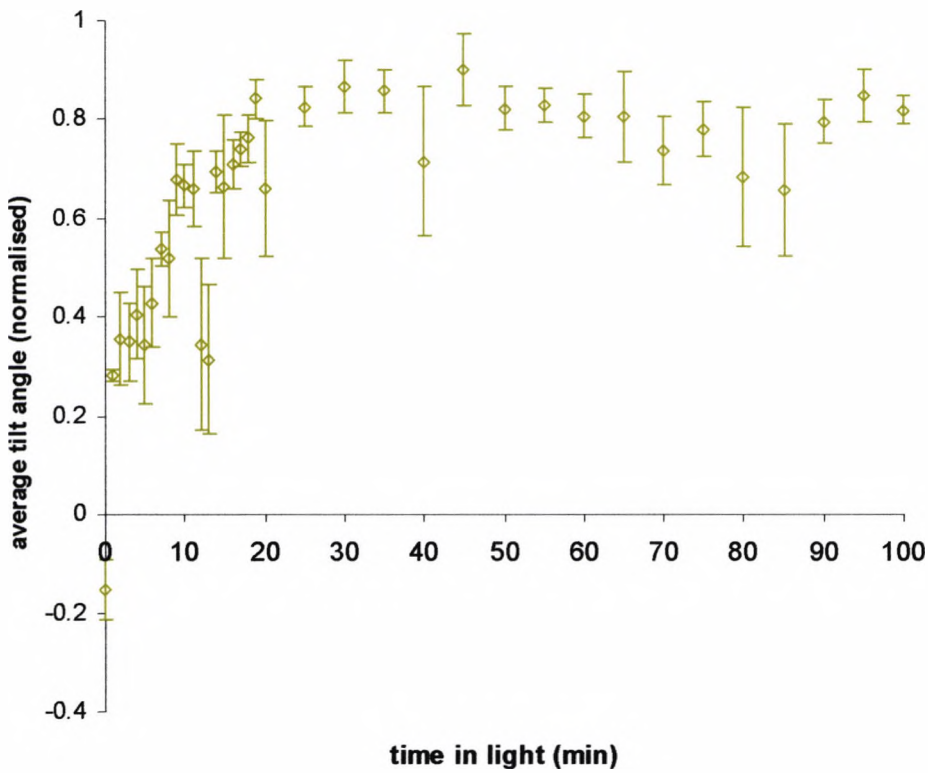

Fig 6-13 Zebrafish normalised tilt angle (light phase)

Averaged normalised tilt angle in zebrafish during 100 minutes of light exposure in the middle of the light phase. Each point represents the mean ±SE of 6 fish.

6.4.2.2 Retinomotor Responses

In both the glowlight tetra and the zebrafish, the cones and RPE melanosomes remained fully light adapted throughout the course of these experiments (Figure 6.14 and 6.15).

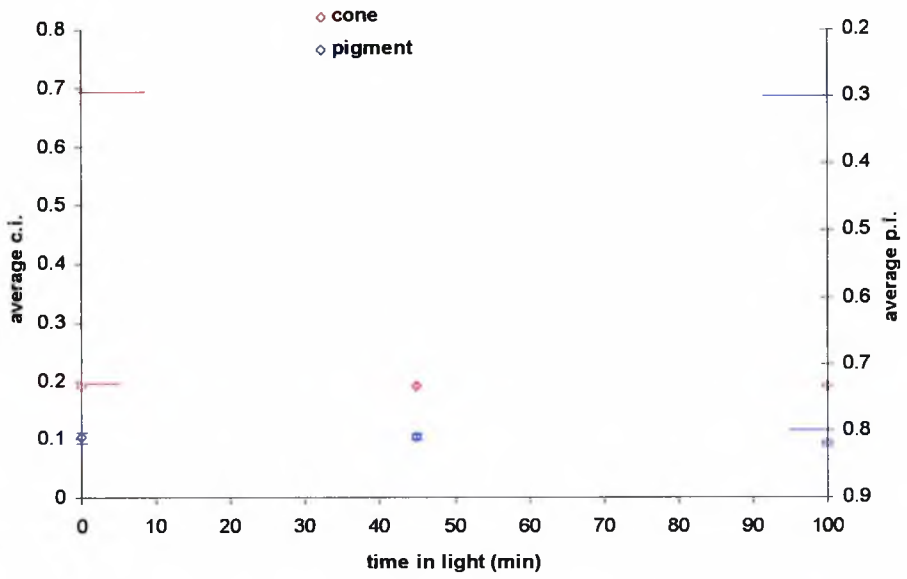

Fig 6-14 Glowlight tetra cone and pigment indices (light phase)

Glowlight tetra cone and pigment indices during 100 minutes of unilateral light exposure in the middle of the light phase. The long bars (red, cones; blue, pigment) represent the normal dark adaptive values while the short bars represent light adapted values. Each point represents the mean ± SE of 6 retinae.

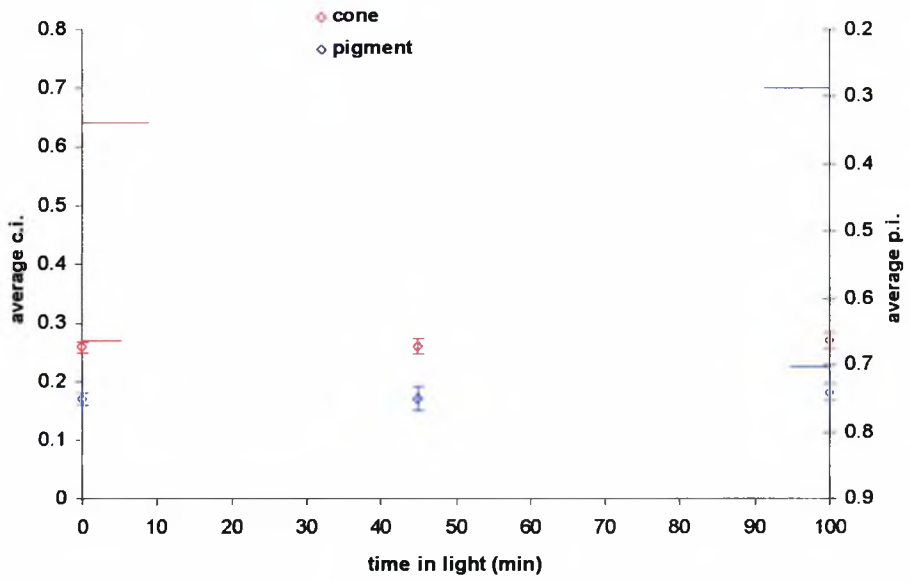

Fig 6-15 Zebrafish cone and pigment indices (light phase)

Zebrafish cone and pigment indices during 100 minutes of unilateral light exposure in the middle of the light phase. The long bars (red, cones; blue, pigment) represent the normal dark adaptive values while the short bars represent the light adapted values. Each point represents the mean ± SE of 6 retinae.

6.5 Discussion

In these experiments, the DLR was measured during 100 minutes of unilateral light exposure during both the middle of the dark and light phases of the light/dark cycle in two species of fish. As outlined in the introduction of this chapter, the angle of tilt during the dorsal light reaction may be an indicator of visual sensitivity. Thus, throughout this discussion alterations in the degree of tilt will initially be taken as an indication of changes in visual sensitivity.

However, although it is probable that changes in the angle of tilt represent changes in visual sensitivity, it is by no means certain. For example, the angle of tilt during the DLR is determined by gravitational and visual inputs. Perhaps rather than the visual input, the vestibular system is adapting and thus responsible for the changes in tilt seen during the course of these experiments. This is, however, unlikely as it has been shown that the utriculus in fish acts as an adaptation free recording mechanism even over long periods of time (von Holst, 1950). Other non-visual reasons that might explain the change in tilt will be discussed at the end of this chapter.

The pattern of changes of the average normalised tilt angle appeared qualitiatively similar in all cases. Generally there was an increase for the first 8-12 minutes before it declined for the next 2-8 minutes and then increased again before levelling off. The same pattern of changes were seen in the tetra (*Gymnocorymbus ternetzi*) where a dip in the tilt angle was noticed in the first 20 minutes of light adaptation before it increased again (Von Holst, 1950). In an earlier experiment, a similar pattern of changes was also noted in the wrasse (*Crenilabrus rostrasus*) although the initial dip in tilt angle was not noted (Von Holst, 1935). Lang (1967) may have noted the initial temporary dip in tilt angle in the guppy (*Lebistes reticlatus*) as he observed that the angle of tilt was changing during the first 5 minutes of light exposure and so decided to ignore this part of each experiment.

If the DLR is an indicator of visual sensitivity, then such sensitivity changed throughout the course of these experiments. In the glowlight tetra, the only time

there is movement of retinomotor elements is during light adaptation in the middle of the dark phase when the RPE melanosomes move. In glowlight tetras, cone movement is therefore unlikely to play a role in determining sensitivity, whereas the RPE melanosomes might. The early dip in sensitivity appears more prominent during light exposure in the middle of the dark phase in comparison to the light phase and this might be due to the migration of RPE melanosomes which reach close to light adapted positions after 15 minutes of light exposure. As left and right eyes were not differentiated during these experiments, if melanosome migration occurred at different rates in each eye, this may possibly contribute to the early dip seen in sensitivity (Figure 7.10).

In the zebrafish during light exposure in the middle of the dark phase, both the cones and the RPE melanosomes migrated to light adapted positions. Thus, these may be involved in determining the sensitivity changes seen during the duration of the experiment. Also, as left and right eyes were not differentiated, then a possible different rate of cone and melanosome movement in each eye may influence the sensitivity. Interestingly, here the initial dip in sensitivity is less obvious. During light exposure in the middle of the light phase, no retinomotor movements in the zebrafish were seen yet the initial dip in sensitivity is present. Thus, in this situation retinomotor movements could not play a part in determining sensitivity and the initial dip in sensitivity cannot be attributed to either movements of cones or the RPE melanosomes.

Although retinomotor responses of cones and RPE melanosomes may change the amount of light being absorbed by the cones, the results of the glowlight tetra and

zebrafish taken together suggest that it is unlikely that they have a role in the determination of sensitivity. This is in agreement with previous findings. In the green sunfish (*Lepomis cyanellus)*, cones contribute a similar amount to the electroretinographically determined spectral sensitivity whether the cones are fully elongated or fully constricted (Dearry and Barlow, 1987). In the midas cichlid (*Cichlasoma citrinellum*) during prolonged darkness, retinas with cones pharmacologically treated to remain in their constricted light adapted positions were found to have the same visual sensitivity, using ERG recordings, as those with cones that were allowed to elongate, again suggesting photomechanical movements do not influence visual sensitivity (Powers et al, 1992).

The pattern of changes in sensitivity in these experiments is difficult to explain on a physiological basis. The recovery of sensitivity in the dark following light bright enough to bleach a significant proportion of both rod and cone photopigment is known as dark adaptation or bleaching adaptation (Fain et al, 2001). In the dark, cone photopigment regeneration takes less than 10 minutes (Rushton, 1963; 1968), while rod photopigment regeneration takes between 20-30 minutes in humans (Campbell and Rushton, 1955). Regeneration of photopigment, and thus recovery of visual sensitivity, also occurs in light, the amount of regeneration depending on the lighting conditions. There will be an equilibrium position reached between the rate of bleaching and the rate of recovery. In bright light, the rate of bleaching will be much quicker than the rate of pigment regeneration. Thus, bleaching adaptation could explain a gradual increase in sensitivity over 100 minutes of light exposure indicating a very slow regeneration of photopigment. However, this does not explain the changes in tilt angle (the initial

dip) found during the first 20 minutes of light exposure observed here and in the black tetra (*Gymnocorymbus ternetzi*) (Von Holst, 1950).

The pattern of changes in sensitivity, including the initial dip, noted here resemble changes in the amplitude of electo-oculogram (EOG) in humans following the onset of light (Figure 6.16). EOG's record the voltage difference between the posterior pole of the eye and the cornea and there is ample evidence to suggest that the retinal epithelium is the major, if not sole, origin of the EOG (Kolder, 1991). EOG's consist of both a fast and a slow phase. The fast phase usually only lasts just over a minute and is responsible for the initial dip seen after light onset and its end is obscured by the rising slow phase. The origin of the fast phase is believed to be ionic changes across the pigment epithelium (Linsenmeier and Steinberg, 1983). The remaining slow phase is the result of excitatory transmission of humoral transmitters from the photoreceptors to the retinal pigment epithelium (Dawis and Niemeyer, 1986; Hoffman and Niemeyer, 1985; Jarkman, 1987). EOG's have also been determined in other animals and also shown to originate in the RPE (Kikawada, 1968; Kolder and North, 1966).

Because the DLR measured here closely resembled the pattern of EOG changes over a similar time span, the DLR may be a reflection of the EOG rather than sensitivity. Thus, the DLR could be a measure of retinal epithelial function. However, previous experiments determining the spectral sensitivity using the DLR contradict this. Although EOGs are dependent on the wavelength of light falling on the eye (Afanador and Andrews, 1978), the spectral sensitivity determined using the DLR was correlated to the absorption spectra of the relevant

cone pigments (Silver, 1974; Powers, 1978; Muntz et al, 1996) indicating that the DLR is more likely to be a measure of photoreceptor sensitivity than RPE function.

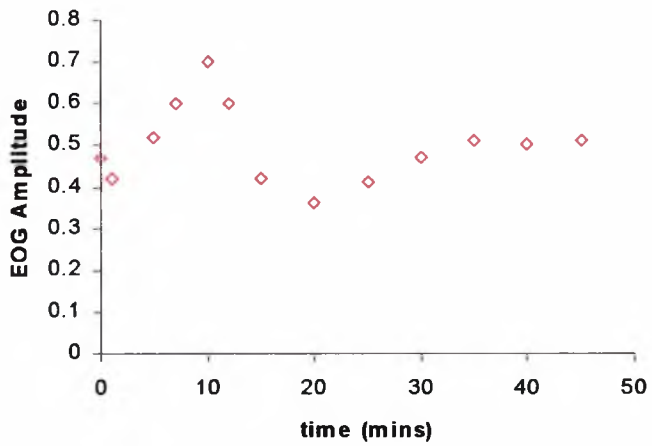

Fig 6-16 The human EOG

Computer simulation of the human EOG in response to light (modified from Kolder, 1991).

There could also be other non visual reasons why fish tilted increasingly over time towards the unilateral light source. The experiments conducted here not only generated a unilateral light source but also a unilateral heat source. The side of the flume which the projected light was aimed at may have gradually become warmer in comparison to the opposite side as the experiment progressed. Thus, it could be argued that fish are tilting their bodies towards a heat source. However this is unlikely for several reasons. Temperature controlled water was continually pumped through the flume and the projector light was filtered by a heat glass thus removing all infrared radiation. Also, fish immediately tilted to the other side of

the flume ('the cooler side') when light was projected from this side at the end of 3 experiments (personal observation).

Muscle fatigue may also play a role in the increased tilt in fish observed over time. The initial tilt towards the light may be a visual phenomenon. However, with time, the tilting may cause muscle fatigue which in turn would negate further tilting towards the light. However, in a previous experiment, the DLR was measured for 4 days and the tilt angle levelled slightly higher than what was found after 4 hours of unilateral stimulation (Von Holst, 1935). If muscle fatigue was causing the tilt, after 4 days it might be expected that the fish either decreased their tilt substantially more or intermittently move upright to replenish or rest the fatigued muscles.

6.6 Summary

The DLR was measured during 100 minutes of unilateral light in the middle of the light and dark phases of a light/dark cycle in 2 species of fish; the glowlight tetra and the zebrafish. The DLR was interpreted as being a measure of sensitivity which was found to change during the course of these experiments. It was unlikely that retinomotor movements played a role in the determination of sensitivity.

Chapter 7-The endogenous control of horizontal cell spinule formation and degradation in the glowlight tetra (*Hemigrammus erythrozonus*)

7.1 Abstract

In this chapter, another retinal rhythm, which has previously been shown to be under control of an oscillator located outside of the eye, is investigated. In teleost retinas, during the light phase of a light /dark cycle the terminal dendrites of horizontal cells show numerous finger-like extensions (called spinules) which invaginate the cone pedicle. During the dark phase of the cycle, spinules are retracted. Spinule formation and degradation are not only influenced by the external lighting conditions but also controlled by a circadian oscillator that is located outside the eye.

Here, the rhythm of spinule formation and degradation in the glowlight tetra is studied. The duration this rhythm continues in constant darkness is taken as an indicator of the circadian drive controlling spinule dynamics. The circadian drive controlling the formation and degradation of spinules in the glowlight tetra shows significant differences to those controlling retinomotor movements. It dampens much earlier in constant darkness indicating that it is less robust.

To quantify further differences between functions under intraocular and extraocular oscillator control, glowlight tetra were exposed to light in the middle of the dark phase and placed into darkness during the middle of the light phase, as in previous experiments (chapter 4). Exposure to light in the middle of the dark phase causes spinule formation to maximum light adaptive levels within 45 minutes. Conversely, placing glowlights into darkness during the middle of the light phase causes a slight withdrawal of spinules. Thus, unlike the cone and

melanosome movements, both light and dark exposure in the middle of the dark and light phase respectively effected the formation and degradation of spinules.

Zebrafish (*Danio rerio*) were also exposed to light in the middle of the dark phase of the light/dark cycle. The time course of spinule formation under these conditions is found to be similar to that of glowlight tetra and other species indicating that the light control of spinule dynamics is similar in different species irrespective of their geographical origin.

7.2 Introduction

In the nervous system of both vertebrates and invertebrates, certain synapses can be characterised by the appearance of finger-like extensions of the postsynaptic membrane that project into the presynaptic terminal. These were first noted in the rat hippocampus (Westrum and Blackstad, 1962). As they were often associated with dendritic spines, they were termed 'spinules' (small spines). Spinules in the teleost retina were first described by Stell (1966), and investigated further by Raynauld et al (1979) and Wagner (1980). Spinules occur most widely in fish (Wagner, 1980) but have also been described in the rat retina (Behrens et al, 1998).

In the teleost retina, spinules have been found in both the inner and outer plexiform layers (Raynauld et al, 1979; Wagner, 1980; Yazulla and Studholme, 1991, 1992). Briefly, spinules in the inner plexiform layer were first recorded in the goldfish retina. Here, mixed rod-cone bipolar cells in sublaminar b (Mb) revealed spinules at synaptic terminals invaginating about 5% of the presynaptic amacrine cell processes (Yazulla and Studholme, 1991). Analysis of mixed rod-cone bipolars cells in different layers of the inner plexiform layer showd different adaptation changes dependent on their morphology. In sublaminar a, terminals of mixed rod-cone bipolars (Ma) were irregular in light, smooth and regular in dark. There were almost three times more spinules in the light compared to the dark. Meanwhile, in sublaminar b, Mb terminals exhibited the opposite behaviour being regular and smooth in the light adapted state and also showing almost three times

more spinules in the dark compared to the light (Yazulla and Studholme, 1992; Behrens and Wagner, 1996).

The different pattern of spinule formation in Ma and Mb bipolar cells agrees with the previous classifications of bipolars cells. Ma bipolars cell give hyperpolarising responses to light and Mb give depolarising responses (Famiglietti and Kolb, 1976). Bipolar cell spinules are thought to contribute to the fine-tuning and regulation of amacrine /bipolar interactions (Yazulla and Studholme, 1992; Behrens and Wagner, 1996).

The remainder of this chapter will be concerned with spinules in the outer plexiform layer of the teleost retina. Here, spinules are found on the dendritic terminals of horizontal cells, which invaginate the dome shaped cavity of the cone pedicle. Horizontal cell spinules are typically 0.3μm long and 0.1μm in diameter. Spinules lack synaptic vesicles and have patch – like submembrane densities at their tips (Wagner, 1980; Wagner and Djamgoz, 1993). Horizontal cell spinules are present during the light phase of the light/dark cycle and are withdrawn during the dark phase (Raynauld et al, 1979; Wagner, 1980; Wagner and Djamgoz, 1993) (Figure 7.1 and 7.2).

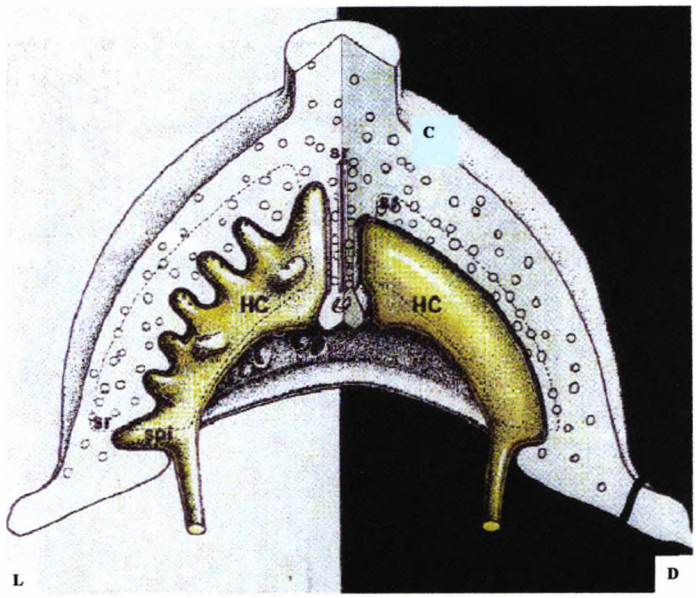

Fig 7-1 Horizontal cell spinule drawing

Drawing showing horizontal cell spinule formation in the light and withdrawal in the dark, inside a cone pedicle. Here; HC, horizontal cell; sr, synaptic ribbon; spi, spinule; C, cone pedicle; L, light phase; D, dark phase (adapted from Wagner and Djamgoz, 1993).

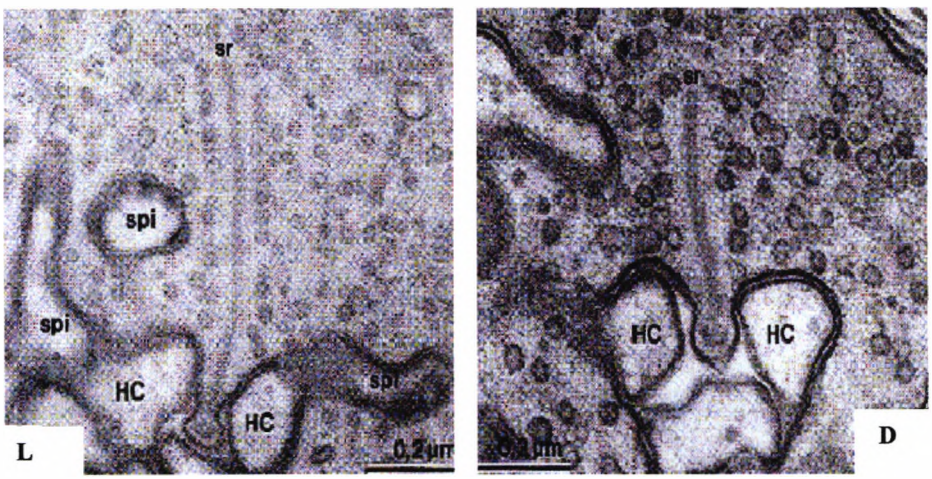

Fig 7-2 Electron micrographs of horizontal cell spinules

Electron micrographs showing horizontal cell spinules during the light phase and lack of spinules during the dark phase of a light/dark cycle. Here; HC, horizontal cell; sr, synaptic ribbon of cone pedicle; spi, spinule; L, light phase; D, dark phase (adapted from Wagner and Djamgoz, 1993).

7.2.1 Horizontal cell spinule function

Transmission from cones to horizontal cells (feedforward transmission) is thought to occur at the tip of the synaptic ribbon (Downing and Djamgoz, 1989). There is morphological evidence suggesting spinules are synaptic structures and physiological recordings showing that feedback transmission from horizontal cells to cones occurs via spinules (Raynauld et al, 1979; Wagner, 1980; Djamgoz, 1984; Djamgoz and Wagner, 1987; Downing and Djamgoz, 1989; Djamgoz and Kolb, 1993). This feedback transmission and the role of spinules have mostly been studied in trying to elucidate the mechanisms underlying chromatic vision (Raynauld et al, 1979; Wagner, 1980; Djamgoz and Greenstreet, 1996; Kröger and Wagner, 1996).

Electrophysiological recordings from horizontal cells in teleost retina give characteristic slow light elicited responses called 'S-potentials', which have been used to divide horizontal cells into two broad classes. Horizontal cells that respond (hyperpolarize) to all wavelengths have been denoted L- type (luminosity), while those that give multiphasic responses to different wavelengths are called C- type (chromaticity). As well as their spectral responses, horizontal cells are also classed according to the connections they make and their morphology (see chapter one). In goldfish, H1 horizontal cells are of the L-type. They have connections with three chromatic types of cones (red, green, and blue) and give a hyperpolarized response to all wavelengths. H2 horizontal cells contact green sensitive, blue sensitive and UV sensitive cones. They depolarise to red and hyperpolarize to green wavelengths and as such are C-type. H3 horizontal cells

are also C-types which show hyperpolarization to red wavelengths and depolarization to green wavelengths but only synapsing with blue sensitive and UV sensitive cones (Stell, 1975; Stell et al, 1975; Stell and Lightfoot, 1975; Stell et al, 1982; Kammerman and Spekreijse, 1995).

Using the above array of connections and spectral responses, Stell and Lightfoot (1975) suggested that centrally located horizontal cell dendrites receive direct input from cones while lateral horizontal cell processes return an opposite (sign-inverting) signal to the appropriate cone. Therefore, H2 type horizontal cells without any input from red sensitive cones will depolarize to red wavelengths due to sign-inverting feedback from H1 type horizontal cells to the green cones. This has been verified using intracellular recordings and specific staining techiniques (Weiler and Wagner, 1984). Furthermore, this feedback signal was accompanied by the appearance of spinules indicating that these may in fact be the sites of the sign-inverting synapse (Weiler and Wagner, 1984). Raynaud et al (1979) also found that the disappearance of horizontal cell spinules was associated with the loss of colour opponency and cone function in ganglion cells, and that spinule return parallels colour opponent recovery. Thus, spinules may play a role as sites for feedback mechanisms in the process of chromatic vision.

More recently, an alternative model has been proposed to explain the horizontal cell–cone connectivity and the spectral responses seen (Kammermans et al, 1991; Kammerman and Spekreijse, 1995). In this model, all horizontal cell types feed back to all cone types. Similarly, all horizontal cells receive input from more than one cone system. This would lead to spectrally broad feedback signals in the

cones. Kraaij et al (1998) used physiological recordings to show that the feedback signal received by a cone has a much broader spectral sensitivity than the cone itself. This result supports the idea that all horizontal cell types must feedback to all cone types and also that the spectral characteristic of the horizontal cell type is lost in the output of the cones. This theory however does not dismiss the possibility that spinules may be acting as the sites of horizontal cell feedback to the cones.

7.2.2 Neurochemical control of horizontal cell spinules

Dopamine has been shown to play a part in horizontal cell spinule formation (Weiler et al, 1988a; Djamgoz et al, 1989; Kohler and Weiler, 1990; Kohler et al, 1990; Kirsch et al, 1991; Behrens et al, 1992). The application of dopamine to dark-adapted retinae causes the formation of spinules, whilst haloperidol, a dopamine antagonist, suppresses the formation of spinules during light adaptation (Weiler et al, 1988a, b; Djamgoz et al, 1989; Kohler and Weiler, 1990; Kirsch et al, 1991). Further, injection of 6-hydroxydopamine (6-OHDA), which destroys dopaminergic retinal cells, also blocks light-induced spinule formation, which is restored by the application of exogenous dopamine (Kirsch et al, 1991; Wagner et al, 1992a and b). In dopamine depleted retinae, the (Cb/H2)-type horizontal cells also lose their biphasic response in light, which is restored after application of exogenous dopamine. This is further evidence that spinules are involved in chromatic spectral responses (see above, 7.2.1) (Kirsch et al, 1991). Changes in retinal dopamine content throughout a light/dark cycle also correlate with spinule variations throughout the cycle. Spinules were numerous during the light phase

(day) when the dopamine content is high, and least at night (dark phase) when the dopamine content is low (Kohler et al, 1990).

Horizontal cells contain dopamine receptors that are positively linked to adenylate cyclase (D_1 receptors) and thus cAMP (Van Buskirk and Dowling, 1981). Dopamine induces spinule formation by elevating the intracellular cAMP level in horizontal cells (Kirsch et al, 1991) and diffuses large distances within the retina to its target site from the interplexiform cells from which it is released (Yazulla and Studholme, 1995).

Other neurochemicals have also been linked to the formation of horizontal cell spinules. In the teleost retina, nitric oxide can be synthesized by several types of cell including cone photoreceptors and horizontal cells (Schumann and Madison, 1994). Application of nitric oxide donor compounds to isolated dark adapted retina caused the formation of horizontal cell spinules, while light induced spinule formation could be suppressed using a nitric oxide scavenger (Greenstreet and Djamgoz, 1994; Pottek et al, 1997). Thus, nitric oxide could be involved in the light adaptive formation of horizontal cell spinules and may be operating via the dopaminergic system (Haamedi and Djamgoz, 2002).

Protein kinase C activation was also found to promote the formation of spinules in dark adapted retinae depleted of dopaminergic neurons, indicating a dopamine independent pathway (Weiler et al, 1991). Retinoic acid injected into the orbits of carp caused spinule development in dark-adapted retina (Weiler et al, 1998). Nerve growth factor was also found to induce spinule formation in the dark, an

effect that may be mediated by both nitric oxide and dopamine (Haamedi et al, 2001).

The neurochemical control of spinule retraction has been studied to a lesser degree. Glutamate, a neurotransmitter found in all vertebrate photoreceptors, is released from turtle cones in the dark (Copenhagen and Jahr, 1989). In goldfish retina, glutamate receptor immunoreactivity has been localised to horizontal cell dendrites invaginating cones and rods and in spinules during light adaptation (Klooster et al, 2001). Incubation of light adapted carp retina with glutamate, or its analogue kainic acid, resulted in a reduction of horizontal cell spinules (Weiler et al, 1988a and b; Weiler and Schultz, 1993). The action of glutamate is mediated by an ionotropic α- amino -3-5-methyl - 4- isoxazoleproprionic (AMPA)/ kainate-type receptor (Weiler and Schultz, 1993). An influx of calcium into horizontal cells in both dark and glutamate induced retraction of horizontal cells indicates a possible role for calcium in spinule retraction (Schmitz et al, 1995;Weiler et al, 1995; Okado et al, 1999).

7.2.3 Cellular mechanisms of horizontal cell spinule formation and degradation

Using immunocytochemistry, horizontal cells were shown to label intensely with phalloidin, indicating the presence of filamentous (F-) actin (Drenckhan and Wagner, 1985). Therefore morphological changes in horizontal cells, including spinule formation, may involve actin. Cytochalasin, an inhibitor of actin assembly, has shown that light-dependent horizontal cell spinule formation and maintenance is an actin dependent process. Inhibiting actin assembly using

cytochalasin suppressed both the formation and maintenance of spinules during light adaptation in fish retina and the biphasic spectral resonses of (Cb/H2)-type horizontal cells (Ter-Margarian and Djamgoz, 1992; Weiler and Janssen-Bienhold, 1993; De Juan and Garcia, 1998). Spinule formation may be caused by actin polymerization at the dendritic terminals of horizontal cells (Weiler et al, 1991). An alternative explanation involves a redistribution of cytoplasm, caused by alterations in the actin network of horizontal cell terminals resulting in spinule protrusion (Schmitz and Kohler, 1993).

7.2.4 Circadian control of horizontal cell spinules

Horizontal cell spinule formation and degradation occurs not only as a result of direct light/dark stimulation but is also under endogenous control. When fish are left in constant darkness, horizontal cell spinules continue their rhythm of dawn formation and dusk degradation for at least 48 hours. Also, horizontal cell spinules anticipate the onset of dawn (Douglas and Wagner, 1983; Wagner et al, 1992a and b). Unlike the plethora of retinomotor movement studies, there have only been two other species (the goldfish and blue acara) used to investigate the endogenous control of spinule formation and degradation (Douglas and Wagner, 1983; Wagner et al, 1992b).

In this chapter, the rhythm of horizontal cell spinule dynamics in the glowlight tetra is examined. First, the pattern of spinule formation and degradation is determined during a normal light/dark cycle and in continual darkness for 24 hours. This is compared to both the cones and RPE melanosome rhythms in the

glowlight tetra as well as spinule dynamics in both the goldfish and blue acara. The effects of light exposure in the middle of the dark phase and dark placement during the middle of the light phase on spinule dynamics are also examined. For further species comparison, zebrafish are also exposed to light in the middle of the dark phase and spinule formation is examined.

7.3 Methods and Material

7.3.1 Fish and Light Schedules

Glowlight tetras and zebrafish were obtained and maintained as previously described. The light schedules used in this chapter are similar to those in both chapters 3 and 4. All fish were entrained to a LD 12:12 light cycle for a minimum of 30 days.

7.3.2 Preparation for electron microscopy

The preparation of tissue for electron microscopy differed in small but significant ways to the methods used for light microscopy, so the full procedure will be described.

After killing the fish by cervical transection and pithing, they were immediately enucleated and eyecups prepared. Posterior eyecups were placed into freshly prepared 1.5% paraformaldehyde and 1.5% glutaraldehyde fixation solution which also contained tannic acid (see appendix). Tannic acid is added to the solution because of its ability to stain thin membranes. Generally, it took 3

minutes to prepare eyecups before they were placed into fixative. Eyecups, rather than pierced corneas as for light microscopy, used to ensure deep and quick penetration of the fixative into the inner retina. After overnight fixation, eyecups were washed three times each time for 20 minutes while rotating with buffer solution, and post-fixed in 2% Osmium tetroxide for 1 hour. To prepare for electron microscopy, eyecups were;

A/ washed in water three times, each time for 20 minutes.

B/ immersed into a 50:50 solution of ethanol:water for 5 minutes.

C/ stained with 2% Uranyl acetate in a solution of 70% ethanol (70:30, ethanol:water solution) overnight and rotated to ensure good penetration.

D/ immersed into a 80:20 solution of ethanol:water for 20 minutes, during the following morning, while rotating. This was repeated using a 90:10, followed by a 95:5 ethanol:water solution and then three times in a 100% ethanol solution.

E/ immersed into a solution of Histoclear for 30 minutes again while rotating.

F/ placed into a 50:50 solution of Histoclear: resin for 30 minutes while rotating.

G/ immersed into 100% resin and kept rotating overnight at room temperature.

H/ placed into an individual baking mould, filled with fresh resin. Eyecups were orientated to allow good radial sections of the retina to be cut. Due to the heavy staining caused by Osmium tetroxide, orientation of the eyecups was more difficult in comparison to light microscopy.

Moulds were placed into an oven set at 60°C for 48 hours to allow polymerisation and hardening of the resin.

As for light microscopy, blocks were trimmed manually with a razor blade. Using glass knifes and the Reichert-Jung Ultracut E microtome, 1μm sections were first

cut and collected for light microscopy. 1μ sections were cut until sections with a good proportion of well-fixed outer plexiform layer were obtained. When this was achieved, ultra-thin sections of 50-80nm were cut using a Diatome diamond knife situated on top of a metal 'boat' filled with doubled distilled water, onto which the sections would float after cutting. The interference colours created by sectioning indicated the actual thickness of the sections. Only those sections that were silver with a tinge of gold were collected onto copper grids (Biorad, 3.05mm, R002GUC) for analysis. Grids were allowed to dry on filter paper before being placed into standard grid holders for storage until electron microscopy could be carried out.

All electron microscopy was carried out using a LEO EM912 electron microscope at the Institute of Anatomy, University of Tübingen, Tübingen.

7.3.3 Spinule identification

The protocol adopted by Kirsch et al, (1991) was used to identify spinules. The following conditions had to be met to be classified as a spinule;

A/ The position of the spinule was crucial in its identification. Spinules have to protude from the lateral or apical aspect of lateral horizontal cell dendrites.

B/ The shape of the spinule is also of importance. To be catergorised as a spinule, it should be circular in cross-section and eliptical to finger-like in oblique or side views.

C/ Ultrastructurally, they carry characteristic, triangular, membrane densities on the cytoplasmic sides of the horizontal cell membranes.

7.3.4 Experimental procedures

7.3.4.1 Glowlight tetras

The experimental procedures were the same as those outlined in chapter 3. Briefly, groups of 3 fish were placed into jars in their home tank prior to experimentation and sampled both during a light/dark cycle and in 24 hours of continual darkness. Fish were also light and dark adapted during the middle of the dark and light phases respectively for 100 minutes as described in chapter 3. The only difference to procedures carried out in chapter 3 and 4, is that during the spinule experiments, eyecups were prepared immediately after killing fish.

7.3.4.2 Zebrafish

In a manner similar to the glowlight tetra, zebrafish were exposed to light in the middle of the dark phase on two separate occasions. During the first experiment, fish were sacrificed at 0, 15, 30, 45, 60, and 100 minutes following light exposure in the middle of the dark phase, and cone and pigment indices were determined. In the second experiment, fish were sacrificed at similar times as in the first experiment but on this occasion spinule numbers were determined. To establish a normal light and dark adaptive spinule value, 3 fish were sacrificed at both the middle of the light and dark phases and prepared for spinule analysis.

7.3.5 Horizontal cell spinule assessment

Retinae were examined at a magnification of 6,300-28,000x. The number of horizontal cell spinules and synaptic ribbons per cone pedicle were counted to give a spinule to ribbon ratio (S/R) per cone pedicle for 50 cone pedicles per retina. Two retinae were counted per fish and at each time point 3 fish were counted giving a total of 300 cone pedicles per time point. Most sections analysed were transverse although a few were tangential. However this should make no difference to the spinule/ribbon ratio (Wagner, H-J, 1980). During analysis the identity of the retina being counted was not known.

Spinule to ribbon (S/R) ratios were used to compensate for the fact that cone pedicles contain numerous ribbons. In larger pedicles belonging to large cone types, more synaptic ribbons are seen and this would mean more spinules would also be present during the light phase. If spinules numbers were counted outright, this could lead to errors. For example, if one counted mostly large pedicles in one retina and small pedicles in another, the former would appear to have more spinules than the latter.

Due to problems with the quality of fixation, all of these experiments were repeated on more than one occasion. If after one hour of counting and analysing it did not look as though 50 pedicles could be counted accurately, the retina was discarded from analysis. Thus, these experiments were repeated until 3 fish per

time point were counted although up to 5 fish were counted at some time points and at one time point, only 2 fish were counted (see below).

As spinule formation and degradation has been shown to be under control from outside the eye (see discussion below), in these experiments 'n' is taken as the number of fish rather than the number of retinae examined as used in the analysis of retinomotor movements.

7.4 Results

7.4.1 Glowlight tetra

7.4.1.1 Basic 24 light/dark cycle of horizontal cell spinule dynamics

Horizontal cell spinules are numerous during the day and far fewer at night. During the day, spinules reach maximum numbers to give a spinule/ribbon ratio of 2.96±0.18, and decline to minimal numbers at night with a spinule/ribbon ratio of 0.79±0.18 (Figures 7.3 and 7.4). These values were used as the light and dark adaptive values for statistical analysis.

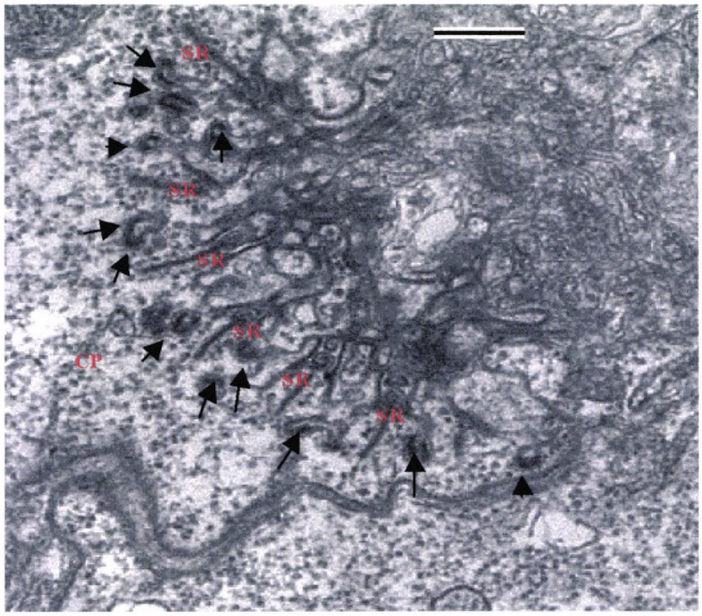

Fig 7-3 Glowlight tetra light adapted cone pedicle

Transverse section of a light adapted glowlight tetra cone pedicle (CP) showing synaptic ribbons (SR) and horizontal cell spinules (arrows) taken during the middle of the light phase. Bar = 1μm.

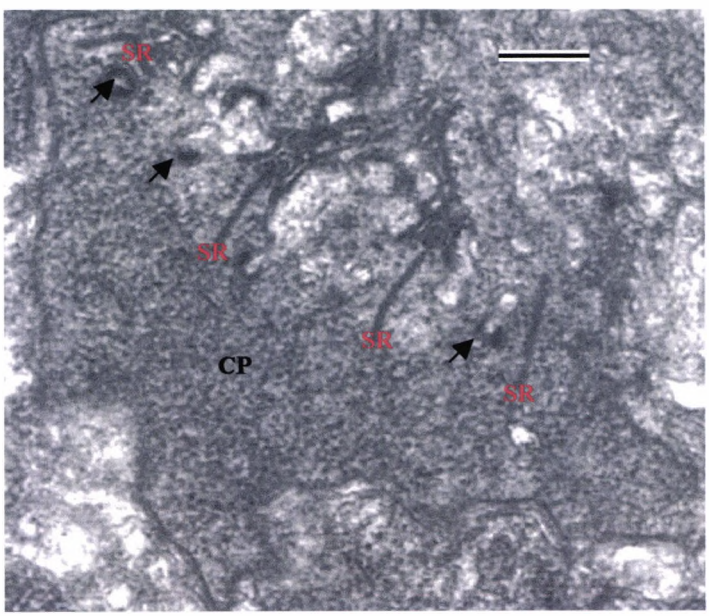

Fig 7-4 Glowlight tetra dark adapted cone pedicle

Dark adapted glowlight tetra cone pedicle (CP) taken during the middle of the dark phase. Note the fewer numbers of horizontal cell spinules (arrows) associated with each synaptic ribbon (SR). Bar =1μm.

Horizontal cell spinule formation predicts the onset of dawn in a similar way to the contraction of cones and dispersion of melanosomes in the retinal pigment epithelium (RPE), indicating they are also under endogenous control. At t = -0.5 (0.5 hours before dawn), there is no statistically significant difference between the S/R value and the maximum light value ($p>0.1$, unpaired Student t test) while at t = 0, there may be a difference from the maximum daytime levels ($p=0.05$, unpaired Student t test). Spinule numbers reach maximum levels in the middle of the day at t=6 and these are maintained until t = 12 ($p>0.05$ at all points, unpaired Student t test). At t =12.5, 30 minutes after dusk, there is no dark adaptive movement of cones or RPE melanosomes yet spinule degradation has began as the S/R ratio is significantly different to the daytime value ($p<0.05$, unpaired Student t test). Spinule numbers reach minimum values at t=15 and these are maintained at t =18 ($p>0.2$ at both times, unpaired Student t test) during the middle of the night. Apart from t=12.5 where spinules have begun to withdraw, the pattern of spinule formation and degradation during a normal light/dark cycle is similar to the cycle of cone and RPE melanosome movements (Figure 7-5).

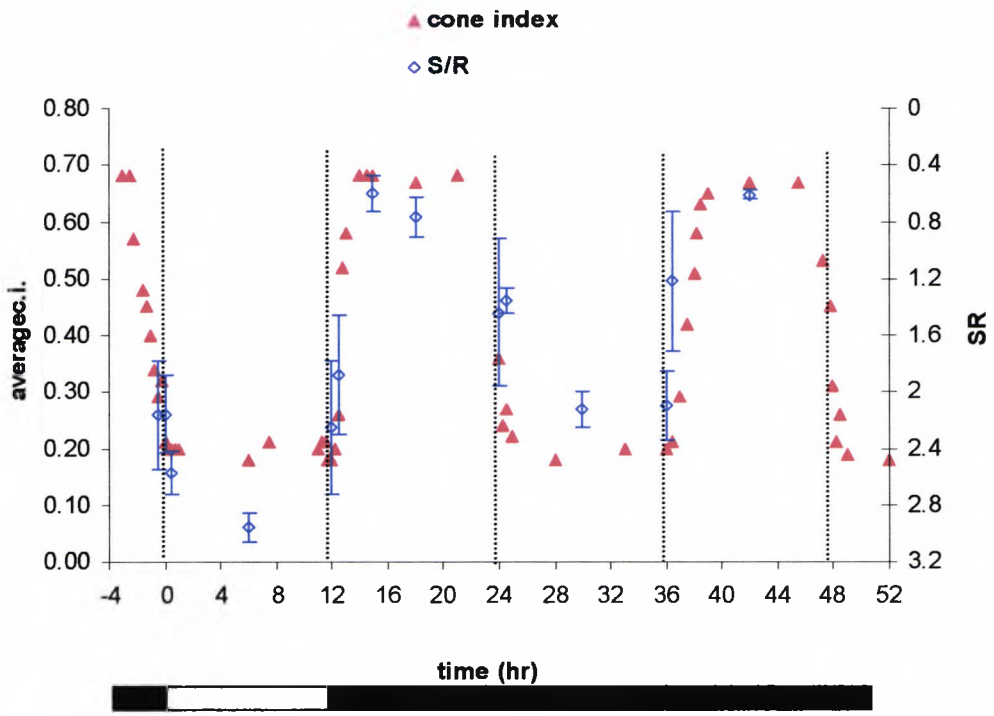

Fig 7-5 Spinule to ribbon ratios during a LD cycle and in continual darkness

Glowlight tetra S/R ratio during a 12 hour light/dark cycle (LD 12:12) and in continual darkness in comparison to the cone index. Here, the red triangles represent cone indices (note that the pattern of cone indices is very similar to the pigment indices over the same period, see Figure 3.4) Each S/R point represents the mean ± SE of between 2-5 fish (2 fish at t=24.5) while the cone data comes from previous work (chapter 3). The light/dark bar gives the light schedule during the experiments while the dotted lines give lights on (t=0), lights off (t=12), expected lights on (t=24, 48) and expected lights off (t=36).

7.4.1.2 First cycle in constant darkness

Both formation and degradation of horizontal cell spinule formation in constant darkness continues, indicating endogenous control (Figure 7.5). However, the amplitude of the rhythm appears damped. Throughout the subjective day from t=24 until t=36, spinule numbers are less than the maximum light adaptation

number ($p<0.05$ for all points except at t=24.5, unpaired Student t test) (at t =24.5, spinule numbers appear to be reduced although as only 2 fish were sampled at this point, no statistical comparison has been made). At t = 36.5 (0.5 hours after subjective dusk) and at t = 42 (subjective midnight), the S/R values are similar to the minimum value ($p>0.10$ at both times, unpaired Student t test). Thus, full minimum values are reached in constant darkness while maximum light values are not achieved, which indicates the beginning of rhythm damping. In comparison, the cycle of both cone and RPE melanosome movement shows no damping which may indicate that both of these components are under stronger endogenous control than the spinules (Figure 7.5).

7.4.1.3 Light adaptation in the middle of the night

Within 15 minutes of light exposure during the middle of the dark phase, spinule numbers have significantly increased ($p < 0.05$, unpaired Student t test). Unexpectedly, following 30 minutes of light exposure, the spinule numbers have decreased as the S/R value is significantly less than that following 15 minutes of light exposure ($p<0.05$, unpaired Student t test). However, after 45 minutes of light exposure, the S/R value is similar to the maximum light value ($p>0.1$, unpaired Student t test). Apart from the anomolous change seen after 30 minutes of light exposure, this pattern is similar to that found for RPE melanosomes, while at the same time no effect can be seen on the position of the cones (Figure 7.6 - 7.8).

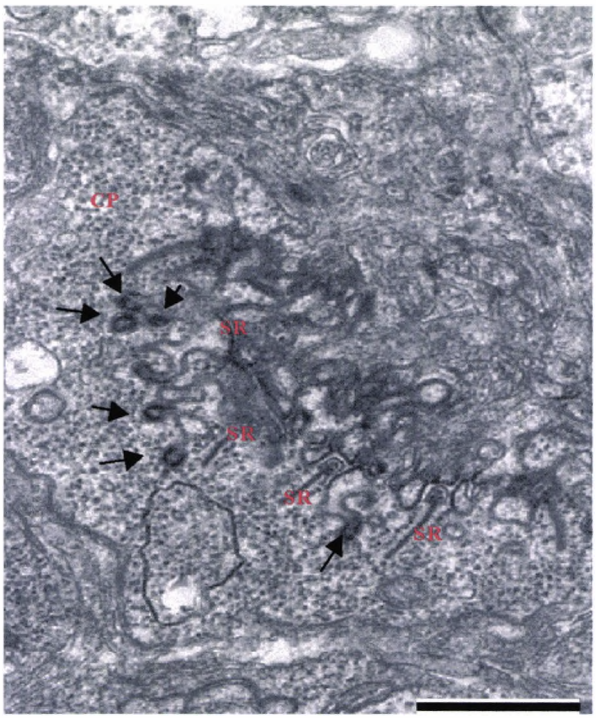

Fig 7-6(a) Glowlight tetra cone pedicle (light exposed)

Transverse section of light adapted glowlight tetra cone pedicle (CP) showing synaptic ribbons (SR) and horizontal cell spinules (arrows) taken after 30 minutes of light exposure during the middle of the dark phase. Bar = 1µm.

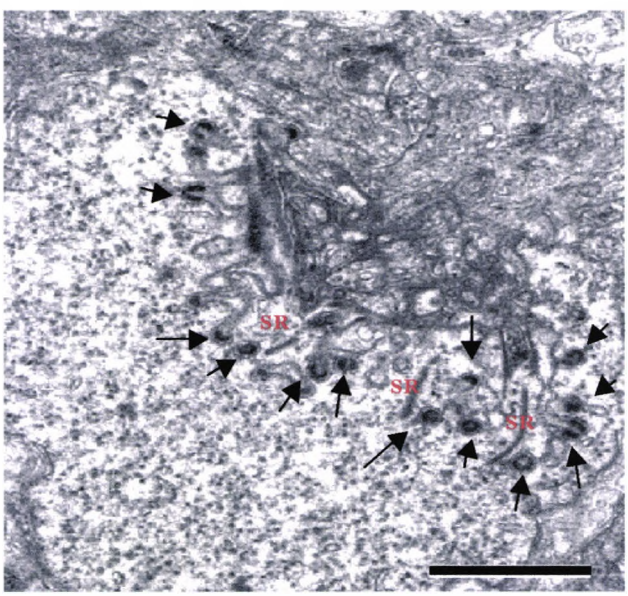

Fig 7-6(b) Glowlight tetra cone pedicle (light exposed)

Transverse section of light adapted glowlight tetra cone pedicle (CP) showing synaptic ribbons (SR) and horizontal cell spinules (arrows) taken after 100 minutes of light exposure during the middle of the dark phase. Bar =1µm.

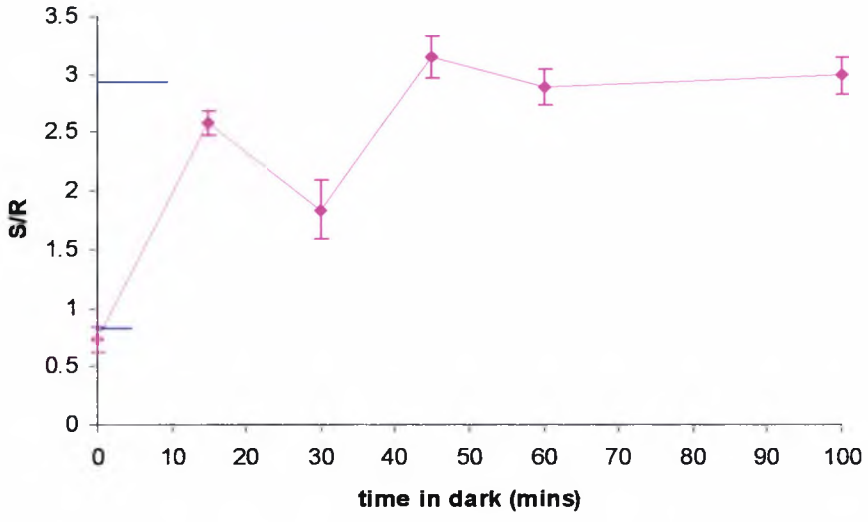

Fig 7-7 Glowlight tetra S/R values during light exposure

Glowlight tetra S/R during 100 minutes of light exposure in the middle of the dark phase. Each S/R point represents the mean ±SE of between 3-5 fish. The long bar represents the normal light adaptation value and the shorter bar represents the normal dark adaptation value.

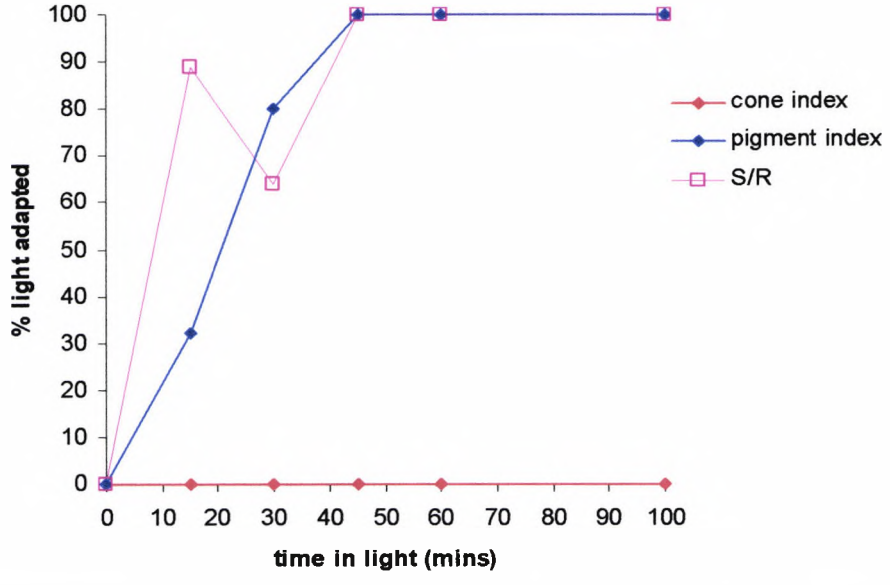

Fig 7-8 Normalised glowlight tetra S/R, cone and pigment indices during light exposure in the middle of the dark phase

Normalised glowlight tetra S/R, cone and pigment indices during 100 minutes of light exposure in the middle of the dark phase. Data for both the cone and pigment indices comes from chapter 4.

7.4.1.4 Dark adaptation during the middle of the day

100 minutes of dark adaptation in the middle of the light phase has produced a slight reduction in the number of spinules in comparison to the maximum light value ($p<0.05$, unpaired Student t test). No effect is seen on either the cones or the RPE melanosomes (Figure 7.9 and 7.10).

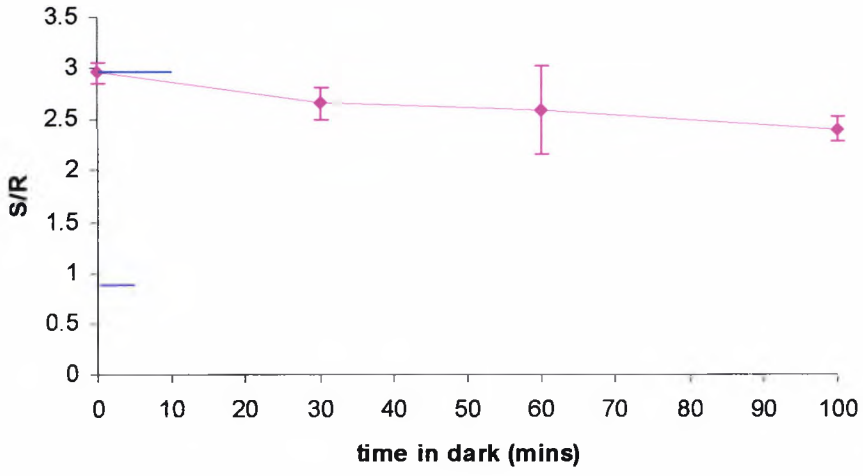

Fig 7-9 Glowlight tetra S/R during 100 minutes of dark placement in the middle of the light phase

Glowlight tetra S/R ratios during 100 minutes of darkness in the middle of the light phase. Each S/R point represents the mean and ±SE for 3 fish except at t=60 minutes where only 2 fish were sampled. The long bar represents the normal light adaptation values and the shorter bar represents the normal dark adaptation value.

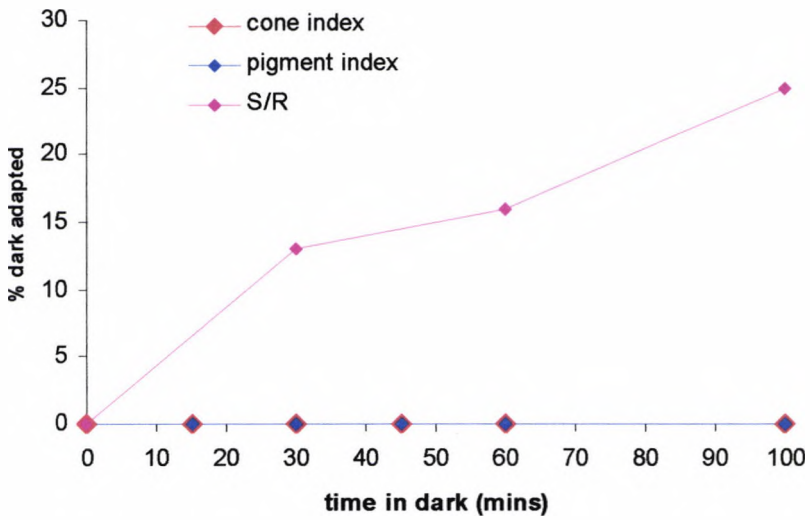

Fig 7-10 Normalised glowlight tetra S/R, cone and pigment indices during dark placement in the middle of the light phase

Normalised glowlight tetra S/R, cone and pigment indices during 100 minutes of dark placement in the middle of the light phase. Data for both the cone and pigment indices are from chapter 4.

7.4.2 Zebrafish

Horizontal cell spinules numbers, cone and pigment indices were first determined in the middle of the day and during the middle of the night and these values were taken as the maximum light and dark adaptive values for statistical analysis (table 7.1).

Table 7.1 Zebrafish S/R, cone and pigment maximum and minimum values

	Maximum light adaptive value	Maximum dark adaptive value
S/R	2.93±0.23 (n=3)	1.16±0.13 (n=3)
Cone index	0.27±0.04 (n=6)	0.63±0.04 (n=6)
Pigment index	0.71±0.06 (n=6)	0.28±0.03 (n=6)

7.4.2.1 Light adaptation in the middle of the night

Spinule numbers reach a maximum within 15 minutes of light exposure as the S/R value is similar to the maximum light value ($p > 0.05$, unpaired Student t test) (Figure 7.11). This is much earlier than the time required for both zebrafish cones and RPE melanosomes to reach full light adaptive levels (Figure 7.12). Horizontal cell spinules in the retina of the zebrafish behave much like those of the glowlight tetra (Figure 7.13).

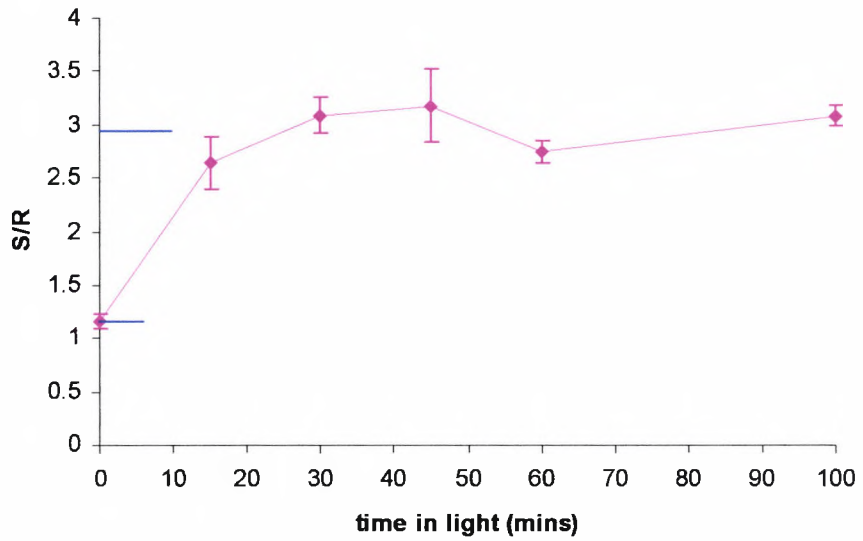

Fig 7-11 Zebrafish S/R, cone and pigment indices (light exposure)

Zebrafish S/R ratios during 100 minutes of light exposure during the middle of the dark phase. Each S/R point represents the mean ±SE of 3 fish except at t=45 minutes where only 2 fish were sampled. The short bar represents the normal dark adaptation value and the longer bar represents the normal light adaptation value.

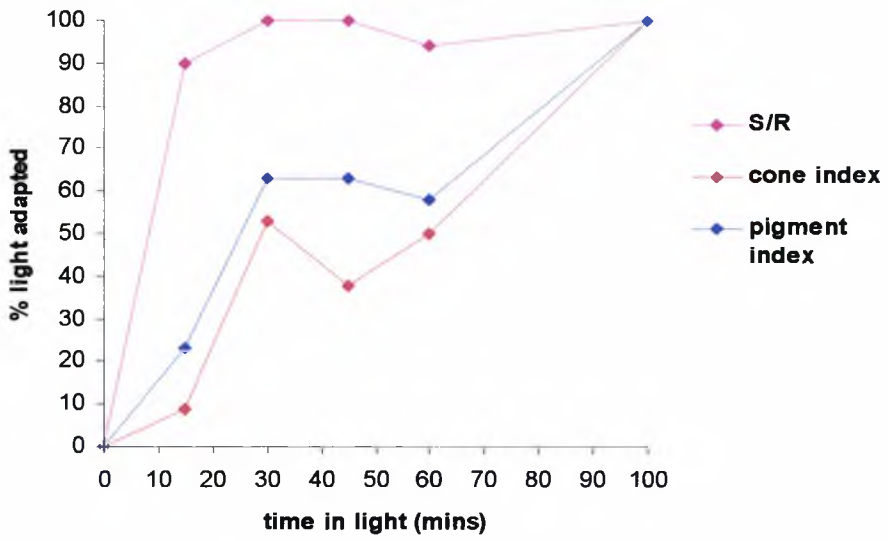

Fig 7-12 Normalised zebrafish S/R, cone and pigment indices during light exposure in the middle of the dark phase

Normalised glowlight tetra S/R, cone and pigment indices during 100 minutes of light exposure in the middle of the dark phase.

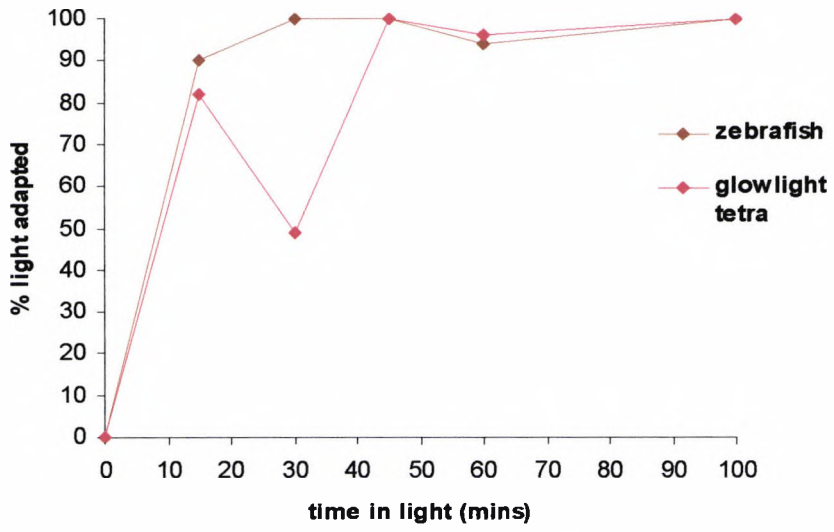

Fig 7-13 Normalised glowlight and zebrafish S/R (light exposed)

Normalised glowlight tetra and zebrafish S/R ratios during 100 minutes of light exposure in the middle of the dark phase.

7.5 Discussion

Earlier (chapter 3), the rhythm of both cones and RPE melanosomes in the glowlight tetra was investigated during both a normal light/dark cycle and in constant darkness. The duration that both the cone and melanosome rhythm continued without damping in constant darkness was taken to suggest strong endogenous circadian control in comparison to other species. There is good evidence to suggest that the oscillators controlling cone and melanosome movement are located within the eye although the evidence for melanosomes is less strong (see chapters 1 and 3).

In this chapter, glowlight tetra horizontal spinule dynamics were also shown to be under circadian control by both their increasing numbers before dawn and their cyclic formation and degradation in constant darkness. However, in constant darkness, although minimum numbers of spinules were seen during subjective night, spinules did not reach maximum light adapted values during the day, suggesting the onset of damping.

Evidence suggests that the site of spinule control is outside of the eye and that different oscillators control spinules, cone and RPE melanosme movement. Optic nerve section did not abolish retinomotor movements in either the green sunfish (Dearry and Barlow, 1987) or the midas cichlid (McCormack and Burnside, 1992). However, in the black bass, optic nerve integrity was required for the formation and degradation of horizontal cell spinules (Dejuan et al, 1996). Gonadotropin-releasing hormone (GnRH), which is found in efferent fibres

connecting the nucleus olfactoretinalis to the retina, caused spinule formation in isolated eyecups and thus also suggested central control of spinule formation (Behrens et al, 1993). Further, destruction of retinal dopaminergic cells using 6-hydroxydopamine (6-OHDA) had no effect on both the light adapted and the circadian movements of cones and RPE melanosomes in blue acara (Douglas et al, 1992). However, in the same fish, 6-OHDA treatment depressed the formation of spinules in light and also the circadian formation of spinules in constant darkness (Wagner et al, 1992a and b), further evidence for independent circadian oscillators for retinomotor movements and spinules.

By comparing the duration of rhythms in constant darkness, the strength of the circadian drive controlling cone, RPE melanosome and spinule dynamics in the glowlight tetra can be compared directly. Assuming that damping did commence in the spinule rhythm, the order of most persistent non-damped rhythm to least in constant darkness is;

Cones > RPE melanosomes > spinules

Therefore, this can be taken to be the order of circadian drive regulating their control. The same pattern was observed for the goldfish (Douglas and Wagner, 1983) and blue acara (Wagner et al, 1992a and b), which represent the only other circadian spinule studies to date.

The nucleus olfactoretinalis at the base of the olfactory bulbs has a role in light-adaptive spinule formation on teleost horizontal cells (Behrens et al, 1993). Efferent fibres connecting the olfactoretinalis to the retina contain GnRH and are presynaptic to retinal dopaminergic interplexiform cells (Behrens et al, 1993).

GnRH-immunoreactive perikarya in the nucleus olfactoretinalis show a marked intrinsic rhythmic activity in their electrical discharges (Oka and Matsushima, 1993), which could represent an oscillator controlling various neural subsystems including the retinal dopamine system (Behrens et al, 1993). Thus, the relatively early damping in constant darkness of the spinule rhythm could be a result of desynchronization between loosely coupled individual GnRH cells. Alternatively, early damping of the spinule rhythm could be due to damping of the oscillator itself as a result of the molecular machinery running down in constant darkness.

Although in all species to date, the circadian drive controlling spinules has been found to be weaker than those controlling cones and melanosome movement, there do appear to be some species differences. In the goldfish, in constant darkness the spinule rhythm only reached 27% of its amplitude seen during a light/dark cycle (Douglas and Wagner, 1983). In comparison, the blue acara showed no damping of the spinule rhythm in constant darkness (Wagner et al, 1992a and b), while the glowlight tetra here showed some evidence of damping (Table 7.2). Clearly, there is a species variation in the robustness of the circadian drive controlling spinule dynamics. As both equatorial species (the glowlight and blue acara) have stronger circadian drive controlling spinule function in comparison to the temperate goldfish, this agrees with the earlier suggestion (section 3.5) that equatorial species may have stronger circadian drive controlling retinal changes (see chapter 3). However, given the paucity of endogenous spinule studies, such a conclusion is tenuous especially as the more equatorial glowlight tetra appears to have a less circadian drive than the blue acara.

Table 7.2 Maximum amplitude of spinule rhythm in constant darkness in comparison to during a L/D cycle

Species	Distribution (Latitude)	% Rhythm Amplitude in constant darkness	Authors
Goldfish (*Carassius auratus*)	35°N to 23°N	27%	Douglas and Wagner (1983)
Blue *acara* (*Aequidens* pulcher)	12°N to 5°N	100%	Wagner et al (1992a and b)
Glowlight tetra (*Hemigrammus erythrozonus*)	5°N to 1°N	62%	Present study

Within 15 minutes of exposure to bright light in the middle of the dark phase in the glowlight tetra, spinule numbers increased significantly although maximum levels were not reached. Thus, the light control of spinule dynamics in glowlight tetras is similar to that controlling RPE melanosomes. In contrast, there is poor light control of cone movement (see chapter 4). Placing fish in the dark had little effect on the position of either the cones or RPE melanosomes (chapter 4), while there was some spinule withdrawal at the same time.

Light exposure in the middle of the dark phase caused the formation of spinules to maximum light adapted levels in the zebrafish within 15 minutes. Similarly, in the goldfish, maximum light adapted spinule numbers were reached within 30 minutes of light exposure (Douglas and Wagner, 1983). These times are similar to those observed for the glowlight tetra and suggest that light control of spinule

dynamics is similar in different species irrespective of their geographical origins (both the goldfish and zebrafish are temperate species). However, after 30 minutes of light exposure, the spinule numbers in glowlight tetra showed a reduction before reaching maximum light levels after 45 minutes. It is difficult to explain why this may have happened or if it is significant. Perhaps the rapid formation of spinules due to the unexpected light exposure caused saturation in the mechanism which needed to 'deplete' a light adaptive neurochemical before replenishing again.

7.6 Summary

In this chapter, glowlight tetra horizontal spinule cell formation and degradation were found to be under circadian control. Although circadian drive controlling both cone and RPE melanosome movement in the glowlight was found to be unusually strong in comparison to other species, the same could not be said for the spinule dynamics. Spinule damping during the first day in constant darkness was seen in the glowlight, whereas in another species (blue acara) no damping was noted under similar conditions, indicating that the glowlight tetra spinule dynamics are under less circadian drive.

Following light exposure in the middle of the day, the formation of spinules in the glowlight tetra followed a similar time course to that in both the zebrafish and the goldfish, indicating that the light control of spinule dynamics might be similar in different species of fish.

Chapter 8-General conclusion and future directions

8.1 General conclusions

This thesis commenced by outlining the basic rhythm of both cone and RPE melanosome retinomotor movements during a normal light/dark cycle and in continual darkness, in the glowlight tetra (*Hemigrammus erythrozonus*). Both the cone and melanosome rhythms continued, without damping, in continual darkness (cones for at least 14 and melanosomes for around 7 days) far longer than any species examined to date. This long duration of rhythm perseverance in continual darkness was interpreted as indicating strong oscillator control of retinomotor movements in comparison to other species. After reviewing previous retinomotor studies, it was suggested that the glowlight tetra and other equatorial species may have evolved stronger circadian drive to control retinomotor movements in order to help prepare for dawn which is of a shorter duration in comparison to temperate regions.

Nearly all previous retinomotor studies have observed damping of the cone or RPE melanosome rhythm within the first 48 hours in darkness. Thus, the glowlight tetra offered a good opportunity to conduct further circadian experiments on the retinomotor movements in particular for the cones. The light control of RPE melanosome movement was found to be strong during light exposure in the middle of the dark phase and very weak following a dark pulse in the middle of the light phase. In comparison, there was poor light control of cone movement during both a light and dark pulse in the middle of the dark and light phases respectively. However, during a full light/dark cycle, some light control of cone movements was shown at two different periods. Thus, light control of cone

movements exhibited a circadian rhythm. Although these two periods of light control are relatively short which give little opportunity for masking by light, entrainment to a 180° phase shifted light/dark cycle occured at a similar rate to that found in other animals.

Glowlight tetras were also entrained to 24 hour light/dark cycles with variable photoperiods ranging from 1-14 hours. As the glowlight tetra is an equatorial species, oscillators controlling retinomotor movements need only serve to put retinomotor elements in the correct positions at the same time each day of the year. In comparison, temperate species oscillators need to put retinomotor elements in the correct positions at times which will vary throughout the year. Thus, while it is important for temperate species to be sensitive to photoperiod, it is less so for those originating near the equator. However, the rhythm of cone movements in the glowlight tetra was found to entrain well to all photoperiods ranging from 1 to 14 hours. Theories involving both one and two-component oscillators could explain the mechanisms by which retinomotor movements entrain to light/dark cycles with variable photoperiods. Unusually, the free-running period was the same (24 hours) in all fish following entrainment to most light/dark cycles with variable photoperiods suggesting either a robust oscillator with little flexibility or perhaps a two oscillator structure where the morning component M, and the evening component E, are coupled so that no matter how one is moved with respect to the other, the overall period of the pacemaker will remain unchanged.

As there was no cone movement during light exposure in the middle of the dark phase, while the RPE melanosome did move, the glowlight tetra was used to examine whether retinomotor movements play a role in determining visual sensitivity as measured by the dorsal light response (DLR). The DLR was also recorded in the zebrafish (*Danio rerio*) as during light exposure in the middle of the dark phase, both cones and melanosomes moved to light adapted positions within 45 minutes of light exposure. By comparing the DLR in these two species during light exposure in the middle of the dark as well as light (during this time, no retinomotor movements are seen as cones and melanosomoes remain fully light adapted, in both species, throughout the course of the experiment) phases, it was concluded that it was unlikely that retinomotor movements play a role in determining visual sensitivity.

Another ocular rhythm, the formation and degradation of horizontal spinule cells, was also investigated in the glowlight tetra. Unlike retinomotor movements, spinule dynamics are under central control with the oscillator residing outside of the eye. In constant darkness, the spinule rhythm showed signs of damping within the first 24 hours, indicating that circadian drive is much weaker than that controlling both cone and RPE melanosome movement. In contrast to both the cone and melanosome movements, spinule dynamics were affected by both light exposures in the middle of the dark phase as well during dark placement in the middle of the light phase. Following light exposure in the dark phase, the formation of spinules in both the glowlight tetra and the zebrafish (*Danio rerio*) followed a time course similar to those seen in the goldfish (*Carassius auratus*) the only other species examined to date.

8.2 Future directions

This thesis poses more questions than it answers. Possibly the biggest conclusion that can be drawn from this study is that the glowlight tetra (*Hemigrammus erythrozonus*) warrants further investigation!

One reason given for the long persistence of retinomotor movements in constant darkness in the glowlight tetra was that the glowlight tetra, and other equatorial species, may have evolved strong oscillators governing their retinomotor movements. Another explanation given may be that different retinomotor movement oscillator strengths are simply species specific. The different strength of these oscillators may in turn be a reflection of both the hierarchy and interaction between different oscillators within a particular species. In fish, circadian oscillators have been shown in the retina (Cahill et al, 1991), the pineal gland (Bolliet et al, 1996) and also other parts of the brain of some fish (Tabata et al, 1991) and the interaction of these oscillators is thought to regulate overall rhythmicity (Tabata, 1992). The coupling and/or hierarchal order of these oscillators is different even in closely related species. For example, in the catfish (*Heteropneustes fossilis*) (Garg and Sundararaj, 1986) pinealectomy causes breakdown of circadian rhythms, whereas to destroy circadian rhythmicity in the catfish (*Silurus asotus*), pinealectomy, removal of both eyes, and shielding the brain from external light is required (Tabata et al, 1991).

The role that oscillators controlling retinomotor movements, the pineal gland, and possibly other oscillators located in the brain play in the locomotor activity of the

glowlight tetra remains to be determined. To do this, locomotor activity following entrainment to a light/dark cycle needs to be investigated during the following conditions;

1/ during a light/dark cycle of normal intact fish. Under this condition, the basic pattern of locomotor activity in constant darkness can be determined.

2/ during a light/dark cycle of fish that have had both lateral eyes removed. This should determine the contribution that ocular oscillators (including those controlling retinomotor movements) have on the overall locomotor activity.

3/ during a light/dark cycle of fish that have undergone pinealoctomy. Thus, the role the pineal gland has in maintaining locomotor activity can be determined.

4/ during a light/dark cycle of fish that have undergone pinealoctomy and have had both lateral eyes removed. If a locomotor rhythm persists, this could indicate the presence of other oscillators located in the brain.

5/ during a light/dark cycle of fish that have undergone pinealoctomy, have had both lateral eyes removed and have had the brain region shielded from light. It is expected that under these conditions that no rhythms in locomotor activity will be seen.

The results of these 5 experiments would help to construct a simple model showing how the two or three independent oscillators influence the overall locomotor activity in glowlight tetra. Previously, in the lamprey (*Morita et al, 1992*) and the catfish (*Heteropneustes fossilis*) (Garg and Sundararaj, 1986) pinealoctomy abolished the locomotor activity rhythm. Thus in these fish, the pineal gland could be considered the 'master clock'. In comparison, in the catfish (*Silurus asotus*) pinealectomy, removal of both eyes and shielding the brain region from external light is required to destroy circadian locomotor activitity (Tabata et

al, 1991). Thus, in catfish the pineal, lateral eyes, and oscillators located somewhere in the brain could all maintain rhythmic locomotor activity. The lamprey (34-71°), the catfish (*Silurus asotus*) (23-53°) and to a lesser extent another catfish (*Heteropneustes fossilis*) (5-33°), are temperate species yet how the different oscillators interact with each other to maintain circadian rhythmicity is not necessarily the same.

Unfortunately neither of the lamprey nor the stinging catfish (*Heteropneustes fossilis*) have been investigated to determine whether retinomotor movements persist in constant darkness. It would be of interest to perform the 5 experiments described above in a temperate species that has only limitied retinomotor movement in constant darkness (for example the bluegill (*Lepomis macrochirus*) (25-50°N) where the rhythm of cone movements is seen to dampen during the first 0-24hours in constant darkness) and compare the results to the glowlight tetra Thus, one could determine whether a strong oscillator controlling retinomotor movements indicates an oscillator that is also capable of influencing locomotor activity.

In chapter 4, the light control of cone movements in the glowlight tetra was shown to be relatively poor during most of the circadian phase. However, two 'small windows' existed where stronger light control of cone movements were seen Neither 100 minutes of bright light in the middle of the night nor 100 minutes of darkness in the middle of the day had any effect on cone position. The lack of cone movement during light exposure and dark placement during the day and night respectively offers a good opportunity for neurochemical studies to

determine the extracellular control of cone movement. Dopamine could be applied to cultured eyecups or retinae in the middle of the night in order to try to light adapt the cones. Similarly, cultured eyecups or retinae could be treated with melatonin in the middle of the day to see if this causes cone elongation. To date, attempts to develop a culture system to analyze cone movements in the glowlight tetra have met with little success. At best, a short term system where cones were seen to light adapt in cultured eyecups at dawn was achieved (personal observation).

In chapter 5, glowlight tetras were found to entrain well to 24 hour light/dark cycles with photoperiods ranging from 1-14 hours. Models using either a one or two component oscillator could explain the mechanisms by which the rhythm of cone movements could entrain to the different light cycles. In constant darkness, following entrainment to all photoperiods, although there was almost no change in the free-running period, the pattern of cone movements did alter. No matter what light/dark cycle fish were entrained to, in constant darkness animals exhibited a rhythm of cone movement that became more similar to the pattern seen in animals following entrainment to a LD 12:12 cycle. This indicates that altering the photoperiod has only produced temporary effects on altering the shape of the cone rhythm. It would have been interesting to determine how long these temporary effects last. Thus, the cone rhythm could be determined during a further 24-72 hours in darkness, following entrainment to the novel photoperiods, to see if the rhythm now follows that found during entrainment to a LD12:12 cycle. Likewise, it would be interesting to repeat these experiments on glowlight tetras bred under the different light regimes. Would these fish, in constant darkness, now exhibit the

same pattern of cone movements as that found during the light/dark cycle in which they were bred and raised, or would the pattern be more similar to that found during a LD 12:12 cycle?

Previous experiments have determined PRC curves to calculate the limits of entrainment to different photoperiods. To determine accurately a PRC curve for the cone movements in glowlight tetra would involve a substantial amount of work (perhaps 1.5 -2.0 years). Thus, one must decide whether such a task is warranted. Considering the relatively insensitivity of the cone oscillator during most of the light/dark cycle, it would be of interest to compare this finding to the shape of the PRC curve. This, taken together with the fact the no PRC curves exist for any retinomotor response, may make the construction of a PRC for cone movement worthwhile.

Appendix 1-Solutions

1/Fixative for light microscopy

2.5% glutaraldehyde (Sigma, St. Louis USA)
1% paraformaldehyde (BDH, England UK)
3% sucrose (BDH, England UK)
in 0.1m phospate buffer, pH=7.4 (Sigma, St. Louis USA)

2/Fixative for electron microscopy

1.5% glutaraldehyde (Sigma, St. Louis USA)
1.5% paraformaldehyde(BDH, England UK)
3% sucrose (BDH, England UK)
0.05% picric acid (Sigma, St. Louis USA)
0.05% tannic acid (Sigma, St. Louis USA)
0.08% $CaCl_2$ (Sigma, St. Louis USA)
in 0.1m cacodylate buffer, pH=7.4 (Fisher Chemical, Leister England, UK)

3/Diluent

50% terpineol (Fluka Chemika, Steinheim, Switzerland)
50% alocohol (BDH, England UK)

4/Toluidine blue

50%, 1% toluidine blue (BDH, England UK)
50%, 2.5% sodium carbonate (BDH, England UK)

Appendix 2-Post-hoc Bonferoni test on melatonin data

Table A1 gives 'time pairs' where the ocular melatonin content was significantly different between the two times, as found using a post-hoc Bonferoni test.

Table A2. Post-hoc Bonferoni test on melatonin data

Time	Significantly different from	Level of significance
t0	t42	0.002
t6	t42	0.013
t14	t42	0.002
t18	t42	0.038
t22	t26	0.011
t24	t42	0.000
t26	t22	0.011
	t34	0.031
	t38	0.044
	t42	0.000
	t46	0.025
t30	t42	0.048
t34	t26	0.031
t36	t42	0.000
t38	t26	0.041
t42	t0	0.002
	t6	0.013
	t14	0.002
	t18	0.038
	t24	0.000
	t26	0.000
	t30	0.048
	t36	0.000
t46	t26	0.025

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