CIRCADIAN ACTIVITY RHYTHMS AND EARLY GENE EXPRESSION IN THE SUPRACHIASMATIC NUCLEUS OF A DIURNAL RODENT, RHABDOMYS PUMILIO

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A thesis submitted in partial fulfillment of the requirements for the degree of Magister Scientiae in the Faculty of Science, University of the Western Cape

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November, 2001

FOR MY PARENTS.

Faith makes all things possible.

Love makes all things easy.

Hope makes all things work.

Abstract

CIRCADIAN ACTIVITY RHYTHMS AND EARLY GENE EXPRESSION IN THE SUPRACHIASMATIC NUCLEUS OF A DIURNAL RODENT, RHABDOMYS PUMILIO

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MSc thesis, Department of Zoology, University of the Western Cape.

Although humans are diurnal in behaviour, animal models used for the study of circadian rhythms are mainly restricted to nocturnal rodents. This study focussed on the circadian behaviour and gene expression in a diurnal rodent from South Africa, the four-striped field mouse (*Rhabdomys pumilio*). In contrast to nocturnal rodents which have rod dominated retinas, the retina of this field mouse contained approximately an equal amount of cones as rods. The cones included short wavelength (blue sensitive) and mid-wavelength (green sensitive) cones.

In order to characterise the behavioural pattern of daily activity, locomotor rhythms were studied under different light regimes using an automated data recording system. Under conditions of natural daylight which include dawn and dusk transitions, *R. pumilio* showed activity restricted to the day time period. Activity was concentrated around morning and evening hours with a decrease during mid-day. A similar diurnal pattern of behaviour was recorded under a light-dark cycle of artificial illumination. Under conditions of constant darkness, the animals showed a free-running circadian rhythm of locomotor activity with activity concentrated during the subjective day. Free running periods varied greatly between individuals from slightly less to slightly more than 24 hours (ranging from 23.10 to 24.80 hours). Under conditions of constant light, animals also showed more activity during the subjective day, but the free running period in all individuals was consistently longer than 24 hours (ranging from 24.30 to 24.79 hours). In contrast to nocturnal animals whose activity is masked by the presence of light, activity in the diurnal *R. pumilio* was masked by darkness.

The photic response of the suprachiasmatic nucleus (SCN) was also assessed using light induced expression of Fos, which is a marker of neuronal activity. Animals were exposed to a 15 min pulse of monochromatic light at different times of the circadian period. The amount of Fos protein expressed was quantified in brain sections of the SCN using immunohistochemistry and quantitative image analysis. The results showed that under conditions of constant darkness, Fos expression was consistently low indicating that the protein showed no endogenous circadian variation. Following a light pulse, Fos was induced in the SCN when the photic stimulation occurred during the subjective night time

with a peak in expression during late subjective night. This differed from nocturnal rodents in which the peak in expression occurs during the early part of subjective night. There was no Fos induction during subjective day, which corresponds to results found in other rodents. This result indicated that as in nocturnal rodents, early gene expression is gated by the phase of the circadian clock. The results supported the idea that in nocturnal and diurnal rodents the basic mechanisms of the circadian clock in the SCN are similar, but the behavioural and physiological outputs are regulated differently downstream from the SCN.

Declaration

I declare that *Circadian Activity Rhythms and Early Gene Expression in the Suprachiasmatic Nucleus of a Diurnal Rodent, Rhabdomys pumilio* is my own work, that it has not been submitted before for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

DESIREÉ M. THOMAS October 2001

SIGNED

Acknowledgements

Dr. Hofmeyr who believed in me and had been a huge support and guiding force. I would like to thank Martin Hendricks, Dr. Scott Ramsay, Quentin Joshua and Goosain Isaacs for their help in trapping *R. pumilio*. Prof. Jarvis (U.C.T.) for tips on how to trap *R. pumilio*. Martin Haupt who built the cages. Gundula Miethe and Marietjie Oosthuizen for caring for the animals when I was unable to. Dr. Philip Bateman for his help in getting my French visa. Prof. Nigel Bennett for allowing me to work in his laboratory and for obtaining the funding for this project. Naura Chounlamountri for helping with the immunohistochemistry. Camille Rieux for sharing his office, and being a great working partner. Rosa Yssaad, for extending her kindness and friendship. Dr. Roelof Hut for discussion, critical input and much needed Dutch bonhomie. Dr. Cooper for allowing me to work in his laboratory in Lyon. I could not have asked for a better person to introduce me to the field of neuroscience. Dr. A. Lukats, for allowing me to use unpublished data on the retinal structure of *R. pumilio*. Greshon de Beer for haranguing finance on my behalf so that I could get my funding. Karen Peterson and Abigail Londt who made being away from home easier by writing all the time, and just for being my friends.

Marc Schumann for help with the programming, teaching me LaTeX 2_ε and all the programs needed to go with it, and for his support and love. My gratitude extends beyond the power of mere words.

This study was financed by the French Embassy in Pretoria (special thanks to Monsieur Patrick Le Fort), the International Brain Research Organisation, National Research Foundation, Royal Society and FRD-MAE. Part of the results of this study was presented at the 5^{th} Society of Neuroscientists of Africa (SONA) meeting financed by a SONA travel grant.

Keywords

1. *R. pumilio*
2. Four-striped Fieldmouse
3. Retinal Projections
4. Suprachiasmatic Nucleus
5. Fos Expression
6. Twilight
7. Activity Pattern
8. Tau
9. Masking
10. Diurnality

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Chapter 1

General Introduction

1.1 Biological Rhythms

> 'Every indication of contrivance, every manifestation of design, which existed in the watch, exists in the works of nature...' (Paley, 1828)

Almost all species adapt their behaviour to the daily fluctuations of light, temperature, competition and predation in the environment. If these fluctuations are regular, such as the light-dark cycle, the species develop an internal program or genetically encoded response to the changes, whereas an irregular fluctuation can elicit an immediate stimulus-response reaction (Daan, 1981). Although persistent daily rhythms had been observed for decades, Pittendrigh and Bruce (1959) were the first to coin the term 'clock' to describe the endogenous rhythms of plants and animals. This internal program or biological clock allows the anticipation of daily and seasonal changes by synchronising the behavioural and physiological systems of the organism to external stimuli (Turek, 1994). Circadian rhythms, which have a period close to 24 hours, are of importance in human medicine, and play a role in the timing of drug administration for the treatment of diseases and diagnostics (Florez and Takahashi, 1995). These rhythms also play a role in the timing of diseases, with eighty percent of asthma attacks occurring at night and deaths due to cardiovascular disease occurring mainly in the morning (Arendt, 1998). The adaptive advantage of biological rhythms in nature is also supported by observations in animals with damaged or no clocks, which become more susceptible to predation because they are active at unfavourable times (Decoursey *et al.*, 1997).

Circadian rhythms are distinguishable from other biological rhythms because they have periods that closely match that of the external day-night cycle and this period remains stable over a wide range of temperatures (Pittendrigh and Bruce, 1959). Ultradian rhythms are rhythms with a frequency of less

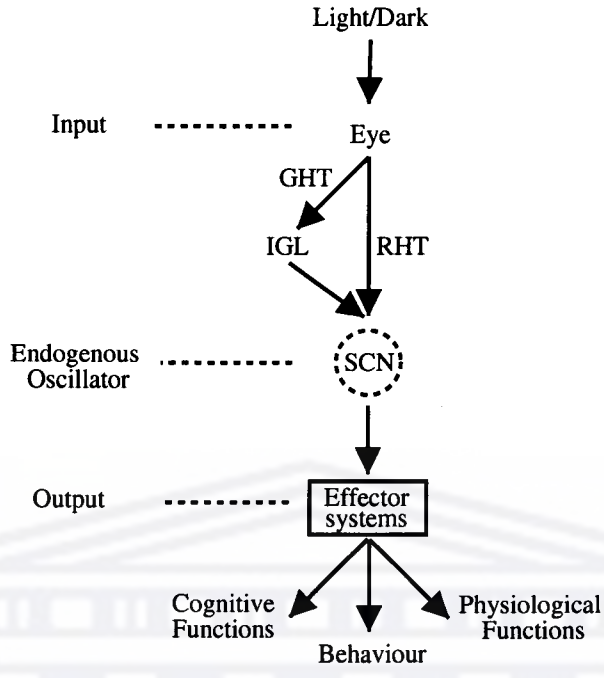

Figure 1.1: Schematic diagram showing the main components of the circadian system in mammals. GHT = Geniculohypothalamic tract, IGL = Intergeniculate Leaflet, RHT = Retinohypothalamic tract, SCN = Suprachiasmatic Nucleus.

than a day e.g. pulsatile secretion of hormones whereas the menstrual cycle would be infradian, since it has a frequency of more than a day (Arendt, 1998). The circadian clock has many characteristics in common with a self-sustaining oscillator, i.e. that the endogenous self-sustaining oscillation is an innate attribute and that the phase and period of this endogenous oscillation can be entrained and corrected by being coupled directly to a light cycle of the environment (Pittendrigh and Bruce, 1959). This initial view of a single oscillator has since been modified to that of a multi-oscillatory system (Shirokawa *et al.*, 2001, for review).

Circadian rhythms are ubiquitous and the basic components of the system are similar in all species in that it has an input pathway, an oscillator and many output pathways as can be seen schematically in Figure 1.1. The format of this General Introduction will follow that of the diagram, giving more details on the basic components of the circadian system.

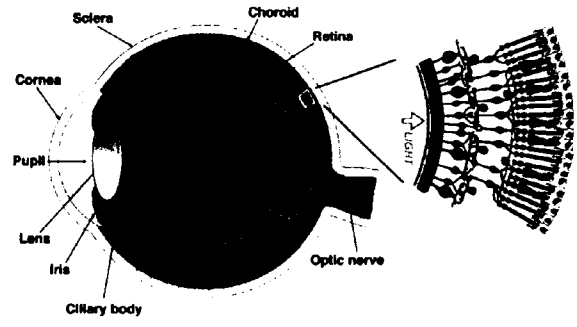

Figure 1.2: Structure of a vertebrate eye with an enlargement of part of the retina, with permission from Kolb *et al.* (2001).

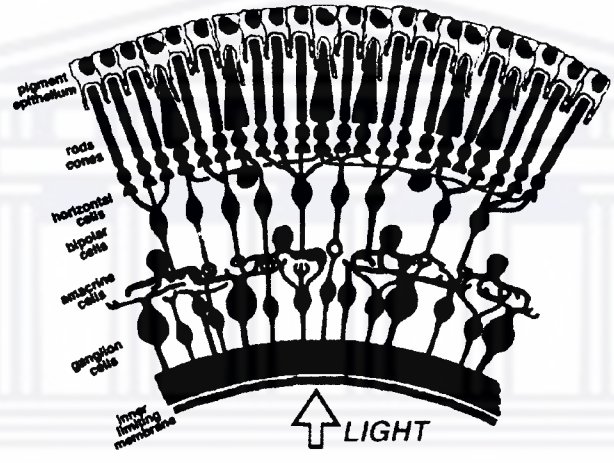

Figure 1.3: Schematic diagram depicting the general structure of a vertebrate retina, with permission from Kolb *et al.* (2001).

1.2 The Eye

1.2.1 Structure

The main components of the eye consists of a pupil, iris, cornea, lens, sclera, choroid, and retina (Figure 1.2), but I will only deal with the retina because it is the most important structure of the eye where circadian rhythms are concerned.

The retina is embryonically derived from the neural tube and thus forms part of the central nervous system. It consists of three nuclear layers (the ganglion cell layer which develops first, the inner nuclear layer and the outer nuclear layer), two synaptic layers (the inner and outer plexiform layers), a photoreceptor layer which develops last, and a retinal pigment epithelium layer (Figure 1.3). The ganglion cell layer contains cell bodies of ganglion cells and displaced amacrine cells, the inner nu-

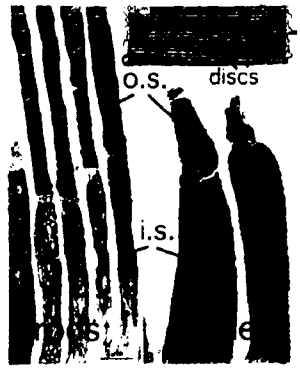

Figure 1.4: Electron micrograph of the classical photoreceptors, rods and cones, with permission from Kolb *et al.* (2001). O.S. = Outer Segment, I.S. = Inner segment.

clear layer contains cell bodies of the bipolar, horizontal and amacrine cells and the outer nuclear layer contains cell bodies of the rods and cones. The rods and cones transduce absorbed photons into neural signals, the bipolar, horizontal and amacrine cells relay these signals to the ganglion cells which transmit the information via the optic nerve (the centre of the retina), to the relevant areas of the brain (Kolb *et al.*, 2001).

1.2.2 Classical Photoreceptors and Transduction

All vertebrate retinae contain the classical photoreceptors, rods and cones. Rods are slim, rod-shaped structures whereas cones are conical-shaped structures (Figure 1.4). The outer segments of both rods and cones consist of discs of folded double membranes which contain the light sensitive visual pigment molecules (Kolb *et al.*, 2001). Rods contain the visual pigment, rhodopsin which consists of a protein called opsin and a chromophore derived from vitamin A, called retinal. Rods are sensitive to blue-green light with a peak sensitivity at 500 nm wavelength and are used for vision under dark-dim conditions at night. Cones contain cone opsins which, depending on their structure, are sensitive to either long wavelengths of light (red light), medium wavelengths of light (green light) or short wavelengths of light (blue light). Humans are trichromatic since all three cone types are present, but most mammals are dichromatic, containing only middle (M-cones) and short wavelength pigments, S-cones (Kolb *et al.*, 2001). The murine retina contains cones sensitive in the green wavelength with the maximum sensitivity at 508 nm and cones sensitive in the ultraviolet range with the maximum sensitivity at 359 nm (Jacobs *et al.*, 1991).

When light enters the eye, it passes through the entire retina before attaining the rods and cones. Photons are thus first transduced into a biochemical message and then into an electrical message. At

the level of the photoreceptors, the detection of light results in hyperpolarization of the cell membrane which is relayed to the bipolar cells. The bipolar cells respond either by hyperpolarizing (OFF) or depolarizing (ON) (Doly, 1994). Rod signals converge on only one type of rod bipolar cell in the outer plexiform layer after which divergence occurs to several types of amacrine cells in the inner plexiform layer (Kolb *et al.*, 2001). The rod signal then converges on the ganglion cells. Cones on the other hand, synapse on various cone bipolar cells which in turn synapse directly with ganglion cells. This is a more direct yet less convergent pathway than the rod pathway since few cone bipolar cells converge onto a single ganglion cell.

Novel Photopigments

Recent evidence suggests that a novel photopigment may be involved in circadian rhythmicity. Retinally degenerate mice (rd/rd) which undergo a gradual loss of rods, lose visual capabilities after a certain period but circadian responses remain unaffected (Nagy and Misanin, 1970; Provencio *et al.*, 1994). A number of other models including coneless mice (cl), rodless-coneless mice (rdta/cl, rdta mice are transgenic mice with specific destruction of rod photoreceptors during early development (Freedman *et al.*, 1999)), rds/rds mice (slow inherited retinal degeneration, (Foster *et al.*, 1993)), and a blind mole rat, *Spalax ehrenberghi* (Cooper *et al.*, 1993), are entrained by light. However Yoshimura and Ebihara (1998) found a decline in circadian photosensitivity associated with retinal degeneration in rd/rd mice, although this difference may be due to the use of a different strain of mice. One problem associated with the transgenic models mentioned above, is that due to neuronal plasticity during development, other photosensitive components can compensate the function of defunct rods and cones.

Five putative novel photopigments have been identified in mammals, such as retinal-binding G-protein-coupled receptor (RGR), peropsin, encephalopsin, melanopsin and cryptochromes (von Schantz *et al.*, 2000, review). None of these photopigment molecules have been shown to be directly involved in circadian photoreception and their capacity to be photoactive still remain to be demonstrated. Melanopsin, expressed specifically in retinal ganglion cells which may be part of the retinohypothalamic tract, is still under investigation. Cryptochromes have been implicated in detecting light and mediating photoentrainment in plants and invertebrates (Cashmore *et al.*, 1999; Lucas and Foster, 1999, for review). In mammals, the results have been contradictory since van der Horst *et al.* (1999) found that transgenic mice lacking both cryptochrome 1 (Cry1) and cryptochrome 2 (Cry2) proteins (Cry-mutant mice), show an instantaneous and complete loss of rhythmicity whereas Selby *et al.* (2000) found that Cry-mutant mice still exhibited rhythmic behaviour, but when mice

lacked both rods and cryptochromes, they were arrhythmic. Lucas *et al.* (2001) recently found that rodless-coneless mice are able to perform another non-image forming light response such as pupillary constriction. The photoreceptor involved uses a single opsin/vitamin A-based photopigment with its maximum sensitivity around 479 nm. This differs from the known murine photopigments which have maximum sensitivity at 500 nm (rods), 508 nm (M-cones) and 359 nm, S-cones (Kolb *et al.*, 2001).

1.2.3 A Functional Dichotomy in the Visual System

Several characteristics distinguish the visual and circadian pathways. Photic information is used for both visual and circadian pathways but the pathways are distinct since only a subset of the ganglion cells innervate the suprachiasmatic nucleus (Mason and Lincoln, 1976; Card *et al.*, 1991). The development of the SCN which is a circadian or non-image forming structure, occurs after that of other primary visual structures (Cooper *et al.*, 1993). Ganglion cells connected with the non-image forming pathway are scattered throughout the retina which results in a low spatial resolution of this system, whereas the ganglion cells involved in the image-forming pathway are typically distributed on a centro-peripheral gradient (Cooper *et al.*, 1993). The circadian system is less sensitive to light than the visual system and the intensity-duration reciprocity relationship holds for stimuli of long duration (Takahashi *et al.*, 1984; Dkhissi-Benyahya *et al.*, 2000). In contrast the visual pathways require high contrast sensitivity, spatial summation and temporal resolution for acuity and detection of movement of visual stimuli (Kolb *et al.*, 2001).

1.3 Suprachiasmatic Nucleus

1.3.1 SCN Structure and Retinal Innervation

The suprachiasmatic nucleus (SCN) in rodents is oval shaped, bilaterally symmetrical, located close to the third ventricle and dorsal to the optic chiasm in the anterior hypothalamus. It is one of the brain structures with the smallest neurons. The SCN can be divided into a small rostral area and a large caudal area with a dorsomedial and ventrolateral part (Reuss, 1996). These different regions may play different roles in the circadian system with neurons in the dorsomedial SCN in the rat containing mainly vasopressin and neurons in the ventrolateral SCN containing mainly vasoactive intestinal polypeptide (Goel *et al.*, 1999; van Esseveldt *et al.*, 2000). The most abundant peptide in the SCN is arginine-vasopressin (AVP) and like the other neuropeptides present, it is under the influence of the circadian pacemaker.

The SCN is the primary circadian pacemaker in mammals as has been shown with lesioning and transplantation experiments (Figure 1.5) (Richter, 1967; Rusak and Zucker, 1979; Moore, 1983; Zucker *et al.*, 1983; Sato and Kawamura, 1984a; Decoursey *et al.*, 1997). Destruction of the SCN was one of the first techniques used to determine its role in circadian rhythms. Although a loss in circadian rhythms was observed, the results were inconclusive for several reasons. The loss of rhythmicity may have been caused by damage to areas other than the SCN and certain circadian rhythms may have been undetected because of the techniques used at the time (Murphy and Campbell, 1996).

To strengthen the evidence that the SCN is the primary pacemaker, it was isolated from the brain (all afferents and efferents were severed), but the SCN still exhibited an endogenous rhythm which means that it acted as a self-sustained oscillator (Inouye and Kawamura, 1979). *In vitro*, the SCN neurons fire with distinct periods from one another which suggests that the SCN is a multioscillatory system (Herzog and Tosini, 2001). These neurons synchronise to form two distinct yet coupled oscillators and it has been hypothesised that the one oscillator is entrained by dawn (M-oscillator) and the second oscillator is linked to dusk (E-oscillator) (Pittendrigh, 1960; Pittendrigh and Daan, 1974; Daan and Berde, 1978; Daan *et al.*, 2001). This hypothesis was based on a behavioural phenomenon known as 'splitting', when the activity pattern of the animal splits into two, and each takes on its own rhythm for a couple of cycles before becoming synchronised again (Pittendrigh, 1960; Pittendrigh and Daan, 1974). Recently there has been additional molecular evidence to substantiate this hypothesis (Albrecht *et al.*, 2001).

The most conclusive evidence was provided by transplantation experiments, where fetal tissue from tau-mutant hamsters was transplanted into arrhythmic animals. The activity rhythm of the recipients was restored, but with the period of the donor animal, i.e. the tau-mutant hamsters (Ralph *et al.*, 1990; Ralph and Lehman, 1991; Ralph *et al.*, 1993). These experiments showed that the SCN itself generates the rhythms of the organism.

The SCN receives entraining photic information via the eye. Bilateral enucleation in mammals results in the abolishment of photoentrainment (Figure 1.5 and Table 1.1), and thus suggests that eyes are essential for the transmission of light to the biological clock (Foster, 1998). Although there is still much confusion as to the photopigments involved, it is known that photic information is relayed to the SCN via at least two pathways. A direct innervation from the retina via the retinohypothalamic tract (RHT) (Moore and Lenn, 1972; Moore, 1973) and an indirect path through the intergeniculate leaflet via the geniculohypothalamic tract (GHT) (Ribak and Peters, 1975). The retinohypothalamic tract is essential for photoentrainment with retinal projections mainly to the ventrolateral SCN (Moore

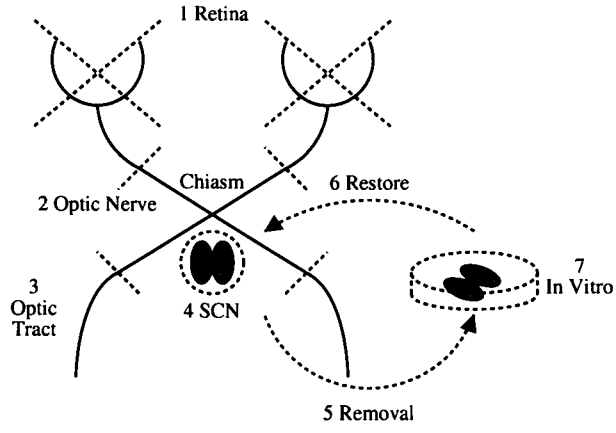

Figure 1.5: Lesioning experiments have been paramount in determining the photic entrainment pathway and the site of the circadian clock. Refer to Table 1.1 for clarification of the diagram.

Table 1.1: Interaction between photic entrainment and endogenous rhythm. The numbers in this diagram correspond to those in Figure 1.5.

Number	Condition	Photic Entrainment	Free-running Rhythm	Vision
1	Eye Removal	No	Yes	No
2	Optic Nerve Section	No	Yes	No
3	Optic Tract Section	No	Yes	No
4	Surgical Isolation of SCN	No	Yes	Yes
5	Lesion or Removal of SCN	No	No	Yes
6	Restored SCN Transplant	No	Yes	Yes
7	In Vitro Culture of SCN	No	Yes	

and Lenn, 1972; Provencio *et al.*, 1998; Tessonneaud *et al.*, 1994). The geniculohypothalamic tract is not essential for photic entrainment, but it has been found to alter the rate of re-entrainment following a change in the light-dark (LD) cycle (Morin *et al.*, 1990), it influences the variability of the offset of activity in the Syrian hamster (de Vries and de Vries, 1995) and it is essential for entrainment of circadian rhythms to skeletal photoperiods (Edelstein and Amir, 1999).

1.3.2 Molecular Mechanisms and Clock Genes

There are many immediate early genes in the SCN, such as c-jun, Jun-B, *c-fos*, Zif-268 but we will deal with *c-fos*. The protein Fos, a product of the *c-fos* gene, is transiently induced and alters gene

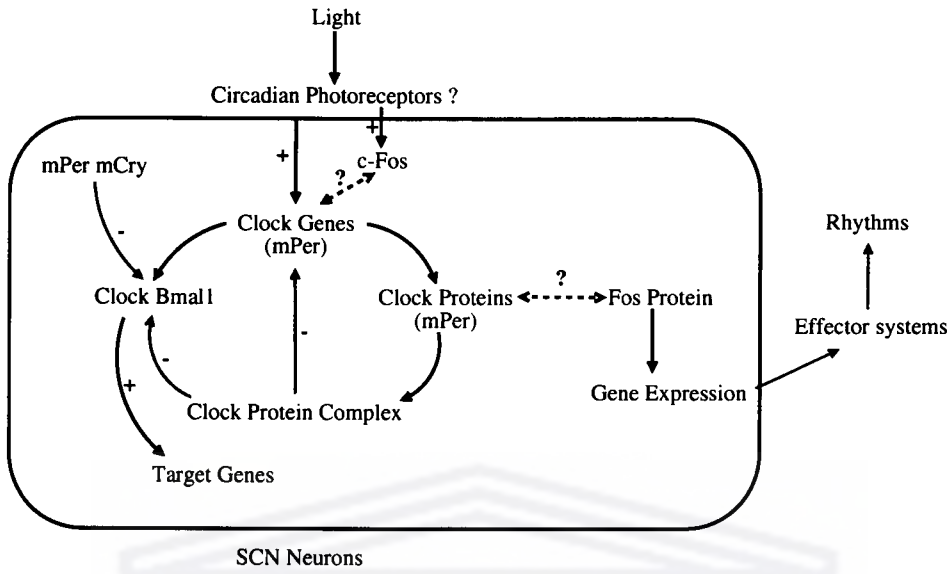

Figure 1.6: Schematic diagram depicting the possible role of Fos in the circadian clock.

expression by regulating transcription (Sheng and Greenberg, 1990). Like the neuropeptides, *c-fos* shows an increase in expression during the day and a decrease at night (Guido *et al.*, 1999) which mirrors *in vivo* and *in vitro* studies on neuronal activity in the SCN (Sato and Kawamura, 1984b). Fos is thus used as a marker for neuronal activity in the SCN. Light increases Fos expression in areas of the SCN that receive retinal input (Rea, 1989; Aronin *et al.*, 1990; Earnest *et al.*, 1990; Rusak *et al.*, 1990; Colwell and Foster, 1992) with the amplitude of expression proportional to the number of photons in the light stimulus (Dkhissi-Benyahya *et al.*, 2000). Fos is only expressed in response to a light pulse at times when the animal is susceptible to a behavioural phase shift i.e. during subjective night (Rusak *et al.*, 1990). In rats, light-induced behavioural phase shifts are blocked by intracerebroventricular injections of antisense oligodeoxynucleotides to both *c-fos* and JunB (Wollnik *et al.*, 1995). Less information is available for diurnal mammals.

The exact role of Fos in the circadian clock is unknown but a hypothetical role for Fos in the circadian clock is schematically given in Figure 1.6. Fos regulates transcription. CLOCK and BMAL1, which are transcription factors, activate transcription of three Period and two Cryptochrome genes resulting in the accumulation of PER and CRY proteins during the day. At dusk, these proteins translocate into the nucleus where they in-turn inhibit the activity of CLOCK and BMAL1. The turnover of these inhibitory proteins during the night then leads to a new cycle of CLOCK and BMAL1 activation (Herzog and Tosini, 2001).

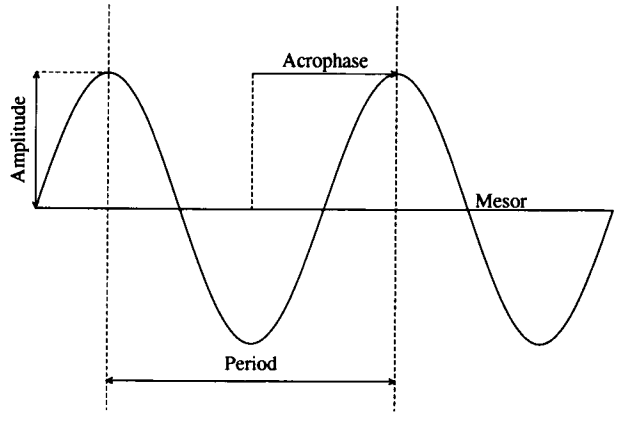

Figure 1.7: Schematic diagram depicting the basic properties of a biological rhythm.

1.4 Properties of the Circadian Clock

The circadian clock drives several different functions including the sleep-wake cycle, the increase in melatonin, prolactin and growth hormone levels at night, with the corresponding decrease in core body temperature and urine volume, an increase in blood pressure and cortisol in the morning and it plays a role in the variation in mood and alertness (Arendt, 1998). This discussion will mainly deal with activity patterns.

To understand the basis of the circadian system, one has to become familiar with the terminology used to describe the rhythms and their measurements. Figure 1.7 depicts the most commonly used terms to describe activity patterns. It is clear that activity patterns show a 24 hour rhythm with three measurements that describe the basic features: **mesor** which is the average value of the fitted curve, **amplitude** is the difference in the curve from the mesor, and **acrophase** corresponds to the maximum value and the **nadir** to the minimum value. The acrophase and nadir are 12 hours apart with the nadir used to define the **phase** of the rhythm (Murphy and Campbell, 1996). The following is a summary of the terms used, ordered alphabetically:

activity onset the time when activity starts.

activity offset the time when activity ends.

alpha, α the period during which the animal is active.

entrained the biological clock is synchronised by external stimuli to the period of the environment.

free-running natural self-sustained rhythm that exists in the absence of all environmental cues (Hunter *et al.*, 1996).

non-parametric entrainment the biological clock is entrained by one or two light pulses, i.e. dawn and dusk.

non-photic stimuli the biological clock can be entrained by stimuli other than light, such as forced wheel running, feeding times, social cues and temperature changes.

period the time it takes for one complete cycle.

phase advance the animal starts its activity earlier relative to the preceding day i.e. the nadir occurs early relative to a reference point (Murphy and Campbell, 1996).

phase delay the animal starts its activity later relative to the preceding day.

phase response curve, PRC the phase of the clock can be advanced or delayed depending on the time the animal is exposed to a light or dark pulse. These advances and delays are graphically depicted in a phase response curve.

phase angle difference, ψ difference between activity onset or offset and lights on or lights off, respectively.

parametric entrainment the biological clock is entrained by continuous light input.

rho the period when the animal is inactive.

tau, τ the period of the endogenous clock.

zeitgeber factor that entrains the biological rhythm to the external environment.

The factors that entrain or synchronise the rhythm to the external environment are termed zeitgebers or time cues. The main zeitgeber is the light-dark transition, but rhythms can be synchronised by non-photic stimuli such as social cues (Goel and Lee, 1995a,b; Zisapel *et al.*, 1999), forced wheel running (Hut *et al.*, 1999a; Kas and Edgar, 1999a) and temperature changes. The biological clock of the animal can be entrained or synchronised to the period of the external environment either by 'discrete' (non-parametric entrainment) or by continuous light input (parametric entrainment) (Pittendrigh and Daan, 1976a). The most commonly used model is that of non-parametric entrainment where one or two light pulses (dawn and dusk), reset the period of the pacemaker to that of the zeitgeber by either advancing or delaying the activity rhythm. The period of the activity rhythm, is not exactly equal to that of the environment. The difference between the activity onset and lights on, or activity offset and lights off, is known as the phase angle difference (ψ). Psi is positive when activity onset (or offset)

precedes lights on (or lights off) and is negative when activity onset (or offset) succeeds lights on (or lights off).

1.4.1 Endogenous rhythm

When placed in constant conditions, such as constant dark or constant light, the biological clock expresses an endogenous or free-running rhythm because there is no zeitgeber to synchronise the period of the rhythm, no restrictions. When an animal is free running, the time corresponding to the endogenous clock is referred to as circadian time (CT) but when the animal is entrained to a light-dark cycle, the time is referred to as zeitgeber time (ZT). The period of the free-running rhythm is termed tau (τ) and it is close to 24 hours but can be longer or shorter. Free-running rhythms can occur in nature, as was found in the Free-living Beaver (*Castor canadensis*) who free ran with a period of about 27 hours when it was trapped under ice and had no photic input (Bovet and Oertli, 1974). Generally τ in constant darkness differs from τ in constant light and Aschoff (1979) hypothesised that these changes were opposite in diurnal and nocturnal mammals. Aschoff's rule stated that in diurnal mammals, τ during constant light (τ_{LL}) was less than τ during constant dark (τ_{DD}) and vice versa in nocturnal mammals.

1.4.2 Phase Response Curve

When an animal is exposed to a light pulse while in constant conditions, the rhythm is advanced or delayed depending on the time of the light pulse. These shifts in the phase of the circadian clock are characterised in a phase response curve (Figure 1.8). In nocturnal mammals, exposure to light during subjective day has little or no effect on the activity rhythm (A in Figure 1.8). Exposure to light during early subjective night delays the activity rhythm (B and C in Figure 1.8) which means that the animal will start its activity later on the succeeding days. Exposure to light during late subjective night advances the activity rhythm (D and E in Figure 1.8) so the animal starts its activity earlier. The beginning of subjective day (CT0 – CT12) corresponds to 'that point in a constant dark rhythm whose normal phase in a light-dark cycle coincides with the dark-light transition' (Pittendrigh, 1960). That means that when the animal is in a light-dark cycle and instead of the lights going on after the dark period, they remain off, the time that the lights were supposed to come on becomes CT0 (Figure 1.9).

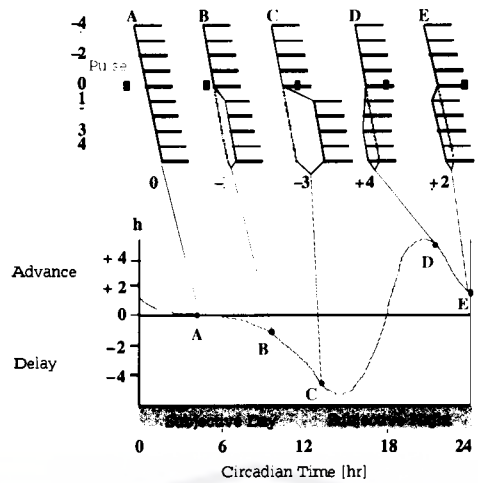

Figure 1.8: Phase Response Curve (PRC) of a nocturnal mammal showing delays in response to a light pulse during early subjective night, and advances during late subjective night.

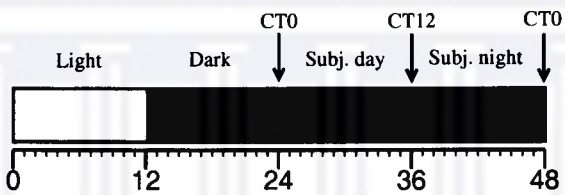

Figure 1.9: Schematic representation defining subjective day and subjective night.

1.4.3 Factors Affecting Circadian Activity Rhythms

A number of factors can modify circadian activity rhythms. For example, *Octodon degus*, a South American hystricomorph rodent (Lee and Labyak, 1997) and *Arvicanthis niloticus*, the unstriped Nile grass rat (Blanchong *et al.*, 1999), become nocturnal when exposed to running wheels. This complicates matters, since in rodents, rhythms of running wheel activity are measured as an overt output of the circadian clock (Kornhauser *et al.*, 1996). The Golden Spiny Mouse, changes its activity pattern from diurnal to nocturnal due to interspecific competition (Zisapel *et al.*, 1999). Nest availability shortens the active period, with onsets starting later, and offsets earlier (Boulos *et al.*, 1996a). In addition, an animal's activity can be masked by a light or a dark pulse. Masking, as defined by Minors and Waterhouse (1989) is 'any process that distorts the original output from the internal clock' though masking is more generally seen as complementing the circadian clock (Mrosovsky, 1999). In nocturnal mammals, a light pulse during the night when the animal is usually active, will block or mask the expression of activity (Mrosovsky, 1994; Redlin and Mrosovsky, 1999a) whereas in diurnal mammals, exposure to a dark pulse during the day, will block their activity. Masking would constitute the immediate stimulus-response reaction mentioned earlier.

1.5 Twilight

Although dawn and dusk play an important role in entraining the biological clock, most experiments use a light cycle with a sharp light-dark transition whereas in nature, twilight consists of a light gradient. The quality of light during twilight changes in 3 aspects.

1. The amount of light the animal is exposed to depends on cloud cover, shadowing or whether the animal looks directly at the sky. The circadian system would therefore have to ignore these minor fluctuations to be able to determine the correct light levels and thus the time of day (Roenneberg and Foster, 1997). To achieve this, the circadian pathway has an intensity-duration reciprocity relationship which holds for stimuli of long duration (Takahashi *et al.*, 1984).

2. The spectral composition changes by approximately a 6 – 8-log unit range of illumination from starlight to midday (Wirz-Justice *et al.*, 1998). During twilight there is a relatively high level of short (blue) and long (red) wavelengths, and a relative decrease in the yellow-orange range (Hut *et al.*, 2000).

3. For practical uses, three definitions of twilight are described based on the position of the sun: astronomical, nautical and civil twilight (Figure 1.10). Astronomical twilight is defined as the period when the centre of the sun is 12 to 18 degrees below the horizon. Nautical twilight occurs when the centre of the sun is 6 to 12 degrees below the horizon and civil twilight occurs during the interval between sunset and the time when the sun is 6 degrees below the horizon. During nautical twilight, objects are distinguishable to the human eye, but no outdoor activities are possible, whereas in civil twilight there is enough illumination to allow outdoor activities (U.S. Naval Observatory, 2001).

   Animals under a twilight cycle appear to have higher upper limits of entrainment (Boulos *et al.*, 1996b) and *c-fos* expression in the SCN peaks at both dawn and dusk (Cooper *et al.*, 1998) compared to animals under a square light-dark cycle.

1.5.1 Diurnal vs Nocturnal

Animals are characterised as being nocturnal, diurnal or crepuscular based on the proportion of their active period (α) that occurs during the night, day or twilight periods, respectively. As mentioned above, many factors can influence the behaviour of an animal to modify the time of activity, such as predation, presence of a running wheel, competition, nest boxes and masking.

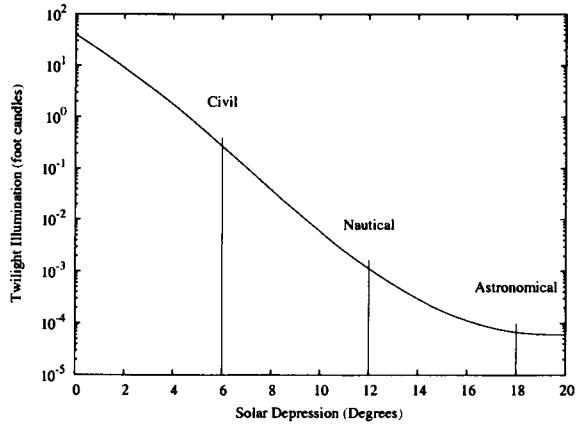

Figure 1.10: Schematic representation defining the different twilight times: civil twilight, nautical twilight and astronomical twilight (Ahmed, 1997).

The mechanisms which determine diurnality and nocturnality are unknown though it is thought that neural activity in areas other than the SCN may be involved (Sato and Kawamura, 1984a; Nunez *et al.*, 1999) because the SCN shows a higher firing rate during the day than during the night in both nocturnal and diurnal mammals (Sato and Kawamura, 1984b; Zlomanczuk *et al.*, 1991). However, multiple unit activity (MUA) outside the SCN is out of phase with the endogenous activity of the SCN but in phase with the activity pattern in nocturnal mammals whereas in diurnal mammals it is in phase both with the SCN and with the activity pattern. It has also been suggested that diurnality and nocturnality between different species may be controlled by the intergeniculate leaflet whereas intraspecific differences may be controlled by the lower subparaventricular zone (Smale *et al.*, 2001). However these hypotheses are mainly conjectural and lack specific evidence.

The relationship between molecular mechanisms, clock genes or Fos expression and diurnality is unknown and findings are contradictory. As most chronobiological research is carried out on nocturnal mammals, the pattern of Fos expression in nocturnal species is well elucidated. Photic induction of Fos occurs only during subjective night, in nocturnal mammals (Rea, 1989; Aronin *et al.*, 1990; Colwell and Foster, 1992) but in certain diurnal mammals, such as the diurnal chipmunk (Abe *et al.*, 1995; Honma and Honma, 1999) and *Octodon degus* (Krajnak *et al.*, 1997), there are changes in Fos expression during both subjective day and subjective night. In *Arvicanthis niloticus*, another diurnal mammal, photic induction of Fos only occurs during subjective night.

Nocturnal and diurnal mammals also differ in retinal structure and the relative number of rods and cones may be related to the period of activity. The European ground squirrel which is active throughout the day, starting its activity after dawn and ending it before dusk, has a high percentage of

cones in relation to rods (Kryger *et al.*, 1998). Hamsters, on the other hand, are 99% active at night, and have a high percentage of rods in relation to cones (Calderone and Jacobs, 1999). Nocturnal mammals are thus more sensitive to light than diurnal mammals as a result of their photoreceptor composition.

The majority of mammalian models used in chronobiology are nocturnal rodents with a few suitable diurnal mammals which include the squirrel, *Arvicanthis niloticus* and *Octodon degus*. *Arvicanthis* is an unstriped Nile grass rat, which is diurnal but becomes nocturnal in certain conditions. *Octodon* is a South American hystricomorph rodent that is diurnal in nature, but shows varying activity patterns once in laboratory conditions. A fundamental issue therefore concerns the distinction between nocturnality and diurnality. Although some species may be described as clearly diurnal such as the European ground squirrel (Hut, 2001) or clearly nocturnal such as the hamster there are species that show activity during twilight or may be partly nocturnal or partly diurnal. In addition most studies are merely descriptive and use a single parameter to classify the pattern of activity.

1.6 Objectives

The objectives of this study were to examine the activity patterns of *Rhabdomys pumilio* and to determine whether the molecular mechanisms correspond to the activity pattern.

This study was instigated by the observation that *R. pumilio* has approximately an equal amount of cones and rods (A. Lukats, pers. communication). In comparison the ground squirrel, which is strictly diurnal, has approximately 97% cones (Kryger *et al.*, 1998) whereas hamsters, which are nocturnal, have less than 5% cones (Calderone and Jacobs, 1999). It was thus hypothesised that based on its retinal composition, *R. pumilio* would express mainly a diurnal activity pattern. Several tests were used to examine this hypothesis.

Objective 1: In order to determine their natural activity pattern, *R. pumilio* were exposed to a 12:12 light-dark cycle and the percentage activity during the light phase and the dark phase was calculated (Refer to Chapter 2). In addition animals were exposed to a natural light-dark cycle including twilight. Previous studies have shown that differences exist between the activity patterns of animals on a square LD cycle and those on a twilight cycle. These differences include an increase in the active period of animals on a twilight cycle, the onset of activity earlier and the offset later, and a greater variation in the activity onset of animals in a square LD cycle (Boulos *et al.*, 1996a,c,b; Tang *et al.*, 1999).

Objective 2: *R. pumilio* were exposed to conditions of constant light or constant dark following entrainment to an LD cycle (Refer to Chapter 2). This was used to determine if the animal expresses a higher percentage of activity during subjective day or subjective night in free-running conditions. Tau was calculated because Aschoff's Rule predicts that in diurnal mammals tau is less in constant light than in constant dark and vice versa for nocturnal mammals (Aschoff, 1979). In addition, it was hypothesised that alpha in constant dark would be less than alpha in constant light. The animals were also placed in constant light to determine whether splitting as observed in other mammals, occurs.

Objective 3: The final behavioural test involved exposing *R. pumilio* to an ultradian light-dark masking cycle of 1 hour light and 1 hour dark (refer to Chapter 3). This LD cycle is too short for the endogenous circadian rhythm to adapt to. Previous studies predict that in nocturnal animals, exposure to a light pulse at night would suppress activity and in diurnal animals, exposure to a dark pulse during the day would have the same effect (Gander and Moore-Ede, 1983; Aschoff and von Goetz, 1988, 1989; Mrosovsky *et al.*, 1999; Redlin and Mrosovsky, 1999a). It was thus hypothesised that if *R. pumilio* was diurnal it would show a suppression of activity during the dark phase and activation of activity during the light phase and vice versa if *R. pumilio* was nocturnal. This masking paradigm has not been studied in a diurnal murid rodent, so if *R. pumilio* were diurnal it would constitute the first diurnal murid rodent model.

Objective 4: Photic induction of Fos in the SCN was investigated to correlate the expression of Fos with the activity pattern of *R. pumilio* (Refer to Chapter 4). There have been inconsistencies in the Fos data for diurnal mammals with respect to Fos expression during subjective day. For example, *Octodon degus* shows a decrease in Fos expression at CT4 and the diurnal chipmunk shows an increase in Fos expression at CT2 – CT10. *Arvicanthis*, which is also a diurnal mammal, shows no Fos expression during subjective day, but shows the same pattern of expression as nocturnal mammals. All mammals studied thus far, show an increase in Fos expression during subjective night in response to a light pulse (Rea, 1989; Aronin *et al.*, 1990; Colwell and Foster, 1992; Abe *et al.*, 1995; Krajnak *et al.*, 1997; Katona *et al.*, 1998). It was therefore predicted that photic induction of Fos would occur during subjective night.

Objective 5: During the course of the study, the problem of defining a species as strictly diurnal or

nocturnal rapidly emerged. This was due to the observation that the activity of *R. pumilio* occurred mainly during the daytime period but partly during the dark period. In addition, there were inter-individual differences in the activity patterns. A final objective was thus to develop a model based on several parameters, to define the degree of nocturnal-diurnal behaviour (Refer to Chapter 5).

1.7 *Rhabdomys pumilio*

The four-striped field mouse, *R. pumilio* (Figure 1.11), is distributed discontinuously from southern Africa (South Africa, Angola, Botswana, Zimbabwe and Malawi) to east Africa (Kenya, Tanzania and Uganda) (Brooks, 1974; David, 1980; Johnson, 1980). They live above ground in heavily vegetated areas and their nest sites are characterised by runways which lead from one bush to another. *R. pumilio* has a highly structured social system based upon a dominance hierarchy (Johnson, 1980). They are grey-brown in colour, with four distinctive black stripes on their back, are approximately 10 – 14 cm in length and weigh 40 – 70 g. Small mammals can be aged using body mass, pelage characteristics, body and tail length. These parameters can be used individually or in combination with each other. Body mass was used to age *R. pumilio* according to the table constructed by Brooks (1974) and only animals 10 weeks and older were used. The breeding season is from September to April and the gestation period ranges from 23 days to 25 days (Brooks, 1974). Reliable indicators of reproductive activity in females and males, respectively, are a perforated vagina and descended testes, (Brooks, 1974).

There have been conflicting reports about the activity patterns of *R. pumilio* and even though most authors consider them diurnal (Brooks, 1974; Marais, 1974; Johnson, 1980), it is based on observational and not quantitative data.

1.7.1 Trapping and Maintenance

The mice used in the behavioural experiments, were live-trapped with Sherman traps, in the Cape Flats Nature Reserve, Bellville, Cape Town (33°55′S, 18°22′E), South Africa during May 2000. The traps were baited with oatmeal and peanut butter rolled into balls. The traps were checked three times daily and on very hot days, four times daily to prevent heat stress in the traps. The animals (23 males) were kept in a temperature-controlled room (±25°C) and were maintained on hamster feed (consisting of various seeds) supplemented with fresh fruit and vegetables before being transported to

Figure 1.11: *R. pumilio* characterised by the four black stripes on its back.

the Department of Zoology, University of Pretoria, where the experiments were to be performed.

At the University of Pretoria, 12 male *R. pumilio* were used for the behavioural experiments. Only male *R. pumilio* were used because of the possibility that the oestrus cycle may cause the females to have more variable behaviour. The animals were individually housed in plastic cages (60×45×35 cm) equipped with running wheels, nests, and infra-red captors in a light-controlled room. The nests were placed so that any movement inside the nest would not be registered by the captors. The captors were placed at the level of the animal and were connected to a minimitter system which recorded activity every minute. Mesh wire was used to prevent the animal from chewing the wires though on several occasions they were able to get behind the mesh. The running wheels were placed directly in-line with the captors since wheel activity is measured as an overt output of the circadian clock (Kornhauser *et al.*, 1996). The animals were fed parrot feed, which is similar to hamster feed, supplemented with fruit and vegetables. Feeding occurred three times weekly at varying times, to prevent the animals from entraining to their feeding times.

Animals used in the experiment to determine photic induction of Fos in the SCN, were trapped during August 2000 in the same manner as above. Forty-eight *R. pumilio* (males and females) were transported to the University of Pretoria, where the experiments were performed. The animals were housed in pairs, a male and a female, in mouse cages and were placed in a light-controlled room in a 12:12 light-dark cycle.

Chapter 2

Circadian Activity Patterns in *R. pumilio*

2.1 Introduction

Almost all species are adapted to change their behaviour on a daily or 24-hr basis, with the main zeitgeber (environmental cue) being light. The circadian pacemaker can be entrained either by 'discrete' changes in light intensity (non-parametric entrainment) where one or two light pulses (one at dawn and one at dusk) reset the period of the pacemaker (τ) to that of the external environment by advancing or delaying its phase, or by continuous light input (parametric entrainment) (Pittendrigh and Daan, 1976a). Although Daan (2000) prefers the parametric entrainment model, a model supported by results found for the European Ground Squirrel, a diurnal burrow-dwelling mammal that does not use dawn or dusk for entrainment (Hut *et al.*, 1999b), the most commonly used model is that of non-parametric entrainment. Most studies of entrainment use abrupt light-dark (LD) transitions (square LD cycles) even though in nature, illumination changes gradually throughout the day. Wirz-Justice *et al.* (1998) predicted that the neurons innervating the suprachiasmatic nucleus from the eye, are specialised for light intensities occurring around dawn and dusk. Differences have been observed in activity rhythms between a square LD cycle and a twilight cycle as twilight transitions may strengthen the LD zeitgeber, i.e. the upper limit of entrainment is raised (Boulos *et al.*, 1996b; Tang *et al.*, 1999) and at the cellular level, it has been shown that *c-fos* expression peaks twice when the animal is on a twilight cycle (Cooper *et al.*, 1998).

Animals can be characterised as nocturnal, diurnal or crepuscular based on the proportion of their activity occurring during the night, day or twilight times, respectively. The mammalian circadian system has been extensively investigated in nocturnal mammals, though it is not known whether they provide an adequate model for human circadian disorders. The neural basis at which nocturnal and

diurnal mammals are differentiated is unknown and the distinction between nocturnality and diurnality is not always fixed or clearly determined. A switch in activity patterns from diurnal to nocturnal can occur in certain mammals when exposed to running wheels, as occurs in *Octodon degus* (Lee and Labyak, 1997) and *Arvicanthis niloticus* (Blanchong *et al.*, 1999), or from diurnal to nocturnal due to interspecific competition, as is the case with the Golden Spiny mouse (Zisapel *et al.*, 1999).

When an animal is entrained, its system adopts a specific phase relationship with the zeitgeber. To experimentally determine entrainment the biological rhythm should be recorded with and without a zeitgeber. When placed in constant conditions, i.e. when the external cues are removed, the biological clock persists with a free-running period, τ, close to 24 hours. Generally, τ in constant dark (DD) differs from that in constant light (LL) and it is hypothesised that these changes are opposite in diurnal and nocturnal mammals (Aschoff, 1979). This hypothesis is termed Aschoff's rule where τ during constant light is less than τ during constant dark in diurnal mammals and vice versa in nocturnal mammals. This rule was based on a limited amount of knowledge, and was refined by Daan and Pittendrigh (1976). The new theory states that τ_{LL} lengthens in species with $\tau_{DD} < 24$ hrs and shortens in species with $\tau_{DD} > 24$ hrs, irrespective of whether the animal is diurnal or nocturnal.

Rhabdomys pumilio is a murid rodent that has been hypothesised to be diurnal based upon observational data (Brooks, 1974; Marais, 1974; Johnson, 1980). In this study, the activity patterns for *R. pumilio* were quantitatively determined as well as the effects of different light schedules on the activity. Half the number of animals used in the experiment were placed under natural conditions to determine the effects of twilight on the activity pattern. At the start of the experiment, the animals were kept without running wheels, since in certain mammals, there is a change in the activity when exposed to running wheels. Tau during constant dark and constant light was calculated to determine the free-running period of *R. pumilio* and to determine how it changed between the two conditions as there are conflicting predictions by Aschoff (1979) and Daan and Pittendrigh (1976).

2.2 Materials and Method

2.2.1 Experimental Procedures

Twelve wild caught male *R. pumilio* were placed under the light schedules as shown in Table 2.1. For the first 12:12 LD cycle (with wheels, LD1), six of the males were placed outside (in natural conditions) in individual cages, and six were placed in a light-controlled room supplied with fluorescent tubes emitting light levels of approximately 500 lux. This was to determine the effects of twilight on

1. Phase-angle difference between the onset of activity and the onset of the light phase during the 12:12 LD cycles.

2. Percentage diurnality in the 12:12 LD cycles (Appendix B.1). Activity was added up for the 12 hours of light and 12 hours of dark. Percentage activity during the light phase was equal to the activity during the 12 hours of light divided by the total activity (i.e. activity during 12 hours of light added to activity during 12 hours of dark) multiplied by 100. If the animal was active 70% during the light phase, then that means that the animal was only active 30% during the dark phase and therefore the animal is diurnal.

3. Percentage diurnality during the subjective day of DD and LL (Appendix B.2). The start of subjective day was regarded as equivalent to activity onset and the end of subjective day (CT12) corresponded to tau divided by two. Activity was added hourly for subjective day and subjective night. Percentage activity during subjective day was equal to the ratio of activity during subjective day and total activity.

4. Duration of the active period (α) during the 12:12 LD cycle, constant dark, and constant light. The active period started with the activity onset and ended with activity offset.

5. Period, τ, of the endogenous clock during the LL and DD cycles.

Statistical Analysis

The active period during constant dark, LD2, and constant light were compared using a one factor repeat measures ANOVA. A t-test was performed to test the difference in activity onset and offset between animals in a square light-dark cycle and animals in a twilight cycle.

2.3 Results

Figure 2.1 was a typical double-plotted actogram for *R. pumilio* during LD1, followed by constant dark, LD2 and constant light. It can clearly be seen that *R. pumilio* were able to entrain to a light-dark cycle.

The start of activity or activity onset was clear and occurred approximately at the same time each day, but the end of activity or activity offset, was less precise. Most animals showed bimodal activity with a rest period in the middle of the day (Figure 2.2). This corresponded to personal observations

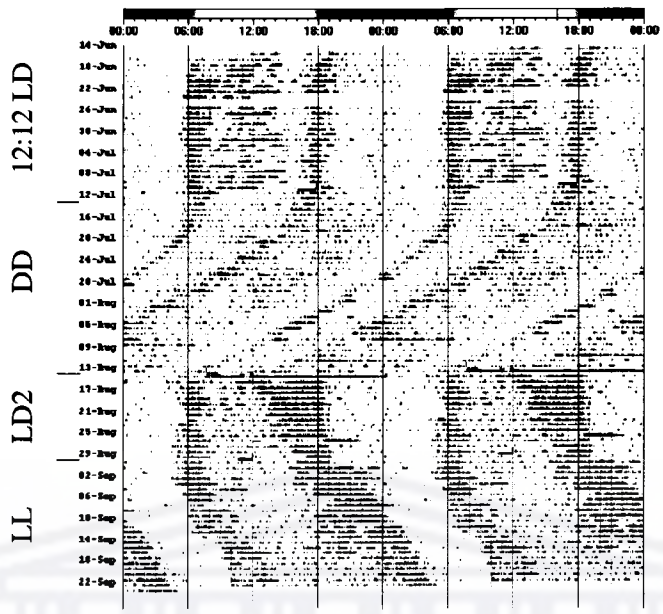

Figure 2.1: Typical actogram for *R. pumilio* (animal no. 11) showing activity during a 12:12 LD cycle (LD1) followed by continuous darkness (DD), a second 12:12 LD (LD2) cycle and constant light (LL). *R. pumilio* were able to entrain immediately to a light-dark cycle after free-running.

made while trapping *R. pumilio* and to observations made by Brooks (1974); Marais (1974) and, Johnson (1980).

The animals were approximately two weeks without a running wheel. Qualitative analysis showed no difference in the activity pattern in the presence or absence of a running wheel. The only effect seen, was in animal no. 1, where the activity appeared to become more concentrated after a running wheel was introduced.

The activity of all the animals was analysed during LD2 and LD3 because the same conditions applied (Figure 2.3). Most of the animals displayed a higher percentage of activity during the light phase than during the dark phase. However, two animals showed a higher percentage of activity during the dark phase of the 12:12 LD cycles. This was evident qualitatively as well (Figure 2.4). It should be noted that only one animal showed 'nocturnal' behaviour in more than one 12:12 LD cycle.

The length of the active period, α, changed from one 12:12 LD cycle to another, though no pattern in the individual changes were discernible (Figure 2.5). There were more variations in α during LD2 (14.06 ± 0.51 hours) which followed DD, than during LD3 (13.41 ± 0.22 hours) which occurred after the animals were in LL. Results were calculated as mean $\pm$ standard error. The changes in α appeared to be due to variability in the activity offset rather than the activity onsets (Figure 2.6). Furthermore,

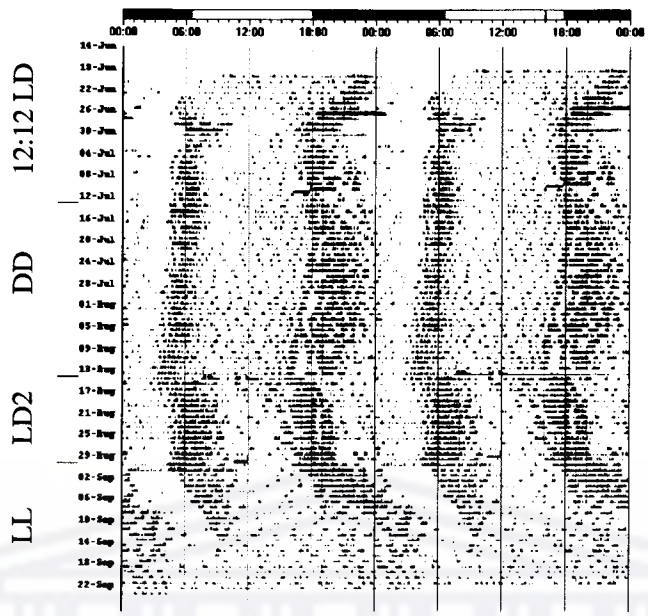

Figure 2.4: Animal no. 7 showed more activity during the dark phase and subjective night than during the light phase and subjective day of a 12:12 LD cycle and DD, respectively.

the variations in α during LD2 could possibly be related to the variation in τ during DD (Figure 2.7).

R. pumilio showed distinct free-running periods in constant conditions. There were large inter-individual variations in τ_{DD} (Figure 2.7). τ_{LL} generally increased from that in DD. Animals with $\tau_{DD} < 24$ hours showed an increase in tau during LL, but animals with $\tau_{DD} > 24$ hours maintained their period or showed a slight decrease in tau during LL. τ_{LL} was more than 24 hours for all the animals. Two animals showed an ultradian rhythmicity 10 – 14 days after they were placed in LL (Figure 2.8).

Alpha also changed significantly when the lighting conditions changed from DD to 12:12 LD to LL (Figure 2.9). There was a significant difference between the length of the activity periods during DD and LD2 ($p = 0.01$), LD2 and LL ($p < 0.01$), and DD and LL ($p < 0.01$).

As stated in the methods, the activity onset of the animal, was regarded as the beginning of subjective day, CT0 (refer to Appendix B.2). More animals showed a higher level of activity during subjective day (87.5%) when in DD than when in LL (70%, Figure 2.10). Once again, it is worth noting that only animal no. 7 showed 'nocturnal' behaviour in both DD and LL (Figure 2.4 and Figure 2.10). It is interesting that the rest period observed during the 12:12 LD cycle (see Figure 2.1, was maintained during constant conditions (Figure 2.11). No splitting of activity was seen in *R. pumilio*.

In LD1, half the animals were placed in natural conditions whereas the other half were placed in

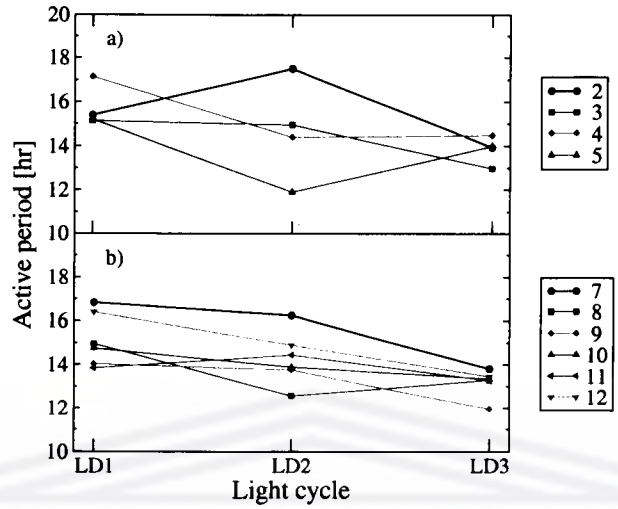

Figure 2.5: Mean activity periods for individual *R. pumilio* in three different light-dark cycles. (a) Individuals exposed in LD1 to a square light cycle and (b) individuals exposed to a natural light cycle in LD1. Alpha changed from LD2 to LD3, though there was no pattern in the changes.

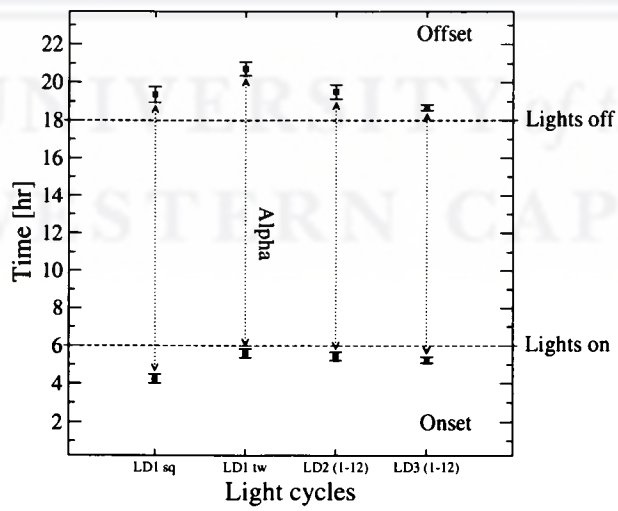

Figure 2.6: Average (mean ± SD) time of activity onsets and offsets for LD1, LD2 and LD3. LD1sq and LD1tw = average time of activity onset and offset for the animals on a square light cycle and twilight cycle, respectively. LD2(1–12) and LD3(1–12) = avg. time of activity onset and offset for all the animals during LD2 and LD3, respectively.

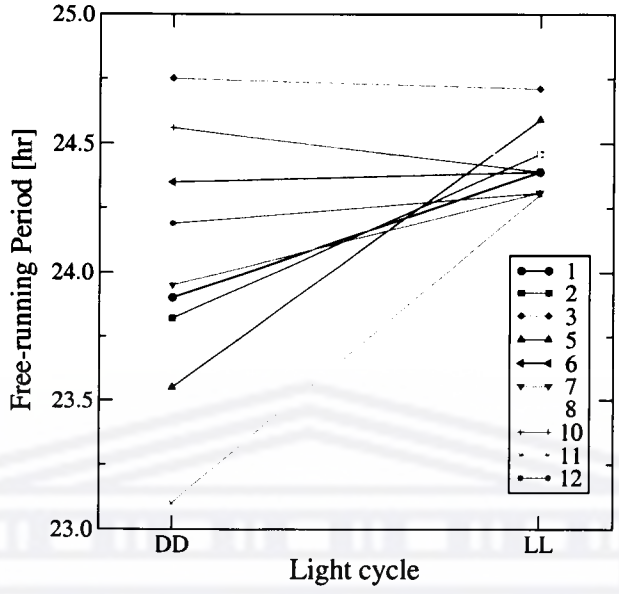

Figure 2.7: Free-running rhythms (τ) during constant dark (DD) and constant light (LL). In LL, $\tau > 24$ hours with less inter-individual variation than in DD.

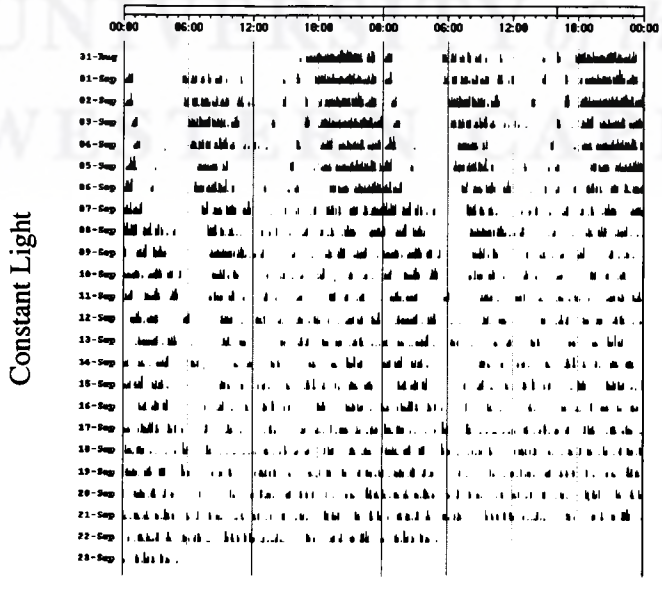

Figure 2.8: After ten days in constant light, animal no. 7 started showing an ultradian activity rhythm.

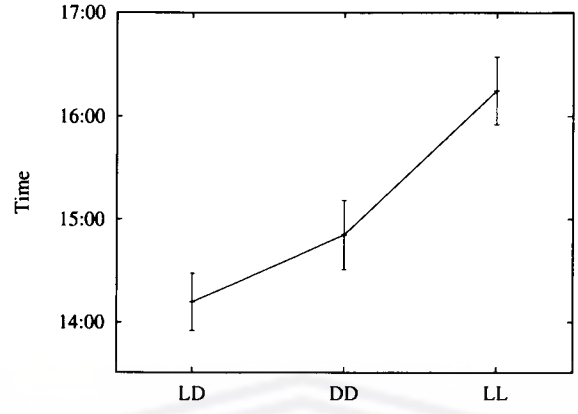

Figure 2.9: Alpha (mean ± Standard error) calculated as the difference between activity onset and activity offset, changed significantly between the different lighting conditions.

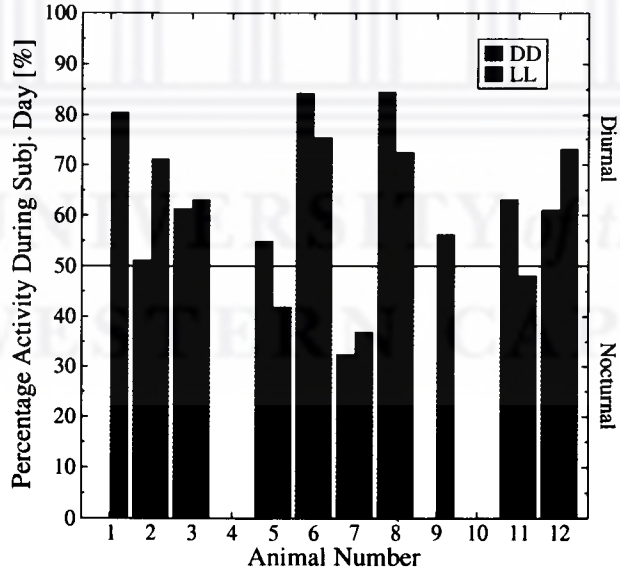

Figure 2.10: Percentage activity during subjective day was calculated as a percentage of the total activity. Only one animal showed activity lower than 50% during subj. day for both DD and LL. This animal was thus nocturnal, being more active during subjective night than during subjective day. Of the 8 animals that have complete data sets, two animals showed contradictory behaviour, being nocturnal in one condition and diurnal in another.

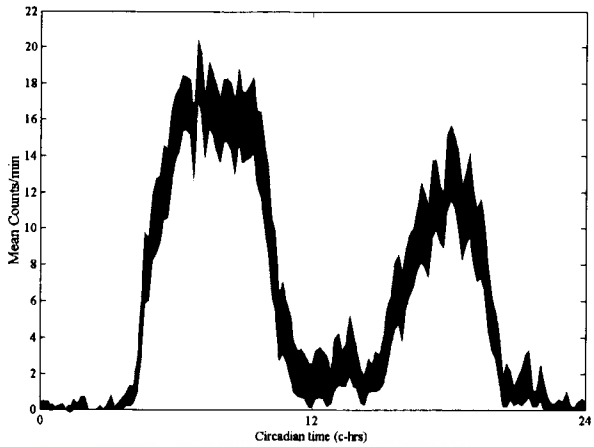

Figure 2.11: Activity profile, calculated using Clocklab, for animal no. 6 during constant light. The rest period during the middle of the day was maintained.

an artificial light cycle. Three twilights are distinguished, and comparing the percentage diurnality for the different twilight conditions showed that choosing the wrong twilight or time of light perception for the calculation, could alter the calculated activity pattern of the animal. On calculating the phase-angle difference for LD2 and LD3, for the animals exposed to natural conditions in LD1, it was found that the animals started on average 0.69 hours before lights on (Figure 2.12) i.e. they had a positive ψ. This calculated phase angle difference was extrapolated to determine a possible time for light perception by the animals in natural conditions by using

$$\text{Time light perceived} = \text{Avg activity onset} + \text{Avg Phase Angle Difference} .$$

and was found to be at nautical twilight (Table 2.2), when the centre of the sun is geometrically 12 degrees below the horizon (Figure 1.10).

There was no difference between the lengths of the average activity period of animals in a square light cycle (avg = 15.10 $\pm$ 0.50 hours) and those in a twilight light cycle (avg = 15.12 $\pm$ 0.50 hours; Figure 2.5) though there was a significant difference in the time of onset and offset (Table 2.3). This is not unexpected since a square light cycle is not equivalent to a twilight light cycle. There was also significant variation of the time of onset ($p < 0.01$) and offset ($p = 0.01$) within the square light-dark cycle group but not within the twilight group.

Referring to Figures 2.13 and 2.14, qualitatively there appeared to be no difference between the animals on a square light cycle and those on a twilight cycle.

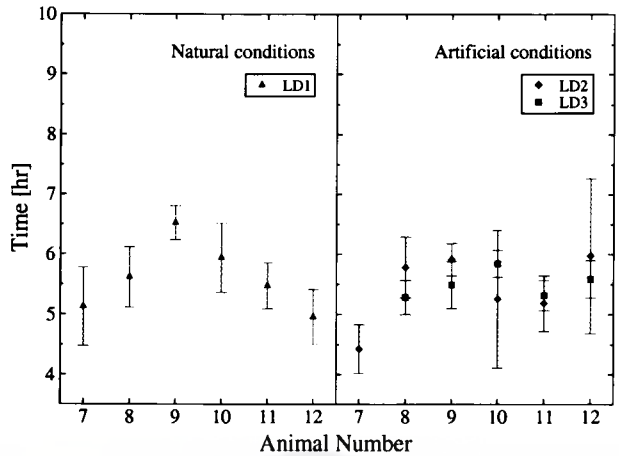

Figure 2.12: Average onsets for animals no. 7–12 in natural conditions (LD1) and in artificial conditions (LD2 and LD3). The average phase angle difference for animals no. 7–12 during LD2 and LD3 was used to determine the time of light perception in LD1. Results were calculated as mean ± SD.

Table 2.2: Times of the different twilights, sunrise and sunset. There was approximately a four minute change in the starting and ending times of the different twilight times, during the period the animals were in LD1.

Position of sun	Begin	End
Astronomical Twilight	5h33	18h50
Nautical Twilight	6h01	18h22
Civil Twilight	6h29	17h54
Sunrise	6h54	
Sunset	17h29	

Table 2.3: Animals in a square LD cycle started and ended activity nearly 1.5 hours earlier than animals in a twilight cycle.

Animal No.	Light Cycle	Onset	Offset
1–6	Square LD cycle	4.22 ± 0.24	19.39 ± 0.39
7–12	Twilight cycle	5.59 ± 0.24	20.70 ± 0.36
		$p < 0.01$	$p < 0.01$

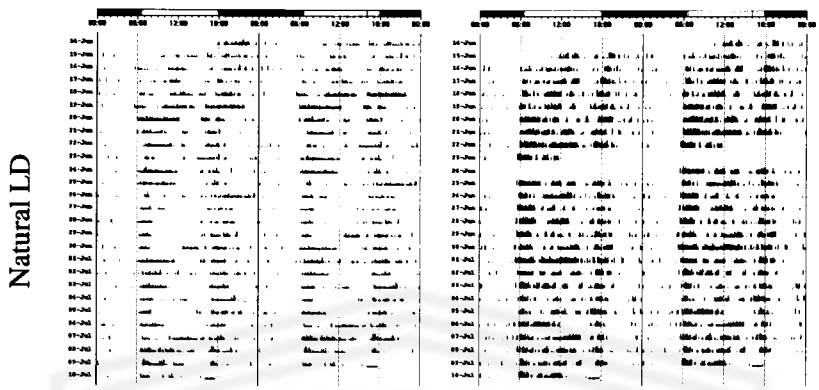

Figure 2.13: Examples of actograms of animals, animal no. 9 (left) and animal no. 11 (right), exposed to the twilight cycle during LD1.

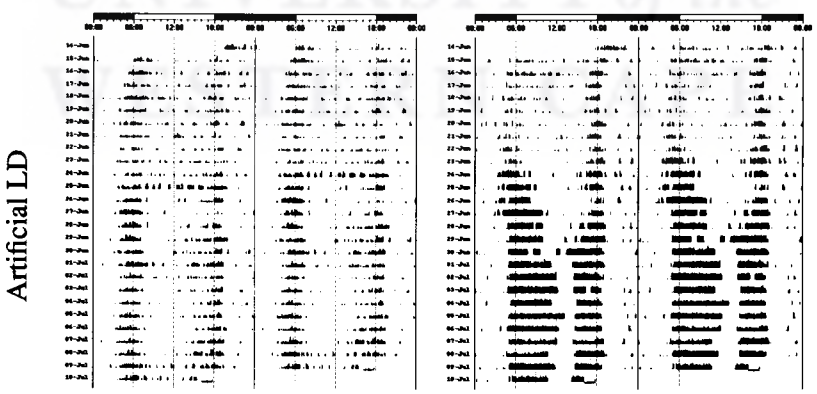

Figure 2.14: Actograms depicting animals, animal no. 2 (left) and animal no. 6 (right), exposed to the square light-dark cycle in LD1.

2.4 Discussion

In rodents, rhythms of running wheel activity are measured as an overt output of the circadian clock (Kornhauser *et al.*, 1996). Although the site of differentiation between nocturnality and diurnality is unknown, animals are characterised as being nocturnal, diurnal or crepuscular based on the percentage of their activity that occurs during the night, day or twilight periods, respectively. This is the first study to determine light-dark entrainment in *R. pumilio*. *R. pumilio* showed stable entrainment which suggested that the light-dark cycle is a potent zeitgeber for this animal as in most other mammals (Rajaratnam and Redman, 1999). Almost all individuals were diurnal in all light cycles even during constant conditions, where *R. pumilio* showed a higher percentage of activity during the subjective day than during the subjective night. The calculation of percentage diurnality during constant conditions had not been performed before, but it could be a good indicator of the animal's activity pattern because it is a reflection of the endogenous clock.

The average percentage diurnality of *R. pumilio* was 68% in a light-dark cycle, compared to the Indian Palm Squirrel, *Funambulus pennanti*, which has an average percentage diurnality of 88% (Rajaratnam and Redman, 1999) and *Arvicanthis* which has an average percentage diurnality of 60% (Katona and Smale, 1997). The similarity in diurnality between *R. pumilio* and *Arvicanthis* could be attributed to both species having a bimodal activity rhythm with a bout of activity in the morning and in the evening with a rest period in the middle of the day. The Indian Palm Squirrel, on the other hand, has a continuous activity rhythm during the day. As with *Octodon degus* the rest period is attributed to the animal's intolerance of high temperatures, but since the same behaviour was apparent in the laboratory and under constant conditions, it is more feasible that the bimodality is an intrinsic feature (Garcia-Allegue *et al.*, 1999).

There were inter-individual differences in α between the three light-dark cycles. There was no pattern in the changes but the variations in α after DD could be related to the variations in τ during DD. Alpha changed as the light cycles changed, with a significant difference in alpha between LD2, DD and LL which corresponded to observations made on *Arvicanthis* (Katona and Smale, 1997). This agreed with predictions made by Aschoff (1979) which states that alpha increases with increasing light intensity.

All the animals showed distinct free-running periods. There were large inter-individual variations in tau during DD, which agrees with results found by Pohl (1982) for diurnal mammals. The average τ_{DD} for *R. pumilio* was 24.06 hours and for τ_{LL} was 24.43 hours. Although average τ_{DD} was close to 24 hours, the variability in tau was large, which does not agree with the findings of Pittendrigh and

Daan (1976b) who state that the variability in tau is smaller in species with average tau closer to 24 hours. In *R. pumilio* τ_{LL} increased when $\tau_{DD} < 24$ hours which disagrees with Aschoff's rule but conforms well to the prediction that if $\tau_{DD} < 24$ hours then it will lengthen in constant light, regardless of whether the animal is diurnal or nocturnal, as is suggested by Daan and Pittendrigh (1976). The prediction which further states that if $\tau_{DD} > 24$ hours it shortens in constant light can possibly be refined because in *R. pumilio* when $\tau_{DD} > 24$ hours it either remained the same or decreased slightly. The fact that tau increased or decreased in LL was not an after-effect of the transient motion that brought the animals into this steady state. Transients are cycles of rapidly changing duration intervening between two steady-states (Pittendrigh and Daan, 1976b). Experiments by Pittendrigh and Daan (1976b) show that hamsters who phase-delay when placed in a 12:12 LD cycle from a DD cycle, also show an after-effect of the sign of the phase shifts in the second free-run i.e the animal would show an increase in tau in the second free-run. This was not apparent for *R. pumilio*. There were less variations in τ_{LL} than in τ_{DD}. Although splitting did not occur during LL, the fact that *R. pumilio* had a bimodal activity pattern is evidence of the presence of a dual oscillator. The M-oscillator was strongly linked to dawn, as the onsets of activity were fairly constant, whereas the E-oscillator was not as strongly linked to dusk, since the offsets were imprecise.

During the first light-dark cycle, half the animals were placed in natural conditions, and half were placed in artificial conditions. Since there are three types of twilight, it was difficult to determine the exact time 'lights on' was perceived since the animals were not equipped with light-sensitive radio collar transmitters as used by Hut *et al.* (1999b) on the European ground squirrel. Civil twilight is used to calculate circadian parameters when the animals are in a twilight cycle because the largest transition between light and dark occurs during this period (Hut, 2001; Hut *et al.*, 1999b), but comparing the percentage diurnality for the three twilights showed different activity patterns in two of the five *R. pumilio*. Pittendrigh and Daan (1976a) state that the choice of twilight is arbitrary because there is no way of knowing when the animal 'perceives' light or the beginning of day. This issue will be discussed further in Chapter 5. Unlike humans or any rodents, *R. pumilio* have 50% cones and 50% rods (A. Lukats, pers. communication). Cones are adapted for phototopic vision and rods are adapted for scotopic vision. Since *R. pumilio* had an abundance of both photoreceptors, they would be able to see well in both dim and bright light. The European ground squirrel, which does not use dawn or dusk for entrainment, has a high percentage of cones.

There was no difference between α for *R. pumilio* on a square LD cycle and those on a twilight cycle. This did not agree with results found by Boulos *et al.* (1996c) or Tang *et al.* (1999), which

could be because simulated and not natural twilights are used in their experiments. Boulos *et al.* (1996c) keeps all conditions stable except for the light cycle, whereas *R. pumilio* were exposed to many other conditions such as changes in temperature, humidity and light levels throughout the day. There was a significant difference between the time of onset and offset of animals on a square light-dark cycle compared to those on a twilight cycle. Animals on a square light-dark cycle started their activity earlier and ended their activity earlier compared to the animals on a twilight cycle. In squirrel monkeys and hamsters, the activity onset occurred later and activity offsets earlier during the square light cycle compared to the twilight cycle (Boulos *et al.*, 1996c; Tang *et al.*, 1999).

Chapter 3

Masking

3.1 Introduction

There are two ways in which an animal can respond to a light pulse, either by synchronising its circadian clock to the light stimulus or by an immediate stimulus-response reaction. The former is termed entrainment and the latter masking (Mrosovsky, 1999). Minors and Waterhouse (1989) define masking as "any process that distorts the original output from the internal clock, whether this originates from inside or outside the body". Masking is a phenomenon that has an effect on activity rhythms, but that has not been researched as extensively as entrainment because it was believed that masking obscured clock-controlled responses and because the masking response of the animal is similar to phase-shifting (Mrosovsky *et al.*, 1999). Three types of masking have been recognised:

Type 1: Masking due to a direct effect of the environment,

Type 2: Masking due to a behavioural change of the animal, e.g. when activity makes the temperature rhythm an imperfect representation of the clock,

Type 3: Masking related to physiological or biochemical changes within the body affecting output of the clock (Mrosovsky, 1999).

Masking can enhance activity (positive masking) or suppress activity (negative masking). For example a nocturnal mammal (a mouse) given a light pulse during the night shows negative masking because a decrease in its activity is observed for the duration of the light pulse (Mrosovsky, 1994). In hamsters, the suppression of activity extends longer than the duration of the light pulse, which could be attributed to their heightened sensitivity to light (Redlin and Mrosovsky, 1999a). A diurnal mammal, the squirrel monkey, exposed to a dark pulse during the day shows negative masking because a

decrease in its activity is observed (Gander and Moore-Ede, 1983; Kas and Edgar, 1999a). The extent of the enhancement or suppression is phase-dependent as was shown in canaries (Aschoff and von Goetz, 1989), hamsters (Redlin and Mrosovsky, 1999a) and squirrel monkeys (Gander and Moore-Ede, 1983). In hamsters the suppression of activity is maximal at dusk, the time when activity onset ordinarily occurs (Redlin and Mrosovsky, 1999a) which shows that masking complements the circadian clock. In wild type mice positive masking (an increase in activity) occurs at levels of irradiance corresponding to that of twilight illumination (Mrosovsky *et al.*, 1999). In nature, the owl monkey (*Aotus lemurinus griseimembra*) has maximal activity when the illuminance is between 0.1 and 0.5 lux (the illumination at full moon). Lower or higher light intensities suppress its activity (Erkert and Gröber, 1986). The brain sites involved in masking are not known, though it has been found that the SCN and IGL are not essential for masking to occur (Redlin *et al.*, 1999; Redlin and Mrosovsky, 1999b).

The most common means of studying masking is to expose the animal to a one or two hour light pulse at a certain time and to calculate the difference in activity compared to the same time the previous day (Aschoff and von Goetz, 1988; Mrosovsky, 1994; Redlin *et al.*, 1999). A second method is to place the animal on an ultradian light-dark cycle to distinguish the masking and entraining effects of light (Gander and Moore-Ede, 1983; Redlin and Mrosovsky, 1999b,a). The latter method has been used to examine masking in *R. pumilio*. The animals were exposed to an ultradian light-dark cycle of 1 hour light and 1 hour dark to further clarify whether they are diurnal or nocturnal. This question is of interest since in Chapter 2 it is shown that *R. pumilio* displays many features of a diurnal activity pattern, although some nocturnal activity was observed in certain individuals.

3.2 Materials and Method

Twelve wild-caught *R. pumilio* were housed individually in cages equipped with a standard running wheel of 17 cm diameter. The animals were placed in a room illuminated by fluorescent tubes emitting light levels of approximately 500 Lux. They were first entrained to a 12:12 LD cycle for 12 days before being placed in a 1:1 LD cycle for a further 12 days. The light cycle was controlled by a timer. Water and food were available *ad libitum*. Refer to Chapter 2 for further details on the recording system.

3.2.1 Analysis

The masking effects of dark were analysed for the entire 12 days by adding the activity counts during the dark and light phases, respectively (Redlin and Mrosovsky, 1999b). Results are given as a percentage of total activity. A result above 50% in the light phase shows that the animal was more active during the light phase, which means that compared to the light phase, the dark phase had a suppressing effect on the activity. Refer to Appendix B.3 for the program used for the calculations.

Activity was calculated for the light and dark periods during the first and second half of subjective day. Activity onset was used as the beginning of subjective day. Refer to Appendix B.3 for the program used for the calculations. A t-test for unequal variance was performed to determine the significance of the differences in activity between the first and second half of subjective day, and between the first and second half of the experiment.

Total activity during subjective day and subjective night was also calculated. This calculation was similar to that used in Chapter 2 to calculate activity during DD and LL.

3.3 Results

Figure 3.1, shows a typical actogram for *R. pumilio* on a masking light cycle (1L:1D). The rest period in the middle of the day was retained in most of the animals, though the length of the rest period varied from one animal to the next. All the animals showed a free running period of more than 24 hours which also occurred when the animals were placed in constant light earlier in this study.

Ten of the eleven animals showed suppression of activity by the dark phase (Figure 3.3). Figure 3.4 depicts three examples of the activity during the dark and light phase over the 12 day period. Activity was analysed for the total light periods and dark periods.

Eight of the nine animals, for which there were sufficient data, showed a higher percentage of activity during subjective day than subjective night (Table 3.1).

It appeared qualitatively that certain *R. pumilio* showed stronger masking effects during the first half of the subjective day compared to the last half, but after quantitative analysis of activity in each portion, no statistical difference was found. Certain animals appeared to show a stronger masking effect during the first six days of the experiment compared to the last six days (Table 3.2) but the difference was not statistically significant. Since in hamsters, the suppressing effect of light on the locomotor activity extends longer than the duration of the light pulse (Redlin and Mrosovsky, 1999a), in *R. pumilio* the percentage activity during the light and dark phases was also calculated minus five

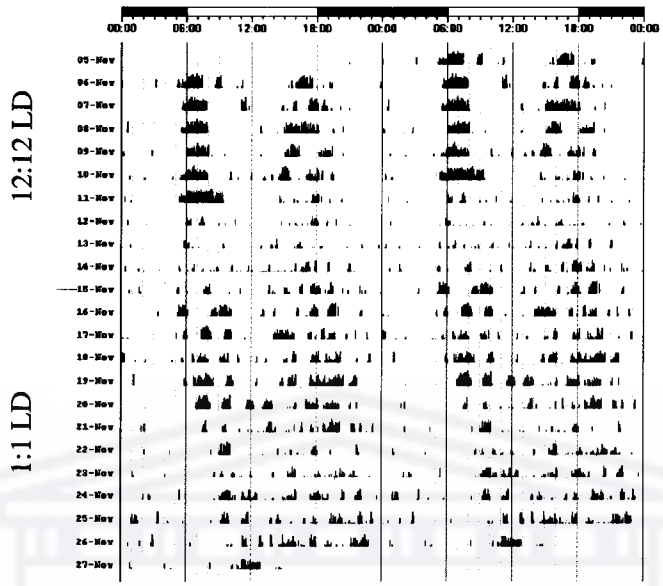

Figure 3.1: Typical actogram for *R. pumilio* (animal no. 12) on a 1:1 LD cycle. The rest period in the middle of the day and the period of inactivity at night were retained.

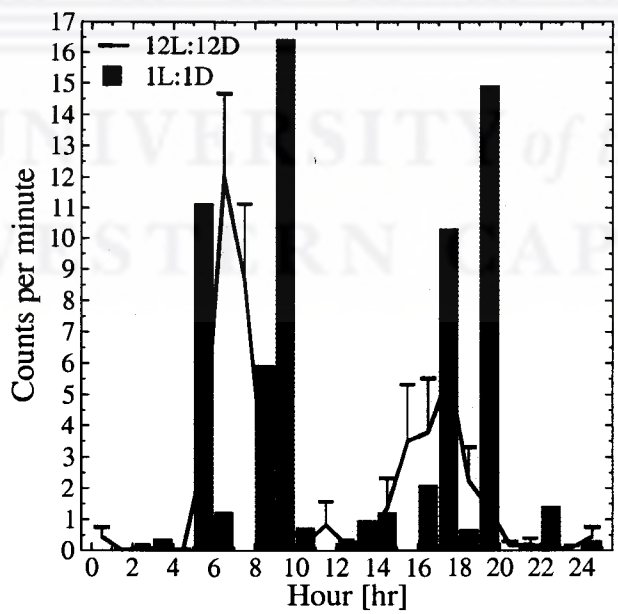

Figure 3.2: Activity profile for animal no. 12 during the first three days of the masking light cycle compared with the activity during the preceding 12:12 LD cycle. The rest period in the middle of the day is maintained, and most of the activity occurs during subjective day.

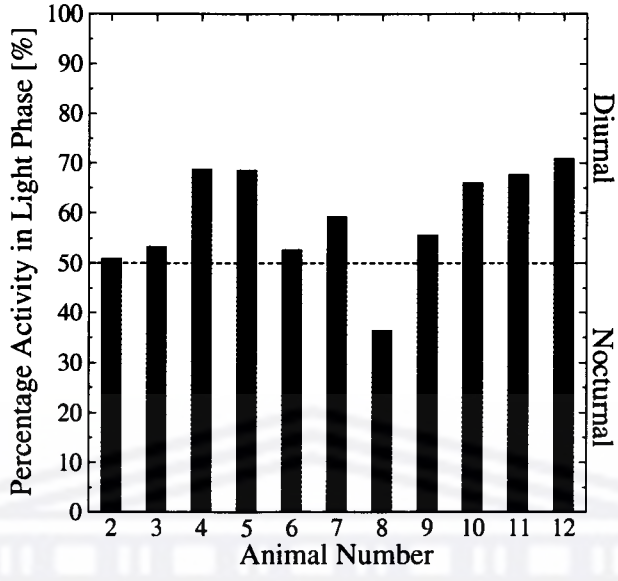

Figure 3.3: Percentage activity during the light phase was calculated as a percentage of the total activity. Activity above 50% in the light phase represented a diurnal animal and activity below 50% represented a nocturnal animal. The values depicted here have been calculated over the full 24 hour period for 12 days. All *R. pumilio*, except one, showed a suppression of activity by the dark phase.

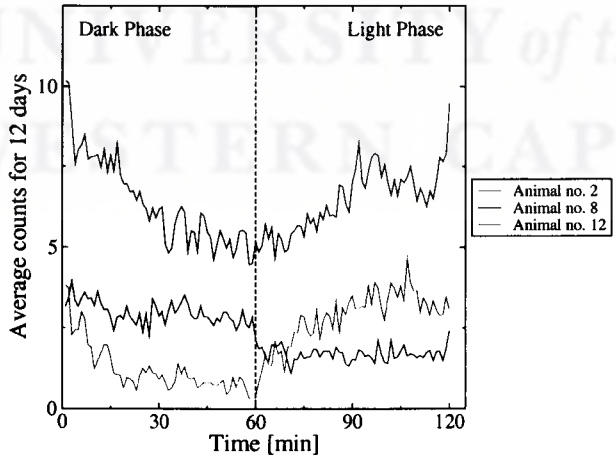

Figure 3.4: Average activity for animals 2, 8 and 12, for the light and dark phases of the masking cycle calculated over 12 days. Referring to Figure 3.3, animal no. 2 had an equal level of activity in the dark and light phases, animal no. 8 showed more activity during the dark phase, and animal no. 12 showed more activity during the light phase.

Table 3.1: Percentage activity during subjective day compared to subjective night. Eight of the nine animals showed a higher percentage activity during subjective day.

Animal no.	Subjective day	Subjective night
2	87.5	12.5
3	90.9	9.1
4	36.9	63.1
5	51.4	48.6
8	92.0	8.0
9	71.0	29.0
10	86.5	13.5
11	52.9	47.1
12	79.9	20.1

minutes at the start and the end of each phase (Table 3.2). Animal no. 7 had no clear onsets, therefore not all data are available.

Animal no. 8, was the only animal that showed suppression of activity by light, i.e. it had a higher percentage of activity during the dark phase of the 1:1 LD cycle. However, it qualitatively (Figure 3.5) and quantitatively (Table 3.1) had the same characteristics as the animals with typical diurnal behaviour. Its activity was mainly during the subjective day with a limited percentage of activity during subjective night and the rest period in the middle of the day was retained. In contrast, animal no. 4 showed a higher percentage of activity during the light phase of the 1:1 LD cycle (Figure 3.6), but instead of mainly being active during subjective day, it showed a higher percentage of activity during subjective night (Figure 3.5 and Table 3.1).

Table 3.2: Percentage activity during the light phase of the masking light cycle. The activity was calculated for different time periods since qualitative analysis of the actograms suggested possible differences. Five minutes were taken off the beginning and end of the phase, only where indicated.

	% Activity During the Light Phase of							
Animal no.	Alpha	CT0 – CT6	CT6 – CT12	CT0 – 24	Day 1 – 6	Day 6 – 12	Day 1– 6[a]	Day 6 – 12[a]
2	53.7	50.0	58.4	51.1	50.7	51.5	50.6	51.5
3	55.4	55.7	56.7	53.5	47.1	60.2	48.3	63.2
4	65.1	64.2	65.6	68.9	65.3	74.6	67.6	76.7
5	68.6	61.3	70.2	68.7	69.9	67.7	73.4	69.7
7				59.4	60.4	58.5	61.5	58.2
8	36.4	37.5	37.1	36.5	35.2	38.2	34.0	38.7
9	58.5	56.1	64.7	55.8	52.0	60.1	49.9	61.8
10	67.9	68.9	65.2	66.2	62.1	72.1	64.3	75.0
11	67.8	68.6	69.0	67.8	69.3	66.7	70.4	68.3
12	71.8	78.0	65.1	71.0	73.2	67.5	75.8	69.3

[a](−5 min)

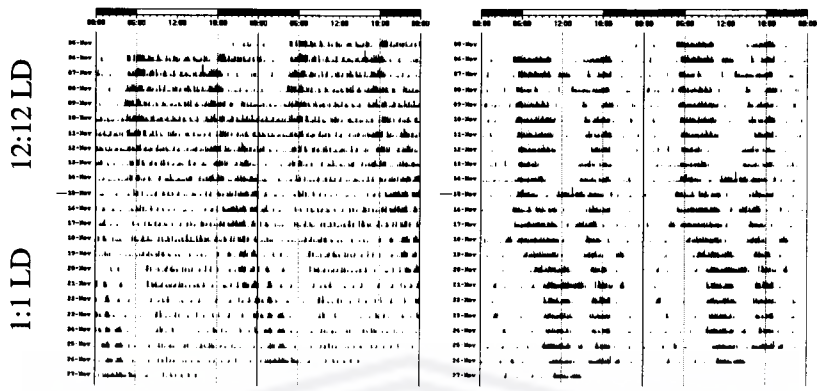

Figure 3.5: Animal no. 4 (left) showed more activity during the light phase of the masking cycle but its activity was not restricted to subjective day. Animal no. 8 (right) showed more activity during the dark phase of the masking light cycle which means that its activity was suppressed by light. Its activity though, was restricted to subjective day.

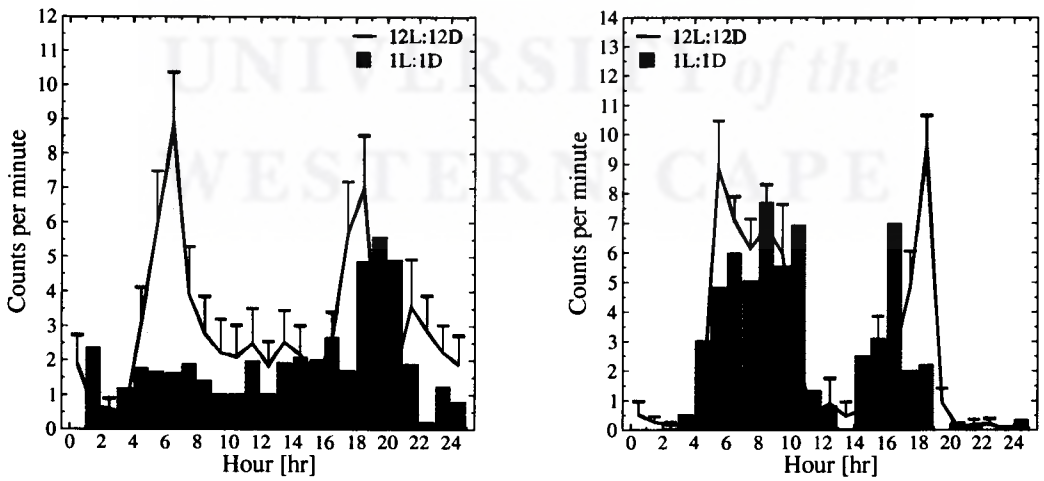

Figure 3.6: Activity profiles for animals no. 4 (left) and 8 (right) during three days of the masking light cycle compared to the activity during the 12:12 LD cycle. Data were plotted in 5 minute bins. The light and dark bars represent the hours of lights on and lights off.

3.4 Discussion

Masking was readily observed in *R. pumilio* and conformed to the hypothesis that in diurnal mammals, activity is suppressed by dark (Gander and Moore-Ede, 1983). The animals retained their rest period in the middle of the day, which has not been reported before in other mammals. All the animals showed a free-running period longer than 24 hours as was observed during the constant light cycle in earlier experiments. This finding may help to elucidate the role of the SCN in masking, since currently there are contradictory results in the lesioning experiments due to accidental damage to the optic chiasm (Stephan and Zucker, 1972; Ibuka *et al.*, 1977; Redlin and Mrosovsky, 1999b).

There are two methods in which masking can be tested, either by exposing the animal to one light pulse and comparing the results to the same time the previous day when the animal was not exposed to a light pulse, or by placing the animal on an ultradian light-dark cycle. Redlin and Mrosovsky (1999a) compared the two methods listing disadvantages and advantages for each. The disadvantage of the ultradian method is that entrainment to the light cycle cannot be completely ruled out. It takes a week for the LD cycle to regain its phase relationship with the circadian cycle so the masking light cycle has to be performed for more than a week. The advantage of the ultradian cycle is that a good representation of masking over successive days is provided. The light pulse on the other hand, is quicker because the animal is able to recover its normal activity rhythm within one or two days. The disadvantage is that the effects of the light pulse may be due to phase shifting and not to masking. In the present study we found another possible disadvantage to using only one light pulse. For example, quantitatively, animal no. 8 was nocturnal, with more activity displayed during the dark phase of the 1:1 LD cycle than during the light phase. However, qualitatively the same activity pattern was shown as the other animals, with the activity period restricted to subjective day, the maintenance of the rest period during the middle of the day and an inactive period during subjective night. Animal no. 4 showed behaviour contrary to that shown by animal no. 8. Although animal no. 4 showed a higher percentage of activity during the light phase, the activity was not entirely restricted to subjective day. This paradoxical behaviour would not have been evident if the animal had only been exposed to one light pulse. In addition, all the animals showed a free-running rhythm with $\tau > 24$ hours, similar to that in constant light which would also not have been evident with only one light pulse.

Four animals (No.'s 3, 4, 9, 10) appeared to show stronger masking effects during the first six days of the experiment compared to the last six days but the difference was not statistically significant.

Overall there was no difference between masking during the first half of subjective day and the second half. Masking is phase-dependent in canaries (Aschoff and von Goetz, 1989), hamsters (Redlin

and Mrosovsky, 1999a) and squirrel monkeys (Gander and Moore-Ede, 1983). It is possible that the time period used in this experiment was too long, therefore, any phase-dependence within subjective day may have been obscured. There was phase dependence in that *R. pumilio* showed higher activity during subjective day than subjective night. In *Octodon degus*, masking appears to have no phase dependence, since activity occurs throughout subjective day and subjective night (Kas and Edgar, 1999b). The pattern of activity in *R. pumilio* during a masking light cycle was completely different from *Octodon* even though both are diurnal mammals.

Unlike hamsters (Redlin and Mrosovsky, 1999a), *R. pumilio* showed no latent suppression of activity during the light phase. This was similar to results found in mice (Mrosovsky, 1994). According to Elliott and Tamarkin (1994), there is a close relationship between melatonin expression and locomotor activity. It would, therefore, be interesting to see whether melatonin suppression due to a light pulse extends beyond the light pulse in *R. pumilio*.

Chapter 4

Photic Induction of Fos in the SCN

4.1 Introduction

The suprachiasmatic nucleus, the site of the primary circadian pacemaker in mammals (Rusak and Zucker, 1979; Moore, 1983; Sato and Kawamura, 1984a; Decoursey *et al.*, 1997), is situated in the anterior hypothalamus dorsal to the optic chiasm. The protein Fos, a product of the *c-fos* gene, is rapidly and transiently induced by light in the SCN. Fos forms a heterodimeric transcription complex with members of the *jun* family which alters gene expression by regulating transcription (Sheng and Greenberg, 1990). The exact mechanism by which gene expression is altered is unknown. It has been suggested that *c-fos* and *mPer1* may be responsible for the photic phase shifting of the clock (Shigeyoshi *et al.*, 1997) or that *c-fos* may instead be involved in the induction of *mPer1* (Best *et al.*, 1999).

The amplitude of Fos induction in the suprachiasmatic nucleus is proportional to the total number of photons in the light stimulus (Dkhissi-Benyahya *et al.*, 2000). Fos induction is phase-dependent and this phase-dependence is similar to that for light-induced phase shifts of locomotor activity (Rea, 1989; Aronin *et al.*, 1990; Earnest *et al.*, 1990; Rusak *et al.*, 1990; Colwell and Foster, 1992; Cooper *et al.*, 1998). For example, in nocturnal mammals, a light pulse given when the animal is in constant dark increases Fos expression in the SCN only during the period of subjective night when light will also induce a phase shift. Further evidence of the link between behavioural phase shifts and photic induction of Fos is shown by the fact that both responses can be blocked by pharmacological agents (Vindlacheruvu *et al.*, 1992; Colwell *et al.*, 1993).

The relationship between Fos expression and behavioural phase shifts, and the pattern of photic induction of Fos is not as clear in diurnal mammals. Honma and Honma (1999) and Abe *et al.* (1995)

found that photic induction of Fos in the diurnal chipmunk, *Eutamias asiaticus* does not correspond with behavioural phase shifts since Fos is expressed during both subjective day and subjective night and the behavioural phase shifts only occur during subjective night. In *Octodon degus* there is a decrease in Fos expression at CT4, when the animal shows a phase shift (Krajnak *et al.*, 1997; Lee and Labyak, 1997). In contrast, in diurnal *Arvicanthis niloticus*, the increase in expression of Fos (during subjective night) corresponds to the times when light also elicits a phase shift (Mahoney *et al.*, 2001). In the present experiment photic induction of Fos expression in the SCN of *R. pumilio*, which I have shown to be diurnal in behaviour, was investigated to determine the pattern of expression.

In addition, since it is known that the photic induction of Fos is mainly in the ventrolateral part of the SCN which receives retinal afferents (Aronin *et al.*, 1990; Earnest *et al.*, 1990; Sumová *et al.*, 1998), three *R. pumilio* were injected with cholera toxin subunit B (CTb) to determine the pattern of retinal innervation to the SCN and other visual structures.

4.2 Materials and Method

4.2.1 Experiment 1: Fos Expression

Forty-eight *R. pumilio* were entrained to a 12:12 LD cycle for three weeks in a room illuminated with fluorescent lights of approximately 500 lux. On the day of the experiment, the lights remained off so that ZT0 (Zeitgeber Time 0) was used as the beginning of subjective day, CT0 (Cooper *et al.*, 1998). Five animals were pulsed with monochromatic green light (10^{14} photons/cm^2/sec) for 15 minutes at the following circadian times: 2, 6, 10, 14, 18, 22 (Figure 4.1). The animals were perfused 60 minutes after the end of the light pulse. Three mice per time period were used for the dark controls and were not exposed to light.

4.2.2 Perfusion and Immunohistochemistry

The animals were anaesthetised first in a jar filled with Halothane vapour and then with an intramuscular injection of 0.3 ml of Sodium pentobarbitol. Once no movement was visible they were perfused intracardially with 0.9% saline which had been warmed to 37°C to retain the dimensions of the vascular system. The saline rinse was followed by Zamboni fixative, a mixture of 4% paraformaldehyde in phosphate buffer (pH 7.4, 0.1 M) with 15% saturated picric acid. The brains were postfixed for 24 hours at 4°C and subsequently placed in 30% sucrose for cryoprotection. Coronal sections (40 μm) were cut on a freezing microtome.

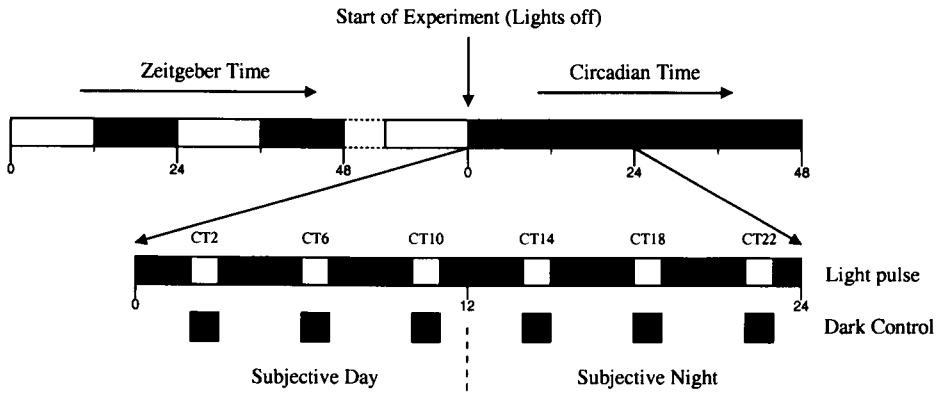

Figure 4.1: Schematic plan of the experiment.

All brain sections were processed simultaneously to prevent any discrepancies in the staining. The procedure was as follows:

1. Two Phosphate buffer (PBS) rinses for 10 min duration at 4°C with continuous agitation.
2. Alcohol-Saline-H_2O_2 for 30 mins at room temperature to suppress endogenous peroxidase.
3. Two PBS rinses, each for 10 min duration at 4°C with continuous agitation.
4. Overnight incubation in Normal Serum which consists of Normal Goat Serum (NGS) and PB-STA (phosphate buffer with triton and azide) at 4°C to block unspecific sites. The normal dilution for the Normal Serum is 150 μl of NGS in 10 ml of PBSTA.
5. Three nights incubation in primary antibody raised in rabbit, of concentration 1:3000 (i.e. 1 μl anti-Fos in 3 ml Normal Serum) at 4°C was used.
6. Two PBST (Phosphate buffer with triton) rinses, 10 min each at room temperature, agitation.
7. Secondary biotinylated antibody for 2 hours at room temperature. Normal dilution is 100 μl anti-rabbit in 10 ml Normal Serum.
8. Two PBST rinses, 10 min each at room temperature, agitation.
9. Incubation in AB complex (normal dilution 100 μl A and 100 μl B in 10 ml PBST) for 2 hours at room temperature. The AB complex needs to be prepared half an hour before.
10. One PBST rinse, 10 min, agitation at 4°C.
11. Two Tris rinses, at 10 min each, agitation at 4°C.

12. DAB-Nickel (240 ml TRIS, 40 mg DAB and 1g Ammonium nickel sulfate), 10 min, agitation at 4°C.

13. 200 μl H_2O_2 added to DAB-Nickel and left to agitate for 6 min.

14. Two Tris rinses at 10 min each. The sections were then left overnight at 4°C in Tris before being mounted on gelatinated slides.

To determine the concentration of the primary antibody needed, a preliminary experiment was carried out. The same protocol as above was used, except that four different concentrations (1:5000, 1:10000, 1:15000 and 1:20000) were used.

4.2.3 Image Analysis

The optical density (OD) of immunohistochemical label was assessed by a computer system consisting of a computer with specific image analysis software (Visiolab, Biocom, Les Ulis, France) connected to a microscope (Aristoplan, Leica) via a cooled digital camera (Photonic Science). The software analyses the gray level (GL) of every pixel of the digitised image. The values of gray level can vary from 0 (black) to 255 (white). OD is calculated from GL as follows:

$$OD = -\log(GL_{object}/GL_{max})$$

where GL_{max} is the mean gray level of a reference region with maximal transmittance. OD and GL are inversely related. The measure of OD affords the most subjective measurement of Fos expression in the SCN (Rieux *et al.*, 2001).

4.2.4 Statistical analysis

The data were statistically analysed using a Kruskal-Wallis ANOVA (data were not normally distributed) to determine differences between the time periods for stimulated and dark control *R. pumilio*. Light stimulated animals were compared to the dark controls for each time period using a t-test.

4.2.5 Experiment 2: Retinal Projections

Three *R. pumilio* received an intraocular injection of 0.5–1.0 μl of 0.2% cholera toxin subunit B (CTb). The animals were anaesthetised with halothane vapour and Ketamine (30 μl) and the pupil of the right eye was dilated with atropine. A small hole was made with a sharpened pipette near the corneal-scleral margin and the cholera toxin solution was injected into the vitreous using a 50 μl tipped glass pipette

sealed to the needle of a Hamilton syringe. The animals were perfused 48 hours later. For fixation methods see Experiment 1 above.

4.2.6 CTb Immunohistochemistry

1. Three 10 min PBS rinses at 4°C with continuous agitation.
2. Alcohol-Saline-H_2O_2 for 60 mins at 4°C to suppress endogenous peroxidase.
3. Three 10 min PBS rinses at 4°C with continuous agitation.
4. Two hours incubation in Normal Serum which consists of Normal Goat Serum (NGS) and PBSTA at 4°C to block unspecific sites.
5. Three nights incubation in primary antibody raised in rabbit, of concentration 1:3000 (i.e. 1 μl anti-Cholera Toxin in 5ml Normal Serum) at 4°C was used.
6. Two PBST (Phosphate buffer with triton) rinses, 10 min each at room temperature, with continuous agitation.
7. Incubation in secondary biotinylated antibody for 2 hours at room temperature. Normal dilution is 100 μl anti-goat in 10 ml Normal Serum.
8. Steps 8–12 same as in Experiment 1.
9. 40 μl H_2O_2 added to DAB-Nickel and left to agitate for 8 min. The sections were examined periodically to determine whether the staining was sufficient.
10. Two 10 min Tris rinses. The sections were left overnight at 4°C in Tris before being mounted on gelatinated slides.

4.3 Results

4.3.1 Experiment 1

There was no significant difference ($p = 0.914$) between the dark controls at the different time periods (Figure 4.2) which showed the lack of an endogenous rhythm of *c-fos* expression in the SCN. Although Fos induction appeared slightly higher in light pulsed animals compared to dark controls

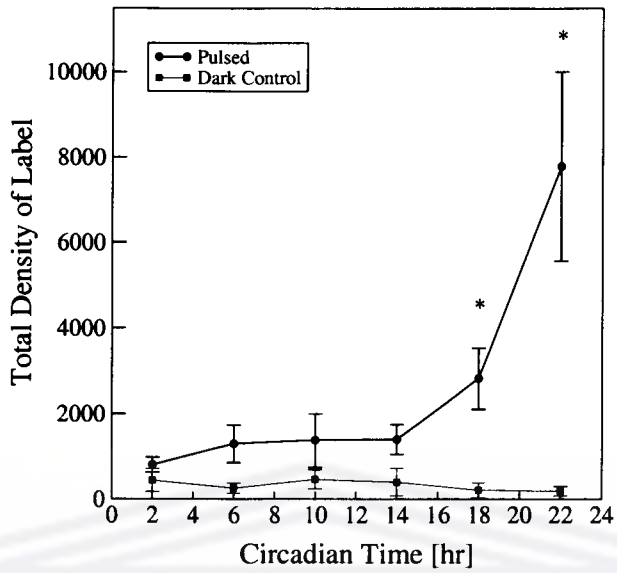

Figure 4.2: Total density of Fos label in the SCN of *R. pumilio* was significantly different from the dark controls at CT18 ($p < 0.05$) and at CT22 ($p < 0.05$) with the peak in expression at CT22. The values are given as mean $\pm$ standard errors.

during CT2 to CT14, although no significant difference was found. There was, therefore, no significant induction of Fos during subjective day (Figure 4.2). There was a significant difference between the light stimulated animals and the dark controls at CT18 ($p = 0.019$) and CT22 ($p = 0.026$, Figure 4.2). Fos induction following a light pulse thus occurred during late subjective night. Figure 4.3 includes representations of coronal sections of the SCN showing photic induction of Fos at different circadian times in the dark control and light stimulated animals.

The expression of Fos occurred mainly in the ventral SCN (Figure 4.3). There was an increase in Fos labelling from rostral to caudal with the highest labelling occurring mid-caudal (Figure 4.4). Differences in the expression of Fos in the dorsal and ventral regions of the SCN were calculated, but no difference was found. The regions were distinguished with the ventral region corresponding to retinal afferent innervation and the dorsal region was distinguished using cytoarchitectural criteria.

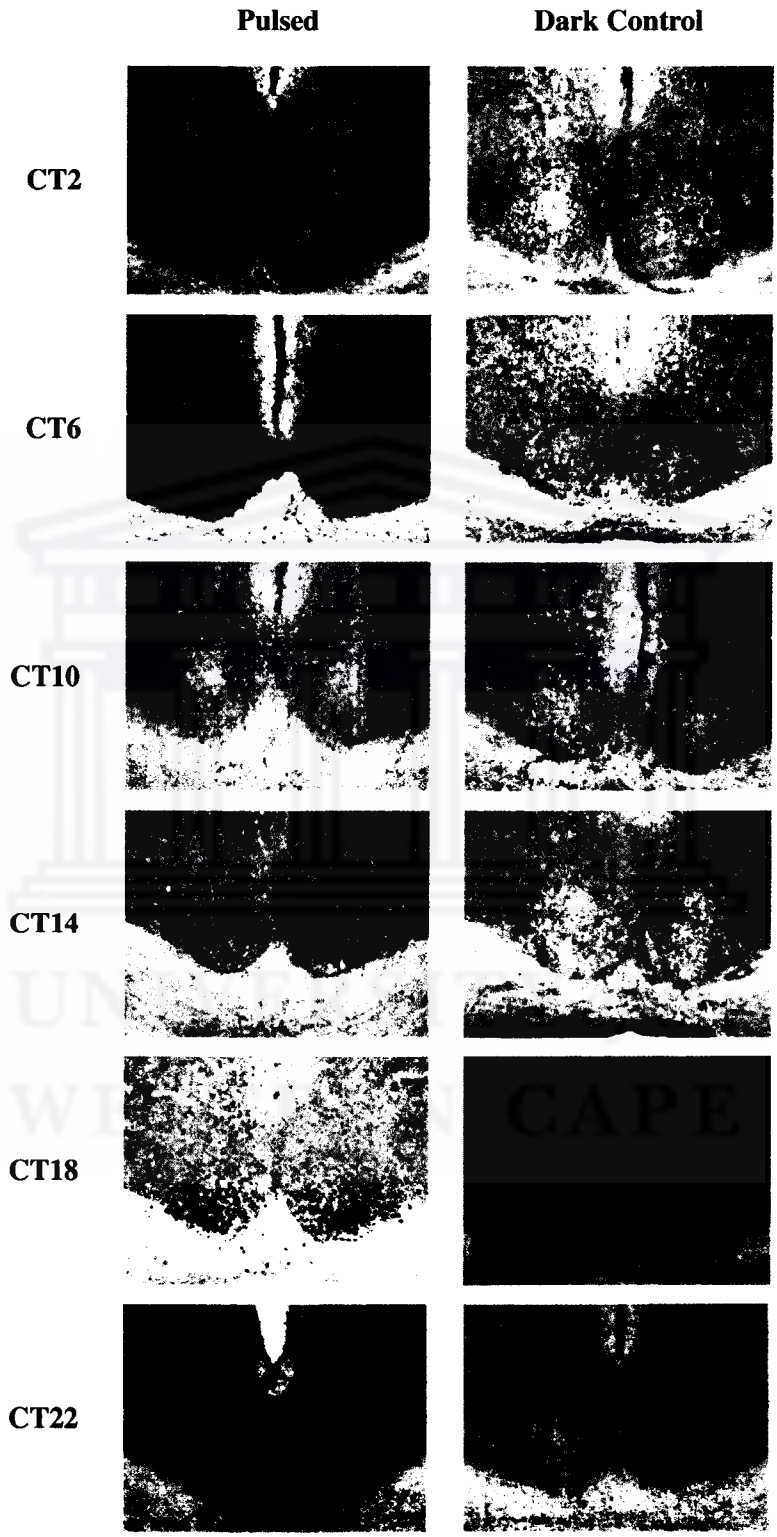

Figure 4.3: Fos labelling is denoted by the black dots and the SCN can clearly be seen because it is a shade lighter than the rest of the section. SCN of animals given a light pulse (pulsed) and animals kept in constant dark (dark controls).

Figure 4.4: Fos expression increased from rostral to caudal in the suprachiasmatic nucleus. These illustrations depict the SCN of a light pulsed animal.

4.3.2 Experiment 2

The retinal fibers in *R. pumilio* projected to all image-forming and non-image forming components of the visual system (Figures 4.5 and 4.6). The suprachiasmatic nuclei were bilaterally innervated, with a slightly higher innervation contralateral to the injected eye, though this was not quantified. The CTb immunohistochemical reaction indicated that the distribution of retinal afferents was mainly in the ventral SCN (Figure 4.5). The fibers were more dense in the rostral and caudal SCN but the pattern of innervation was unusual because rostrally the innervation was more ipsilateral whereas caudally the innervation was more contralateral.

The retinal fibers projected to several structures in the thalamus and tectum. The intergeniculate leaflet (IGL) is a thin horizontal band of fibers between the dorsal lateral geniculate nucleus (dLGN) and the ventral lateral geniculate nucleus (vLGN). The dLGN and vLGN received innervation almost exclusively contralateral to the injected eye, whereas innervation to the IGL showed a complementary overlap in ipsilateral and contralateral label (Figure 4.6).

There was intense labelling contralateral to the injected eye in both the dLGN and the superior colliculus (Figure 4.6).

Figure 4.5: Retinal projections to the SCN from rostral to caudal. Retinal afferents projected bilaterally, with a slightly higher innervation on the contralateral side, and with distribution mainly in the ventral SCN.

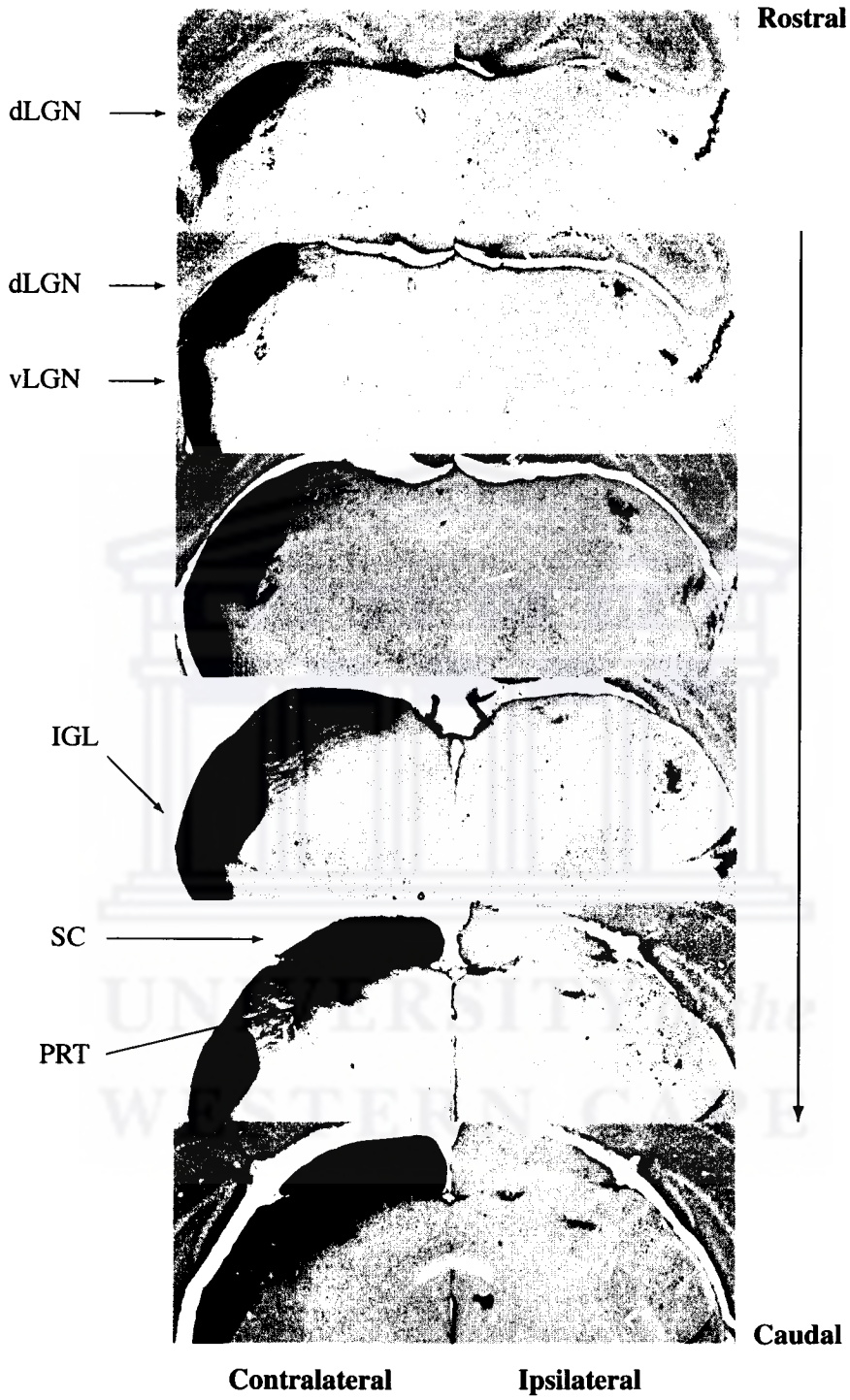

Figure 4.6: Retinal projections to the dorso-lateral geniculate nucleus (dLGN), the ventro-lateral geniculate nucleus (vLGN), the intergeniculate leaflet (top four pictures), the pretectum (PRT) and the superior colliculus (SC, bottom two pictures). The intergeniculate leaflet (IGL) was bilaterally innervated with complementary label on the contralateral and ipsilateral side.

4.4 Discussion

In the first experiment it was shown that Fos was photically induced in the SCN of *R. pumilio* during late subjective night. The fact that Fos was expressed during subjective night and not during subjective day conforms to patterns of Fos expression in diurnal *Arvicanthis niloticus* (Mahoney *et al.*, 2001) and nocturnal mammals but not in diurnal *Octodon degus* (Krajnak *et al.*, 1997) and the diurnal chipmunk *Eutamias asiaticus* (Abe *et al.*, 1995). For all mammals during subjective night, there is a gradual increase and decrease in Fos expression in response to a light pulse (Rea, 1989; Aronin *et al.*, 1990; Colwell and Foster, 1992; Abe *et al.*, 1995; Krajnak *et al.*, 1997; Katona *et al.*, 1998). In *R. pumilio*, there was a sharp increase in Fos expression at CT22 and since Fos expression returned to basal levels at CT2, it is probable that there is a sharp decline in Fos expression after CT22. This would predict a narrow window within which phase shifts could occur, either late night or early morning. This pattern can be related to the activity results found in the earlier experiments. As mentioned previously, the onset of locomotor activity in *R. pumilio* was stable whereas the offset of activity was less precise. The M-oscillator appeared to be more strongly coupled to the zeitgeber than the E-oscillator, therefore it could be predicted that the animal was driven more by its M-oscillator and might be more sensitive to any changes in the period. No phase shifting experiments were performed, but this will be the next set of experiments to determine whether the expression of Fos in *R. pumilio* corresponded with its behavioural phase shifts.

In other diurnal mammals, such as *Octodon degus* (Krajnak *et al.*, 1997) and the diurnal chipmunk (Abe *et al.*, 1995), there is still confusion as to the relationship between Fos and phase-shifting. In nocturnal mammals, it has been shown that Fos expression and phase-shifting have the same phase-dependence (Aronin *et al.*, 1990; Rusak *et al.*, 1990; Colwell and Foster, 1992).

There was no endogenous rhythm of Fos in *R. pumilio*. This result correlates well with results found in mice, rats and the diurnal chipmunk (Earnest *et al.*, 1990; Colwell and Foster, 1992; Abe *et al.*, 1995). Sumová *et al.* (1998) and Guido *et al.* (1999) found a spontaneous rhythm of Fos in the rat in the dorsomedial part of the SCN with an increase in Fos expression during early subjective day. In *R. pumilio* there was no phase dependent difference between Fos expression in the dorsal or ventral areas of the SCN .

In most rodents, photic induction of Fos is expressed throughout the SCN but more strongly in the ventral SCN (Aronin *et al.*, 1990; Earnest *et al.*, 1990; Rusak *et al.*, 1990; Sumová *et al.*, 1998). The same pattern occurred in *R. pumilio* which correlated well with the distribution of retinal afferents. In *R. pumilio* there was an increase in Fos labelling from rostral to caudal with the highest

labelling occurring in the mid-caudal region which corresponded with retinal input. The labelling for *R. pumilio* differs compared to *Arvicanthis niloticus* where the highest labelling occurs in the central SCN (Mahoney *et al.*, 2001).

The SCN of *R. pumilio* received bilateral projections with a slight contralateral predominance. This is similar to results found in *Octodon degus* (Goel *et al.*, 1999) and in sheep (Tessonneaud *et al.*, 1994).

The intergeniculate leaflet is a functionally and anatomically distinct subdivision of the lateral geniculate complex, which received bilateral innervation with overlapping between the ipsilateral and contralateral label in *R. pumilio*. This is similar to data found in the blind mole rat (Cooper *et al.*, 1993), and in mice (Provencio *et al.*, 1998) but in degus, there is significantly less innervation on the ipsilateral than the contralateral side.

Chapter 5

General Discussion

The majority of mammalian models used in chronobiology are nocturnal rodents with a few diurnal mammals from the Families Muridae and Sciuridae. Animals are characterised as being nocturnal, diurnal or crepuscular based on the proportion of their activity occurring during the night, day or twilight times, respectively. *R. pumilio* were diurnal both when in artificial lighting conditions and when in a natural light-dark cycle. They showed a bimodal activity pattern, with activity peaks at dawn and dusk or the corresponding time periods in the artificial light cycle. The activity pattern related well to the retinal innervation because an approximately equal number of rods and cones would allow *R. pumilio* to see in both bright and exceedingly dim light.

When all the individuals were placed in a 12:12 LD cycle, most showed diurnal behaviour with only animal no. 7 consistently showing a nocturnal activity pattern. *R. pumilio* had an average percentage diurnality of 68%. Unlike *A. niloticus* and *O. degus*, there was no difference in the activity patterns when *R. pumilio* were exposed to running wheels or when they were without running wheels. There were large inter-individual differences in alpha during LD2. No conclusive evidence is available, but the possibility that these differences are correlated with the large inter-individual differences in tau during constant dark exists.

When placed in constant conditions, *R. pumilio* showed stable free-running periods. Most of the animals displayed a higher percentage of activity during subjective day than during subjective night, re-inforcing results found during the 12:12 LD cycle which suggested that *R. pumilio* were diurnal. There were large inter-individual variation in tau during constant dark (range = 23.10 – 24.80 hours), similar to large fluctuations observed in other diurnal mammals (Pohl, 1982). Contrary to Aschoff's rule (Aschoff, 1979) but in accordance with results found for *A. niloticus* (Katona and Smale, 1997), all *R. pumilio* displayed tau longer than 24 hours during constant light (range = 24.30 – 24.79 hours).

These results also agreed with predictions made by Daan and Pittendrigh (1976) which state that if tau is less than 24 hours in constant dark, it will be more than 24 hours in constant light. Although tau did not agree with Aschoff's rule, alpha which increased from constant dark to constant light, agreed with predictions by Aschoff (1979).

Some interesting results were forthcoming during the masking experiment. Referring to Figure 3.5, qualitatively it appears that animal no. 8 was diurnal. The active period was restricted to subjective day, and the rest period during the middle of the day was retained (Figure 5.2). Quantitative analysis, however, showed that this animal was more active during the dark phase of the 1:1 LD cycle than during the light phase. Another animal, no. 7, which showed nocturnal behaviour during all the lighting conditions (refer to Figure 5.1 and 5.4) quantitatively showed diurnal behaviour during the masking light cycle. Qualitatively, however, it was active throughout subjective day and subjective night whereas it would be expected to be active during subjective night only. This pattern of being active throughout subjective day and subjective night, was an exception and not the rule for *R. pumilio*, but for *Octodon degus*, it appears to be the rule (Kas and Edgar, 1999b). *O. degus* has a variable activity pattern. With a running wheel *O. degus* is nocturnal and without a running wheel, diurnal. This ability to be active during the day or night, is once again manifested during the masking light cycle. It is possible that *O. degus* and animal no. 7 are still in a transitory stage between nocturnality and diurnality. A slight delay in the M-oscillator or a slight advance in the E-oscillator may result in the animal being diurnal and vice versa for the animal to be nocturnal (Daan *et al.*, 2001). Considering the results, i.e. that tau during constant light was longer than 24 hours for all *R. pumilio* individuals, and that the peak in Fos expression occurred during late subjective night, it will be interesting to see the shape of the phase response curve for *R. pumilio*.

More research is needed to determine the pattern of photic induction of Fos in the SCN of diurnal mammals. In *R. pumilio* the pattern of Fos induction in the SCN is similar to that in other mammals, in that there was an increase in Fos expression during subjective night but not during subjective day. In two diurnal mammals, *O. degus* and the diurnal chipmunk, a change in Fos expression in the SCN occurred during subjective day as well as subjective night (Abe *et al.*, 1995; Krajnak *et al.*, 1997). These inconsistencies may be related to a limited sample number since Abe *et al.* (1995) only used three individuals and Krajnak *et al.* (1997) only gave two light pulses, one at CT16 and one at CT4. Fos expression in *R. pumilio* differed from other mammals, in that the peak of expression occurred during late subjective night at CT22, with a rapid decrease in Fos to low levels at CT2. In other mammals (nocturnal and diurnal) the increase and decrease in Fos expression is gradual, with a peak

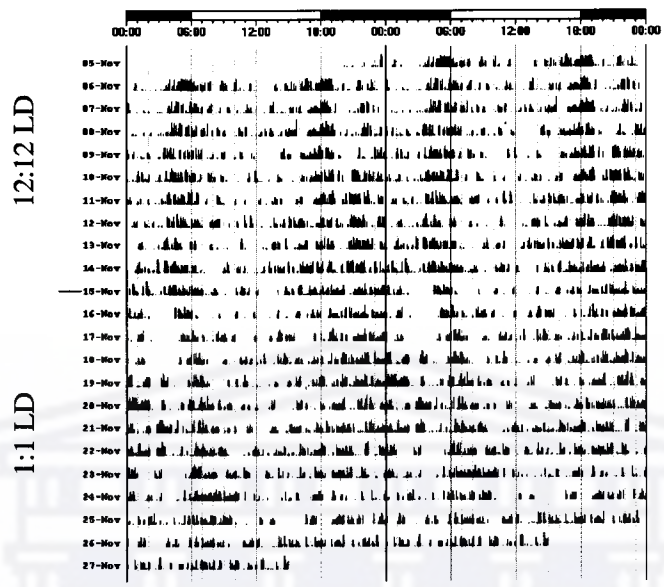

Figure 5.1: Animal no. 7, was active throughout subjective day and subjective night, but quantitatively had more activity during the light phase of the 1:1 LD cycle.

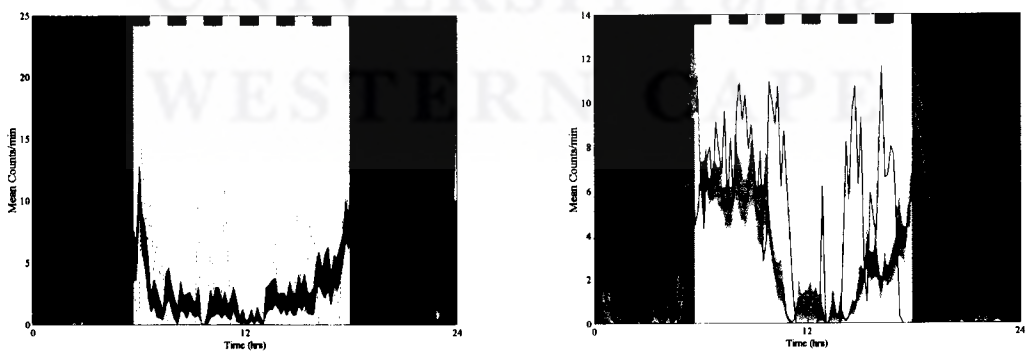

Figure 5.2: Activity profile for one day of the masking cycle of animals no. 7 (left) and 8 (right) superimposed on their respective activity profiles for LD3 (value ± SEM). The black bars depict the hours of darkness and the white bars depict the hours of light. Animal no. 7 was active throughout subj. day and subj. night whereas animal no. 8 was mainly active during subj. day.

around CT14–CT16 (Rea, 1989; Aronin *et al.*, 1990; Colwell and Foster, 1992; Abe *et al.*, 1995; Krajnak *et al.*, 1997; Katona *et al.*, 1998). There was no endogenous rhythm of Fos in *R. pumilio* which corresponds to observations made in nocturnal and diurnal mammals (Earnest *et al.*, 1990; Colwell and Foster, 1992; Abe *et al.*, 1995).

It is not known at which level nocturnal and diurnal mammals are differentiated and the distinction between nocturnality and diurnality is not always fixed or clearly determined. A switch in activity patterns from diurnal to nocturnal can be context specific and occurs in certain mammals when exposed to running wheels, as in *O. degus* (Lee and Labyak, 1997) and *A. niloticus* (Blanchong *et al.*, 1999), or from diurnal to nocturnal due to interspecific competition, as in the case of the Golden Spiny mouse (Zisapel *et al.*, 1999). It may, therefore, be important that one or more parameters should be used to determine whether an animal is diurnal or nocturnal. A simple comparison of this type is illustrated in Figure 5.3 which attempts to define the degree of nocturnality/diurnality along two axes corresponding to the percentage diurnal activity during a 12:12 LD cycle and the masking cycle. In *R. pumilio*, if only one parameter is used for classification then the two individuals in the lower right square would be classified as nocturnal based on their activity during a 12:12 LD cycle, but diurnal based on their activity in the 1:1 LD cycle and vice versa for the individual in the top left square. In my study of *R. pumilio*, since the same animals were used for different light cycles, I was able to determine the percentage diurnal activity in several different conditions (Figure 5.4). Although some animals displayed nocturnal behaviour in certain conditions, all the animals were diurnal (more active during the light phase or subjective day), except for animal no. 7. The periods of 'nocturnal' behaviour could be ascribed to paradoxical masking. Referring to Figure 5.5, it is obvious that although the animal was active the entire day, the activity increased once the lights went off. According to Mrosovsky (1999) paradoxical masking occurs when there is an increase in activity after a decrease in illumination, for a diurnal mammal. The significance of paradoxical masking is unknown but the occurrence thereof should be taken into account.

The type of comparison illustrated by the summary Figures 5.3 and 5.4 could lead to an interesting multi-factorial analysis for refining the notion of nocturnality and diurnality in mammals. This approach is currently being pursued in several different nocturnal and diurnal species in order to test this concept.

A final point of interest involves estimating when the beginning of the day time period is perceived by *R. pumilio*. In square wave lighting conditions, activity started on average 0.69 hours before light onset (daytime phase). Extrapolating to activity rhythms in natural conditions, if we assume that

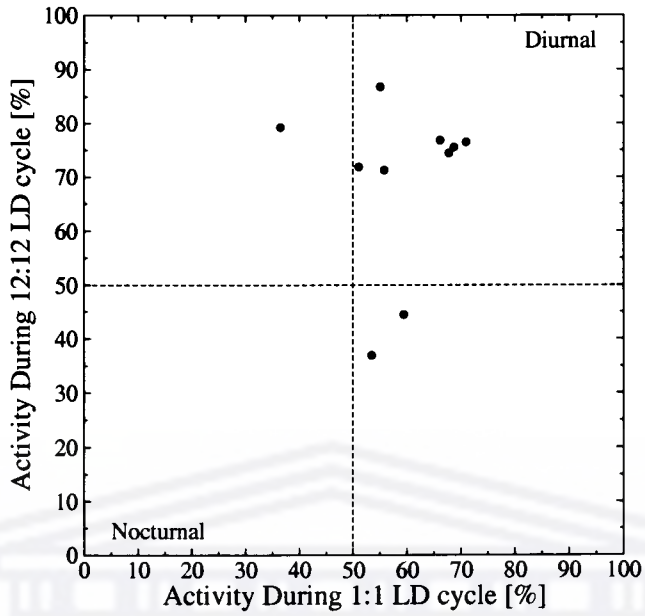

Figure 5.3: Average percentage of activity during the light phase of LD2 and LD3 compared to the average percentage of activity during the light phase of a 1:1 LD cycle. Activity during the light phase was a percentage of the total activity. Seven of the ten animals were diurnal and three animals showed contradictory behaviour.

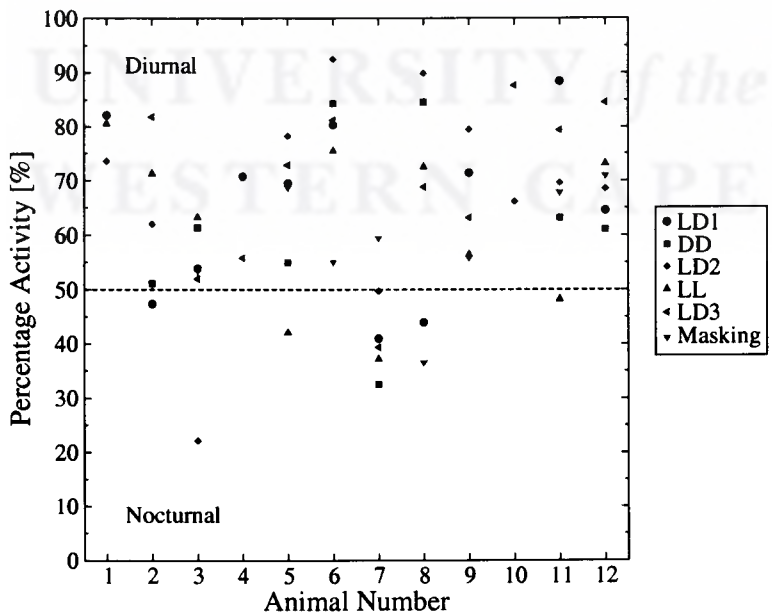

Figure 5.4: Percentage activity during the light phase of LD1, DD, LD2, LL, LD3, and masking. One animal showed nocturnal behaviour in nearly all the parameters tested.

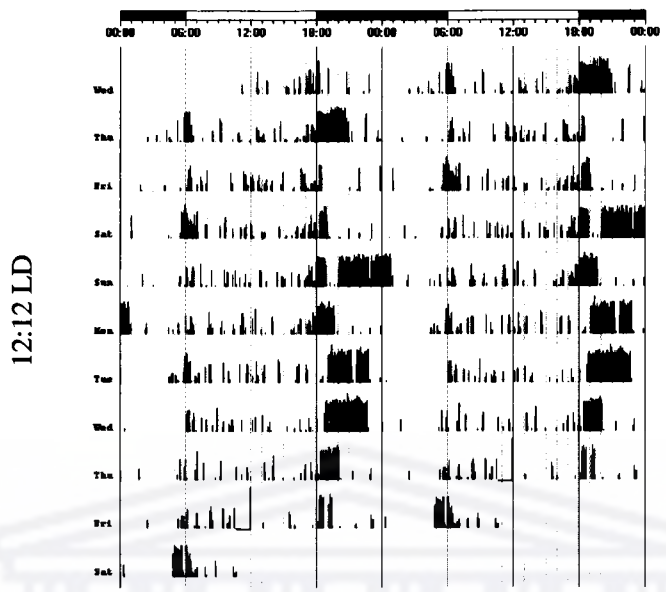

Figure 5.5: Animal no. 3 showed more activity during the night than during the day. This occurred in only one 12:12 LD cycle.

activity also begins 0.69 hours before the beginning of day, then it is possible to estimate at what light level the animal perceives daytime. *R. pumilio* began activity at 5.59 hours, which corresponds to nautical twilight and a light level of approximately 10^{-3}foot candles. Humans are unable to perform outdoor activities at this irradiance (U.S. Naval Observatory, 2001). At 0.69 hours following activity onset, light levels are approximately 10^{-3}foot candles which can thus be defined as the animals 'real' perceived beginning of day. This approach assumes that the influence of light on activity is the same in artificial and square wave light conditions, which may not be the case, as found in squirrel monkeys (Boulos *et al.*, 1996c) and hamsters (Tang *et al.*, 1999) where there was a significant difference in the activity onset and offset of animals in a square light-dark cycle compared to those in a twilight cycle. *R. pumilio* in an artificial light cycle, started and ended activity approximately 1.5 hours earlier than *R. pumilio* in a twilight cycle. This difference in onsets and offsets could be due to the fluctuating temperatures in natural conditions. When all the individuals were in an artificial light cycle, the difference in onset and offset compared to the individuals in natural conditions was 0.6 hours. There was no difference in the length of the active period between the two lighting conditions. This does not agree with evidence found in the hamster (Boulos *et al.*, 1996a; Tang *et al.*, 1999). The difference in results could be due to the fact that *R. pumilio* were in natural conditions and therefore exposed to other factors such as humidity, temperature and light fluctuations throughout the day, whereas the hamsters were placed in a simulated twilight cycle.

Appendix A

Actograms

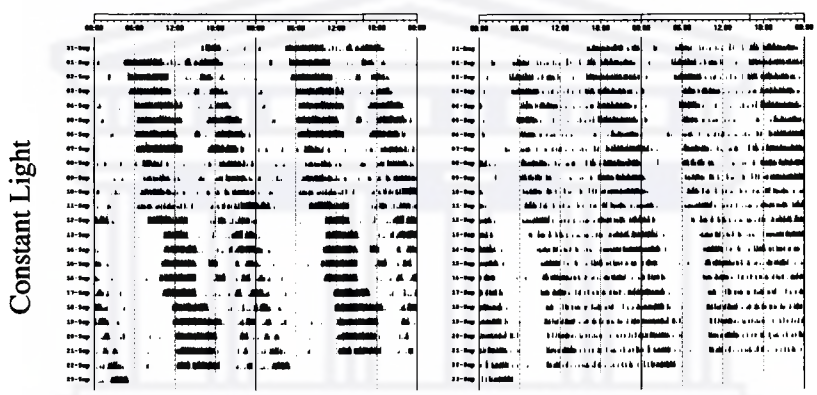

Figure A.1: All animals showed a period greater than 24 hours during constant light. These actograms show the activity pattern during constant light for animals no. 6 and 11.

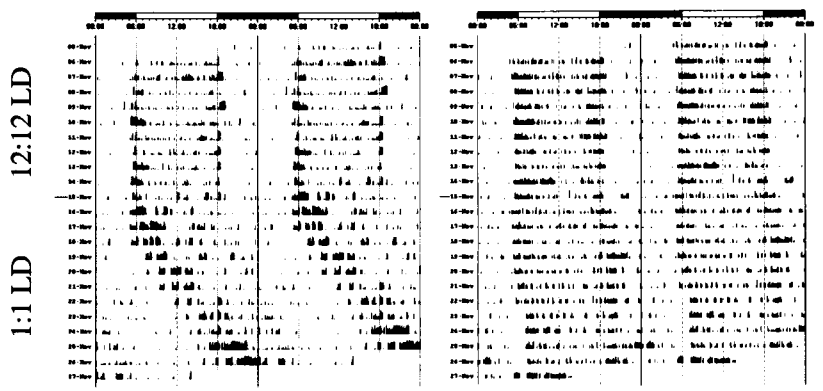

Figure A.2: Actograms depicting animals (no. 3 and 11) in an ultradian light-dark cycle of 1:1 LD.

Appendix B

Programs

The following sections depict the source code of the programs (macros) used to calculate percentage diurnality using Visual Basic, a programming language used in Microsoft Excel.

B.1 Diurnality during LD cycle

This macro was used to calculate the percentage diurnality during the 12:12 LD cycles.

```
Sub LD()


' Clear columns E to K
' And write labels
  Columns("E:K").Select
  Selection.ClearContents
  Cells(7, 6).Value = "Day"
  Cells(7, 7).Value = "Night"

' Initialize row index for totals
  j = 7
  k = 7

' Loop through 12 hour periods for the number of days needed, here 9.5 days
  For counter = 0 To 18
   'Calculate cell number i in minutes and 596 depicts where the program
   'actually starts since I did not start on the first day of the LD cycle.
    i = counter * (60 * 12) + 596
   'Add up 12*60 minutes of activity
    Set curCell = ActiveSheet.Cells(i, 5)
    curCell.FormulaR1C1 = "=SUM(RC[-4]:R[719]C[-4])"
    curCell.Select
    Selection.Copy
   'Write result to day or night column
    If (counter Mod 2) < 1 Then
```

```
    ' Day
      j = j + 1:
      Cells(j, 6).Select
    Else
    ' Night
      Cells(j, 7).Select
    End If
    Selection.PasteSpecial Paste:=xlValues, Operation:=xlNone,
    SkipBlanks:= _ False, Transpose:=False
  Next counter

  'The headings for the different columns
  Cells(7, 9).Value = "Day Total"
  Cells(7, 10).Value = "Night Total"
  Cells(10, 9).Value = "Day Ratio"
  Cells(10, 10).Value = "Night Ratio"

  Cells(8, 9).Value = "=SUM(F:F)"
  Cells(8, 10).Value = "=SUM(G:G)"
  Cells(11, 9).Value = "=I8/(J8+I8)"
  Cells(11, 10).Value = "=J8/(J8+I8)"
  Range("I11", "J11").Select
  Selection.NumberFormat = "0.0%"

End Sub
```

B.2 Diurnality during DD and LL cycle

To calculate percentage activity during subjective day, one has to take into account that the animal is free-running, which means that its day is either starting earlier or later depending on whether its period is less than or more than 24 hours. This macro is therefore more complicated than the previous one and it was used to calculate diurnality during the constant dark and constant light photoperiods.

```
Sub DD()

  Range("F7:T62000").Select
  Selection.ClearContents
 'Type in values in these cells wt c0=cell number of first onset, cf=cell number
 'of last onset, df=number of days over which program must run and it is read
 'by macro
  c0 = Cells(3, 3).Value
  cf = Cells(3, 4).Value
  df = Cells(3, 5).Value
 'Slope (period) or number of cells from one onset to the next is calculated
 'from last onset minus first onset divided by number of days
  s = (cf - c0) / df
  Cells(3, 6).Value = s
  Cells(4, 6).Value = s / 60
 'Loop through days and basically defining what everything is
  k = 8
  For d = 0 To df
   'c_on is onset on day d
    c_on = c0 + d * s
   'Onset hour which is obtained from the row of the onset cell, same with
   'minutes
    h_on = Cells(c_on, 3).Value
    m_on = Cells(c_on, 4).Value
   'The onset cell may be between hours, so shift it to nearest full hour
    c_on_shifted = c_on - m_on
   'The hour is therefore shifted too
    h_on_shifted = Cells(c_on_shifted, 3).Value
   'To calculate ct12 we divide slope by two and add this to the original onset
   'Time, not the shifted onset
    c_mid = c_on + Round(s / 2)    ' or _shifted
   'The minutes are derived from the row of CT12
    m_mid = Cells(c_mid, 4).Value
   'If minutes is less than 30 then shift hour or cell to previous hour
   'otherwise shift it to that full hour
    If (m_mid < 30) Then
      c_mid_shifted = c_mid - 60 - m_mid
    Else
      c_mid_shifted = c_mid - m_mid
    End If
    h_mid_shifted = Cells(c_mid_shifted, 3).Value
```

```
'This is to find CT24
 c_end = c_on + (s - 60)
 m_end = Cells(c_end, 4).Value
'Same reasoning as for m_mid
 If (m_end < 30) Then
   c_end_shifted = (c_end - 60) - m_end
 Else
   c_end_shifted = c_end - m_end
 End If
 h_end_shifted = Cells(c_end_shifted, 3).Value

'Determines offset cell and thus time
 c_off = c_on + Cells(5, 3).Value
 m_off = Cells(c_off, 4).Value
 c_off_shifted = c_off - m_off
 h_off_shifted = Cells(c_off_shifted, 3).Value

 Cells(k, 9).Value = d
'To correct for when the offset is lower than the onset which would occur
'after midnight
 If (h_off_shifted < h_on_shifted) Then
   h_off_shifted = h_off_shifted + 24
 End If
 If (h_mid_shifted < h_on_shifted) Then
   h_mid_shifted = h_mid_shifted + 24
 End If
 If (s >= 1440) Or (h_end_shifted < h_on_shifted) Then
   h_end_shifted = h_end_shifted + 24
 End If

'Loop through hours of day d
 For h = h_on_shifted To h_end_shifted
   Set curcell = Cells(c_on_shifted + (h - h_on_shifted) * 60, 6)
   curcell.FormulaR1C1 = "=SUM(RC[-5]:R[59]C[-5])"
   curcell.Select
   Selection.Copy

  'Dark periods for the CT0 to CT12 period is pasted in a different column
   If (h <= h_mid_shifted) Then
     k = k + 1
     Cells(k, 7).Select:
     Selection.PasteSpecial Paste:=xlValues, Operation:=xlNone,
     SkipBlanks:= _False, Transpose:=False
   Else
    'The dark hours for CT12 to CT24 is pasted in yet another column
     k = k + 1
     Cells(k, 8).Select:
     Selection.PasteSpecial Paste:=xlValues, Operation:=xlNone,
```

```
        SkipBlanks:= _False, Transpose:=False
      End If

    Next h
    k = k + 1
  Next d

 'Headings for the different columns
  Cells(7, 7).Value = "CT0-12"
  Cells(7, 8).Value = "CT12-24"
  Cells(7, 9).Value = "Day"
  Cells(7, 10).Value = "Total CT0-12"
  Cells(7, 11).Value = "Total CT12-24"
  Cells(10, 10).Value = "CT0-12 Ratio"
  Cells(10, 11).Value = "CT12-24 Ratio"

  Cells(8, 10).Value = "=Sum(G:G)"
  Cells(8, 11).Value = "=Sum(H:H)"
  Cells(11, 10).Value = "=J8/(J8+K8)"
  Cells(11, 11).Value = "=K8/(J8+K8)"

  Columns("J:M").ColumnWidth = 12.75
  Columns("O:P").ColumnWidth = 15.75
  Columns("Q:S").ColumnWidth = 12.25
  Columns("N:N").ColumnWidth = 10
  Range("J11", "K11").Select
  Selection.NumberFormat = "0.0%"

End Sub
```

B.3 Masking of activity by dark

To determine whether the activity of *R. pumilio* was suppressed by dark, the activity counts during the dark and light phases, respectively are calculated. The results are expressed as a percentage of total activity. Five minutes were taken off the beginning and end of the light and dark phases, to determine whether there were post-pulse effects.

```
Sub Maskingactivity()

 'Clear columns B to M
 'And write labels
  Columns("B:M").Select
  Selection.ClearContents
  Cells(7, 4).Value = "Dark"
  Cells(7, 5).Value = "Light"
  Cells(7, 7).Value = "Dark (-5)"
  Cells(7, 8).Value = "Light (-5)"

 'Initialize row index for totals
  j = 7
  k = 7

 'Loop through hours adding activity for each hour
  For counter = 0 To 289
    i = counter * 60 + 43
    Set curCell = ActiveSheet.Cells(i, 2)
    curCell.FormulaR1C1 = "=SUM(RC[-1]:R[59]C[-1])"
    curCell.Select
    Selection.Copy
    If (counter Mod 2) < 1 Then
     'Dark
      j = j + 1:
      Cells(j, 4).Select
    Else
     'Light
      Cells(j, 5).Select
    End If
    Selection.PasteSpecial Paste:=xlValues, Operation:=xlNone,
    SkipBlanks:=False, Transpose:=False

   'Same for -5
    Set curCell = ActiveSheet.Cells(i, 3)
    curCell.FormulaR1C1 = "=SUM(R[5]C[-2]:R[54]C[-2])"
    curCell.Select
    Selection.Copy
    If (counter Mod 2) < 1 Then
      k = k + 1:
      Cells(k, 7).Select
```

```
  Else
    Cells(k, 8).Select
  End If
  Selection.PasteSpecial Paste:=xlValues, Operation:=xlNone,
  SkipBlanks:=False, Transpose:=False

Next counter

Cells(7, 10).Value = "Dark Total"
Cells(7, 11).Value = "Light Total"
Cells(7, 12).Value = "Dark (-5) Total"
Cells(7, 13).Value = "Light (-5) Total"

Cells(8, 10).Value = "=SUM(D:D)"
Cells(9, 10).Value = "=SUM(D8:D79)"
Cells(10, 10).Value = "=SUM(D80:D152)"
Cells(8, 11).Value = "=SUM(E:E)"
Cells(9, 11).Value = "=SUM(E8:E79)"
Cells(10, 11).Value = "=SUM(E80:E152)"
Cells(8, 12).Value = "=SUM(G:G)"
Cells(9, 12).Value = "=SUM(G8:G79)"
Cells(10, 12).Value = "=SUM(G80:G152)"
Cells(8, 13).Value = "=SUM(H:H)"
Cells(9, 13).Value = "=SUM(H8:H79)"
Cells(10, 13).Value = "=SUM(H80:H152)"

Cells(8, 9).Value = "full time"
Cells(9, 9).Value = "first half"
Cells(10, 9).Value = "second half"
Cells(13, 9).Value = "full time"
Cells(14, 9).Value = "first half"
Cells(15, 9).Value = "second half"

Cells(12, 10).Value = "Dark Ratio"
Cells(12, 11).Value = "Light Ratio"
Cells(12, 12).Value = "Dark (-5) Ratio"
Cells(12, 13).Value = "Light (-5) Ratio"

Cells(13, 10).Value = "=J8/(J8+K8)"
Cells(13, 11).Value = "=K8/(J8+K8)"
Cells(13, 12).Value = "=L8/(L8+M8)"
Cells(13, 13).Value = "=M8/(L8+M8)"
Cells(14, 10).Value = "=J9/(J9+K9)"
Cells(14, 11).Value = "=K9/(J9+K9)"
Cells(14, 12).Value = "=L9/(L9+M9)"
Cells(14, 13).Value = "=M9/(L9+M9)"
Cells(15, 10).Value = "=J10/(J10+K10)"
Cells(15, 11).Value = "=K10/(J10+K10)"
```

```
  Cells(15, 12).Value = "=L10/(L10+M10)"
  Cells(15, 13).Value = "=M10/(L10+M10)"

  Range("J13", "M13").Select
  Selection.NumberFormat = "0.0%"
  Range("J14", "M14").Select
  Selection.NumberFormat = "0.0%"
  Range("J15", "M15").Select
  Selection.NumberFormat = "0.0%"
  Columns("I:M").ColumnWidth = 12.71
End Sub
```

Masking during Subjective Day

Qualitative analysis suggested that there may be a difference between the first and second half of subjective day. This program calculates the percentage activity during the two halves of subjective day as well as for the active period of the animal, for the light and dark phases.

```
Sub active_period()

  Range("G7:T16500").Select
  Selection.ClearContents
 'Type in values in these cells and it is read by macro
  c0 = Cells(3, 3).Value
  cf = Cells(3, 4).Value
  df = Cells(3, 5).Value
 'Slope or number of cells from one onset to the next is calculated
 'from last onset minus first onset divided by number of days
  s = (cf - c0) / df
  Cells(3, 6).Value = s
 'Loop through days and basically defining what everything is
  k = 8
  For d = 0 To df
    c_on = c0 + d * s
   'Onset hour which is obtained from the row of the onset cell, same
   'with
   'minutes
    h_on = Cells(c_on, 3).Value
    m_on = Cells(c_on, 4).Value
   'The onset cell may be between hours, so shift it to nearest full
   'hour
    c_on_shifted = c_on - m_on
   'The hour is therefore shifted too
    h_on_shifted = Cells(c_on_shifted, 3).Value
   'To calculate ct6 divide slope by four and add this to the original
   'onset time, not the shifted onset
    c_mid = c_on + Round(s / 4)    ' or _shifted
   'The minutes are derived from the row of CT6
    m_mid = Cells(c_mid, 4).Value
   'If minutes is less than 30 then shift hour or cell to previous
   'hour otherwise shift it to that full hour
    If (m_mid < 30) Then
      c_mid_shifted = c_mid - 60 - m_mid
    Else
      c_mid_shifted = c_mid - m_mid
    End If
    h_mid_shifted = Cells(c_mid_shifted, 3).Value
   'This is to find CT12, so divide slope by 2
    c_end = c_on + Round(s / 2)
    m_end = Cells(c_end, 4).Value
```

```
'Same reasoning as for m_mid
 If (m_end < 30) Then
   c_end_shifted = (c_end - 60) - m_mid
 Else
   c_end_shifted = c_end - m_end
 End If
 h_end_shifted = Cells(c_end_shifted, 3).Value

'Determining whether the hour is dark or light
 If (h_on_shifted Mod 2 = 0) Then
   Light = 0
 Else
   Light = 1
 End If
'Determines offset cell and thus time
 c_off = c_on + Cells(5, 3).Value
 m_off = Cells(c_off, 4).Value
 c_off_shifted = c_off - m_off
 h_off_shifted = Cells(c_off_shifted, 3).Value

 Cells(k, 9).Value = d
'To correct for when the offset is lower than the onset
'which would occur after midnight
 If (h_off_shifted < h_on_shifted) Then
   h_off_shifted = h_off_shifted + 24
 End If
 If (h_mid_shifted < h_on_shifted) Then
   h_mid_shifted = h_mid_shifted + 24
 End If
 If (h_end_shifted < h_on_shifted) Then
   h_end_shifted = h_end_shifted + 24
 End If
'Loop through hours
 For h = h_on_shifted To h_off_shifted
   Set curcell = Cells(c_on_shifted + (h - h_on_shifted) * 60, 6)
   curcell.FormulaR1C1 = "=SUM(RC[-5]:R[59]C[-5])"
   curcell.Select
   Selection.Copy
   If (Light) < 1 Then
     k = k + 1:
     Cells(k, 7).Select:
     Selection.PasteSpecial Paste:=xlValues, Operation:=xlNone,
     SkipBlanks:=False, Transpose:=False:
    'Dark periods for the CT1 to CT6 period is pasted in a
    'different column
     If (h <= h_mid_shifted) Then
       Cells(k, 10).Select:
       Selection.PasteSpecial Paste:=xlValues, Operation:=xlNone,
```

```
        SkipBlanks:=False, Transpose:=False
      Else
       'The dark hours for CT6 to CT12 is pasted in yet another
       'column
        If (h <= h_end_shifted) Then
          Cells(k, 12).Select:
            Selection.PasteSpecial Paste:=xlValues,
            Operation:=xlNone,SkipBlanks:=False, Transpose:=False
        End If
      End If
    Else
   'The same for the light hours
      Cells(k, 8).Select:
        Selection.PasteSpecial Paste:=xlValues,
        Operation:=xlNone,  SkipBlanks:=False, Transpose:=False:
      If (h <= h_mid_shifted) Then
        Cells(k, 11).Select:
          Selection.PasteSpecial Paste:=xlValues,
          Operation:=xlNone, SkipBlanks:=False, Transpose:=False
      Else
        If (h <= h_end_shifted) Then
          Cells(k, 13).Select:
            Selection.PasteSpecial Paste:=xlValues,
            Operation:=xlNone, SkipBlanks:=False, Transpose:=False
        End If
      End If
    End If
    Light = (Light + 1) Mod 2
  Next h
  k = k + 1
Next d

Cells(7, 7).Value = "Dark"
Cells(7, 8).Value = "Light"
Cells(7, 9).Value = "Day"
Cells(7, 10).Value = "Dark CT0-CT6"
Cells(7, 11).Value = "Light CT0-CT6"
Cells(7, 12).Value = "Dark CT6-CT12"
Cells(7, 13).Value = "Light CT6-CT12"
Cells(7, 15).Value = "Avg Dark"
Cells(7, 16).Value = "Avg Light"
Cells(10, 15).Value = "Avg Dark CT0-CT6"
Cells(10, 16).Value = "Avg Light CT0-CT6"
Cells(13, 15).Value = "Avg Dark CT6-CT12"
Cells(13, 16).Value = "Avg Light CT6-CT12"
Cells(7, 18).Value = "Dark Ratio"
Cells(7, 19).Value = "Light Ratio"
Cells(8, 17).Value = "CT0-CT12"
```

```
  Cells(9, 17).Value = "CT0-CT6"
  Cells(10, 17).Value = "CT6-CT12"

  Columns("J:M").ColumnWidth = 12.75
  Columns("O:P").ColumnWidth = 15.75
  Columns("Q:S").ColumnWidth = 12.25
  Columns("N:N").ColumnWidth = 10

  Cells(8, 15).Value = "=Average(G:G)"
  Cells(8, 16).Value = "=Average(H:H)"
  Cells(11, 15).Value = "=Average(J:J)"
  Cells(11, 16).Value = "=Average(K:K)"
  Cells(14, 15).Value = "=Average(L:L)"
  Cells(14, 16).Value = "=Average(M:M)"

  Cells(8, 18).Value = "=O8/(O8+P8)"
  Cells(8, 19).Value = "=P8/(O8+P8)"
  Cells(9, 18).Value = "=O11/(O11+P11)"
  Cells(9, 19).Value = "=P11/(O11+P11)"
  Cells(10, 18).Value = "=O14/(O14+P14)"
  Cells(10, 19).Value = "=P14/(O14+P14)"

  Range("R8", "S8").Select
  Selection.NumberFormat = "0.0%"
  Range("R9", "S9").Select
  Selection.NumberFormat = "0.0%"
  Range("R10", "S10").Select
  Selection.NumberFormat = "0.0%"

End Sub
```

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