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AN INTEGRATED APPROACH TO THE ANALYSIS OF THE CIRCADIAN CLOCK OF THE BLOW FLY Lucilia cuprina

Guy Robert Warman

ABSTRACT

The Australian sheep blow fly *Lucilia cuprina* is an economically important dipteran pest whose circadian behavioural rhythms have been the subject of considerable scrutiny. The underlying biochemical nature of these rhythms however, has remained a mystery. The primary objective of this thesis was therefore to investigate the molecular control of circadian rhythms in *L. cuprina* using an integrative approach. To these ends, a dynamic molecular simulation model for *L. cuprina* was formulated using existing biochemical data on insect circadian clocks. The validity of this simulation model was subsequently tested at both molecular and behavioural levels.

The basic molecular assumptions of the simulation model were tested by cloning a full length *L. cuprina per* cDNA and analysing its mRNA and protein expression levels. Isolation of the 4 Kb *L. cuprina per* cDNA revealed the conservation of three functional domains known to be important for circadian clock function; namely the PAS dimerisation motif (with 92% identity to *D. melanogaster* at the amino acid level), and the cytoplasmic and nuclear localisation domains (with 85% and 80% identity respectively). A fourth domain, the threonine-glycine (TG) repeat region, was also found to be conserved, but severely truncated in *L. cuprina*. No length variation was found in the TG repeat of flies collected from several different latitudinal zones, and no correlation was detected between sequences flanking the repeat and latitude of collection of flies. Thus, the contention that the TG repeat region plays a role in temperature compensation of the circadian clock is cast in doubt. Expression analyses (using quantitative RT-PCR) showed *per* mRNA levels to undergo diel oscillations with a period (24 h) and peak phase (Zt 12) consistent with the *Drosophila* data. PER-immunoreactive protein oscillations were also demonstrated, with peak immunoreactivity lagging approximately 3 h behind peak mRNA levels.

The behavioural predictions of the model were tested by recording adult locomotor activity under different light regimes. The simulation model successfully predicted free-run, entrainment, the effect of short light pulses, and the effects of constant
lighting on behavioural rhythms. Disparities between the simulated and real phase response curves for *L. cuprina* are hypothesised to be indicative of an earlier nuclear entry time of the PER-TIM dimer in *L. cuprina* compared with *D. melanogaster*.

The three different approaches of simulation modelling, molecular analysis and behavioural investigation are integrated in the discussion in order to help provide a comprehensive explanation of circadian function in *L. cuprina*. The benefits of an integrated approach to the analysis of circadian function are discussed, as is the relevance of the present findings to the development of a clock-based control strategy for this economically important pest species.
Nothing puzzles me more than time and space; and yet nothing troubles me less, as I never think about them (Charles Lamb, Letter to Southy, 9 Aug. 1815).
ACKNOWLEDGEMENTS

There are a number of people I should like to thank for their continual assistance throughout the course of this thesis. Firstly I should like to extend my gratitude to my supervisor Dr. R. D. Lewis whose knowledge, interest, help and constant encouragement have been invaluable. Thanks are also due to my main supervisor Assoc. Prof. C. Evans for welcoming me into his laboratory which enabled me to conduct the molecular portion of this thesis and for providing an ever-helpful and amazingly diverse understanding of molecular biology.

This work was supported by a PhD scholarship from the Agricultural and Marketing Research and Development Trust (AGMARDT). I should like to thank the trust for this stipend which made the present work possible.

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I am particularly grateful to Dr. C. Millar for adopting me while I was trying to get radioactive techniques going, and for his limitless and good humoured encouragement and advice.

Thank you also to Dr. R. Newcomb conveying his knowledge of cloning genes from flies and for giving me a home during and after the electricity crisis. Thanks also to Anna Fitzgerald for making HortResearch more fun and for introducing me to the HortResearch scones.

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Finally I should like to thank my mother Patricia Warman for supporting me both financially and emotionally through my eternal studenthood, and for spotting the newspaper article about the ‘Ozzie blowfly’ which started this whole thing.
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GLOSSARY OF CHRONOBIOLOGY TERMS

The terminology used here is based on that of Saunders (1982) with modifications.

Active phase ($\alpha$) The time during the sleep-wake cycle during which an animal is active.

Aschoff’s Rule The period of the free-running oscillation ($\tau$) lengthens on transfer from DD to LL in dark active animals, and shortens for light active animals.

Circadian (rhythm) An endogenous oscillation with a natural period close to, but seldom equal to 24 h.

Circadian time (Ct) Time scale covering one full period of an oscillation. Ct 0 is defined arbitrarily (in the present study Ct 6 is defined as the midpoint of the active phase and therefore Ct 0 occurs 6 hours prior to this point).

Desynchronisation Loss of synchrony between two populations of endogenous oscillators involved in the generation of a rhythm.

Diel rhythm A 24 h rhythm that has been measured only in natural or artificial day-night cycles, and not yet known to persist in constant conditions.

Diurnal Active during the day (photophase)

Endogenous rhythm A self-sustained rhythm which continues in the absence of external entraining factors (zeitgeber).
**Endogenous oscillator**  
A self-sustained and temperature compensated oscillator responsible for endogenous rhythmicity.

**Entrainment**  
The synchronisation of an endogenous oscillation to the period of a zeitgeber.

**Free-running**  
A rhythm in its unentrained state (isolated from zeitgeber).

**Free-running period ($\tau$)**  
The period of a free-running rhythm.

**Oscillator**  
The unseen ‘driving’ organ (the biological clock) whose influence from within the organism causes the measurable changes seen as the overt rhythm.

**Period**  
The length of time between the same phase point on two consecutive oscillations.

**Phase ($\Theta$)**  
The instantaneous state of an oscillation within a period. eg. onset of activity.

**Phase advance ($+\Delta\Theta$)**  
The shortening of the period of the rhythm in response to a light or temperature perturbation.

**Phase angle ($\psi$).**  
The relationship between two phase points on the same or different oscillations (phase relationship).

**Phase delay ($+\Delta\Theta$)**  
The lengthening of the period of the rhythm in response to a light or temperature perturbation.
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<td><strong>Singularity ($T<em>S^</em>$)</strong></td>
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<td><strong>Subjective day</strong></td>
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temperature perturbations.

**Ultradian (rhythm)**
An endogenous oscillation with a period many times shorter than the solar day and unrelated to any geophysical cycle.

**Zeitgeber**
The forcing geophysical oscillation which entrains a biological oscillation.

**Zeitgeber time (Zt)**
Time (in hours) relative to the zeitgeber. (i.e. in LD 12:12 Zt 0 is defined as the L-D transition and Zt 12 as the D-L transition).
## GLOSSARY OF MOLECULAR TERMS

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<td>Base pair (bp)</td>
<td>A single pair of complementary nucleotides from opposite strands of the DNA double helix. The number of base pairs is used as a measure of a length of double stranded DNA.</td>
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<td>cDNA clone</td>
<td>A DNA clone derived from a complementary DNA (cDNA) transcript of a mRNA.</td>
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<tr>
<td>cDNA library</td>
<td>A collection of phage containing complementary DNA (cDNA) clones of all of the mRNA species represented in a particular tissue at a particular developmental stage.</td>
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<td>Cloning</td>
<td>The isolation and multiplication of a particular gene by incorporating it into specifically modified phage or plasmid and introducing it into a bacterial cell where the DNA of interest is replicated along with the phage or plasmid DNA and can subsequently be recovered from bacterial culture in large amounts.</td>
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<td>Dimer</td>
<td>A protein made up of two subunits.</td>
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<td>DNase</td>
<td>Deoxyribonuclease: An enzyme which degrades DNA.</td>
</tr>
<tr>
<td>DNA polymerase</td>
<td>Any of several enzymes which catalyse DNA synthesis by addition of deoxyribonucleotide units to a DNA chain using DNA or (in the case of retroviruses) RNA as a template.</td>
</tr>
</tbody>
</table>
Electrophoresis

A technique for separating molecules such as proteins or nucleic acid fragments on the basis of their net charge and mass, by their differential migration through a paper, polyacrylamide or agarose gel in an electric field.

Kilobase (Kb)

Unit of length used for nucleic acids and polynucleotides corresponding to 1000 base pairs or bases.

Kilodalton (kD)

Unit of mass equal to 1000 daltons. One dalton is the unit of mass almost equal to the weight of a hydrogen atom and is used interchangeably with molecular weight.

Northern Blotting

A technique in which RNAs (usually separated by electrophoresis) are transferred to a suitable medium for subsequent hybridisation with radioactive probes for the identification and isolation of RNAs of interest.

Phage (bacteriophage)

A virus infecting bacteria, such as lambda (which infects *E. coli*).

Plasmid

Small self-replicating circular DNA independent of the chromosome in bacteria and unicellular eucaryotes such as yeast, which is maintained at a characteristic stable number from generation to generation. Plasmids typically carry genes for antibiotic resistance and are widely used in genetic engineering as vectors into which foreign genes are inserted for subsequent cloning or expression in bacterial cells.
Poly (A) tail  A stretch of polyadenylic acid residues found at the 3' ends of many eucaryotic messenger RNAs which is added in the nucleus by the enzyme poly (A) polymerase after transcription.

Reverse transcriptase  A DNA polymerase found in retroviruses which synthesises DNA on an RNA template.

Reverse transcription  The synthesis of DNA on an RNA template, catalysed by the enzyme reverse transcriptase.

RNase  Ribonuclease: an enzyme which degrades RNA or cleaves it into shorter oligonucleotides.

RNA polymerase  Any of several enzymes which catalyse the synthesis of RNA from a DNA template by the process of transcription.

Southern Blotting  A technique in which DNA fragments separated by gel electrophoresis in an agarose gel are transferred by blotting to a nylon or nitrocellulose filter for subsequent hybridisation with radioactively labelled nucleic acid probes for the identification and isolation of sequences of interest.

Transcription  Copying of a DNA strand to an RNA strand by an RNA polymerase.

Translation  Process by which RNA directs the synthesis of specific proteins.

Vector  Specifically modified plasmid or phage into which
foreign genes can be inserted for introduction into bacterial or other cells for multiplication or studies of gene expression.

**Western Blotting**

A technique used to transfer the pattern of proteins separated by electrophoresis to a medium in which they can be further analysed.
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>α</td>
<td>alpha (active phase)</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>Ct</td>
<td>circadian time</td>
</tr>
<tr>
<td>ºC</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>DD</td>
<td>constant dark</td>
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<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>Kb</td>
<td>kilobase</td>
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<tr>
<td>kD</td>
<td>kilodalton</td>
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<tr>
<td>L</td>
<td>litre</td>
</tr>
<tr>
<td>LL</td>
<td>constant light</td>
</tr>
<tr>
<td>LD</td>
<td>light-dark cycle (numbers following indicate hours occupied by each)</td>
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<tr>
<td>µg</td>
<td>microgram</td>
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<tr>
<td>µL</td>
<td>microlitre</td>
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<td>µM</td>
<td>micromolar</td>
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<td>M</td>
<td>molar</td>
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<td>mg</td>
<td>milligram</td>
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<td>min</td>
<td>minute</td>
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<td>mM</td>
<td>millimolar</td>
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<tr>
<td>ng</td>
<td>nanogram</td>
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<tr>
<td>g</td>
<td>gram</td>
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<tr>
<td>per</td>
<td>period gene (italicised)</td>
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<tr>
<td>PER</td>
<td>period protein (uppercase, plain face)</td>
</tr>
<tr>
<td>pers. comm.</td>
<td>personal communication</td>
</tr>
<tr>
<td>pg</td>
<td>picogram</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PRC</td>
<td>phase response curve</td>
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<tr>
<td>RT</td>
<td>reverse transcription</td>
</tr>
<tr>
<td>sec</td>
<td>second</td>
</tr>
<tr>
<td>T</td>
<td>period of the zeitgeber</td>
</tr>
<tr>
<td>tim</td>
<td>timeless gene (italicised)</td>
</tr>
<tr>
<td>TIM</td>
<td>timeless protein (uppercase, plain face)</td>
</tr>
<tr>
<td>τ</td>
<td>tau (free-running period)</td>
</tr>
<tr>
<td>Zt</td>
<td>zeitgeber time</td>
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<tr>
<td>Ø</td>
<td>phase</td>
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CHAPTER ONE: GENERAL INTRODUCTION

1.1 An Introduction to Circadian Rhythms

'The earth has made some $15 \times 10^{11}$ turns on its axis since the origin of life. Daily changes in light and temperature have thus accompanied evolution throughout its course......Inevitably, 24 h rhythmicity has become a nearly ubiquitous characteristic of life' (Daan, 1982).

When in 1729 the French astronomer J.J. Ortonts De Mairan reported that daily leaf movements of the heliotrope plant *Mimosa* continued in the absence of external time cues, his colleagues could well have been forgiven for thinking he was losing his mind. What De Mairan had done, however, was make the ground-breaking observation which gave birth to the discipline known as chronobiology. This startling revelation was the first demonstration that organisms possess the innate ability to record the passage of time. It was built on over the next century by the discoveries of DeCandolle (1832), Pfeffer (1875 and 1915) and Darwin (1880) who showed that the period of the rhythm in constant conditions (the free-running period) did not adhere to exactly 24 h (reviewed in Bünning, 1960). The 1960s and 1970s produced a wealth of behavioural and physiological data on the biological clock which was used to formulate mathematical models of clock function. Accompanying clock location experiments disclosed the physical site of oscillatory pacemakers controlling specific elements of behaviour and physiology. The field of chronobiology has now evolved to a point where investigators are beginning to unravel the genetic basis of these timing systems in bacteria, fungi, plants and animals.

Two hypotheses have been proposed to explain the evolution of biological clocks. The first and most commonly held opinion, is that natural selection has operated to increase the survival of those organisms which are able to predict the onset of rhythmic environmental phenomena. The second hypothesis operates at a rather more basic
level in suggesting that biological clocks made available a temporal coordination mechanism which was necessary for symbionts to contribute to the development of eucaryotic organisms (Kippert, 1985). Whatever the driving force behind the evolution of endogenous rhythmicity, it has resulted in a ubiquity almost unparalleled in nature. Virtually all organisms, from the simplest unicellular bacteria through to humans, possess temperature compensated biological clocks which act over a wide range of thermal environments to provide a consistent measure of time.

The fact that most organisms possess a biological clock does not preclude the action of other factors on its output. In fact, geophysical oscillations in particular play an essential role in the regulation of the endogenous clock to a period of 24 h (Büning, 1960). The most commonly accepted entraining agent (zeitgeber) is light, however, a wide range of other factors can be listed, from temperature (Bruce, 1960; Balzer and Harland, 1988) and feeding cycles (Kennedy et al., 1990) through to social interaction (Mrosovsky, 1988).

Circadian timing systems can therefore be seen to result from the action of endogenous clocks with a period close to, but seldom exactly 24 h, which are entrained to 24 h on a daily basis by zeitgeber such as light and temperature cycles. By achieving steady-state entrainment to the zeitgeber, organisms can predict the occurrence of biologically important events and thereby perform certain activities at the most appropriate phase of the daily cycle (Saunders, 1977).

Biological clocks are complex and extensive systems whose output can be measured at numerous different levels and in different tissues. The best analogy to use when describing how the structure of the circadian system is currently viewed, is one which involves a hierarchical assemblage of interacting populations of oscillators. At the top of the regulatory tree are central pacemakers, which themselves can be seen to consist of populations of mutually coupled sub-oscillators (Kronauer et al., 1982; Christensen and Lewis, 1982). These pacemakers are responsible for mediating the oscillatory rhythm of other tissues and organs which act as ‘slaves’. The spontaneous oscillation
of these ‘slaves’ is not precluded (Plautz et al., 1997; Balsalobre et al., 1998), but under normal circumstances they are strongly influenced by the central pacemaker.

The central aims of chronobiology are to elucidate the biochemical nature and physical location of these oscillators, and to determine how they interact with each other to produce the overt processes we recognise as rhythmic behaviour and physiology. The achievement of these goals leads to the development of models to explain how the clock works. These models are continually being renewed and refined, and provide insight into the intricate nature of a complex behaviour.

Since the predominant geophysical cycle on Earth is the day-night cycle, circadian rhythms (those rhythms with a period approximating 24 h) are accordingly the most evident in nature, and are those rhythms which have received the largest amount of research attention. The mechanism underlying the circadian system of the Calliphorid dipteran Lucilia cuprina is the subject of the present work.

1.2 Strategies for Clock Analysis

Circadian clocks have traditionally been studied by measuring clock controlled processes and inferring the status of the clock from the analysis of these driven rhythms. This approach has historically used ‘dry physiology’ or whole organism behavioural techniques in which clock behaviour is deduced from the examination of the effect of environmental manipulation on overt processes (Menaker et al., 1978). A more recent strategy for the analysis of clock function involves the use of ‘wet approaches’ to investigate the anatomical location and biochemical nature of the clock, and its physiological relationships to other systems.

While the whole organism approach has proceeded steadily, and has been aided by the use of non-linear mathematical modelling to infer clock function, the more reductionistic ‘wet approach’ started very slowly. It is a subsection of the latter approach, involving the biochemical and molecular analysis of clock function, which is
now the driving force of circadian rhythm research and the one which is providing the
greatest insight into circadian function in both vertebrate and invertebrate systems.
When viewed together, the tortoise and the hare approaches to circadian investigation
provide a holistic and comprehensive knowledge of how circadian clocks tick.

1.3 Localisation of the Circadian Pacemaker

An obvious approach to investigation of the circadian clock is to attempt to localise
particular elements within an organism. The question of where the central pacemaker
resides has been answered in the past mainly by ablation and transplantation
experiments which, together with the analysis of circadian rhythms in isolated tissue
and organ cultures, has pinpointed the location of pacemakers in a number of different
species.

In mammals, the first demonstration that the suprachiasmatic nuclei (SCN) of the
hypothalamus contained circadian oscillators controlling behaviour and neurosecretion
was shown by selective lesioning experiments (Stephan and Zucker, 1972; Moore and
Eichler, 1972). Proof of the pacemaker role of the SCN came from the use of tau
mutant hamsters (which exhibit a characteristic altered free-running period (see Section
1.7.2)). By reciprocal transfer of SCNs between wild-type and tau mutant hamsters
Ralph et al. (1990) found that the genotype of the donor always determined the period
of the free-running rhythm. The role of the SCN as a circadian pacemaker was thus
firmly established.

Insect pacemakers have also been shown to reside in the brain by a series of ablation
and transplantation experiments, and research to date has led to the generalisation that
exopterygotes (paurometabolous insects whose immature stages resemble adults) have
an optic lobe-based pacemaker (Cymborowski, 1981; Page, 1982; Fleissner, 1982),
while endopterygotes (holometabolous insects with distinct larval pupal and adult
stages) possess a pacemaker located in the midbrain (Cymborowski et al., 1994).
Evidence for the optic lobe location of pacemakers in exopterygotes comes from the demonstration that severance of the optic nerve results in free-run in light cycles, and severance of the optic tract (between the optic lobe and the mid brain) results in arrhythmicity (Nishiitsutsuji-Uwo and Pittendrigh, 1968). Further support is provided by the demonstration that optic lobes transplanted into lobeless Leucophora maderae result in the recipient adopting the behavioural period of the donor (Page, 1982). Roberts (1974) pinpointed the clock to the inner-most layers of the optic lobe (the medulla and the lobula), as removal of the lamina did not cause the destruction of the rhythm. The exact localisation of the cockroach clock to the accessory medulla was recently shown by Reischig and Stengl (1997). The exopterygote circadian pacemaker is now seen to result from the action of bilaterally paired pacemakers in the optic lobes which are coupled via the pars intercerebralis.

In endopterygotes the situation is rather different, as the locomotor oscillator appears to reside in the mid brain. Extirpation of the optic lobes in silk moths (Truman, 1974) and the blow fly Calliphora vicina (Cymborowski et al., 1994) have no effect on the free-running locomotor activity rhythm. Removal of the cerebral lobes, however, results in arrhythmicity (Truman, 1974). In Drosophila brain mutants with reduced optic lobes, such as 'sine oculis' and 'small optic lobes', rhythmicity also persists (Helfrich and Engelman, 1983). The pacemakers of the ground beetle Anthia sexguttata reside in the left and right optic ganglia (in or close to the lobula). These pacemakers can function independently from the rest of the brain and control circadian rhythms of physiological events (Fleissner, 1982; Helfrich-Förster et al., 1998).

A recent publication by Helfrich-Förster et al. (1998) suggests a slightly different branching of the circadian clock location in insects. This comprehensive review of insect clock location research concludes that the accessory medulla is the sole circadian pacemaker controlling locomotor activity in cockroaches and beetles, that in crickets and flies both optic lobe and central brain pacemakers exist, and that in moths the circadian pacemaker resides solely in the central brain (Helfrich-Förster et al., 1998).
Chapter One: General Introduction

The measurement of circadian rhythms in isolated organs and tissues has provided another approach to the elucidation of pacemaker location. The compound action potentials of isolated eyes of the marine gastropods *Aplysia californica* and *Bulla gouldiana* show clear circadian rhythms, and have provided good evidence that these organs contain circadian oscillators (Jacklet, 1969; Block and Wallace, 1982). The measurement of rhythms in *N*-acetyl transferase (which catalyses the conversion of serotonin to melatonin) and melatonin from isolated pineal glands has shown this organ to be the anatomical location of the circadian pacemaker in birds and reptiles (Gaston and Menaker, 1968; Zimmerman and Menaker, 1979).

In the past, further clock location work relied mainly on the measurement of rhythmic SCN electrical activity and metabolic rate *in vivo* (Inouye and Kawamura, 1979) and *in vitro* (reviewed in Gillette, 1991) (reviewed in Schwartz, 1991; Newman, 1991). The advent of molecular genetic techniques has, however, changed the focus of clock location research. The expression patterns of clock genes such as *per* are now used to localise pacemakers. A particularly elegant example of this was the localisation of the locomotor activity pacemaker of *D. melanogaster* to the lateral neurons of the midbrain (Helfrich-Förster, 1996; Helfrich-Förster et al., 1998) (See Section 1.8.3).

1.4 Tracing the Clock Input and Output Pathways

Localisation of the input pathways for clock entrainment and the output pathways which mediate the clock signal to overtly rhythmic processes is another essential element in understanding biological clock function.

1.4.1 Mammalian Clock Input and Output Pathways

The best understood input pathway to a circadian oscillator is that of the input to the mammalian SCN. The main photic input to the SCN from the retina is through the retinohypothalamic tract (RHT) whose glutamatergic afferents provide the primary
light entrainment pathway for the circadian clock (Card and Moore, 1991). The role of the RHT in photic input to the SCN was firmly established by the demonstration that bilateral enucleation or knife cuts result in the loss of entrainment but maintenance of the free-running period (Johnson et al., 1983). Although the RHT is sufficient for photic entrainment, a second pathway appears to be involved in the entrainment process, namely the geniculohypothalamic tract (GHT). The GHT, which projects into the SCN from the retina and the intergeniculate leaflet (IGL), is a common feature of all mammals and has also been shown to participate in the entrainment of circadian rhythms (Rusak et al., 1989). These neuropeptide Y/GABAergic afferents from the IGL are also thought to play a role in the non-photic entrainment of the circadian system (Maywood et al., 1997).

The use of tritiated amino acids and Phaseolus vulgaris leucoagglutinin (PHA-L) as anterograde markers made it possible to trace the efferent projections from the SCN (Watts, 1991). These projections run dorsal to the paraventricular nucleus (PVN) of the hypothalamus. Cells from the PVN project through the medial forebrain bundle to the intermediolateral cell column of the spinal cord. Here they synapse with preganglionic cells that innervate the superior cervical ganglion (SCG) (Watts, 1991). The primary function of the SGC is the sympathetic innervation of the pineal gland, which then produces a rhythm in N-acetyl-transferase. This in turn produces a circadian rhythm in melatonin production which is known to affect a multitude of behavioural and physiological rhythms (Turek, 1991; Illnerová, 1991). As a result of this well-defined pathway, the output from the SCN has been seen in the past to be mainly neural. This proposition has been supported by data showing that an intact PVN is vital for the rhythmic production of melatonin (Hastings and Herbert, 1986). Recent work however, contradicts the theory that SCN output is purely neural by showing the transfer of rhythmicity to result solely from a humoral signal generated in the SCN. SCN tissue which is transplanted into a recipient in a semipermeable copolymer capsule alters the behavioural period of the recipient. This period alteration occurs despite the fact that the graft is prevented from forming neural connections with the host brain (Silver et al., 1996). Rather than providing conclusive evidence that under normal circumstances the output from the SCN is exclusively humoral, these
finding may in fact be implying the existence of redundancy in the clock output pathway.

1.4.2 Insect Clock Input and Output Pathways

As with the insect clock location experiments, the hunt to determine the input and output pathways to and from the clock revealed different systems in exo- and endopterygotes. The photoreceptors for entrainment in exopterygote insects have been localised to the compound eye (Helfrich-Förster et al., 1998). Painting of the compound eye with a mixture of lacquer, bees-wax and carbon black, results in the loss of entrainment in the cockroach (Roberts, 1965), as does bilateral section of the optic nerves (Nishiitsutsuji-Uwo and Pittendrigh, 1968 reviewed in Saunders, 1977, 1982). Removal of the outer synaptic (lamina) region of the optic lobe does not destroy rhythmicity, but causes loss of entrainment, which further clarifies the role of this region in the photoreceptive pathway (Roberts, 1974).

In endopterygotes, extraretinal photoreceptors have been shown to mediate the entrainment signal to the mid-brain clock. Removal of eyes and optic lobes in endopterygotes does not alter the entrainment or free-running period of the circadian locomotor activity rhythm (Truman, 1974; Cymborowski et al., 1994; Helfrich et al., 1985). The existence of extraretinal photoreceptors was confirmed by covering the entire head of three species of silk moth (Antheraea pernyi, Hylaphora cecropia and Samia cynthia) with opaque wax and leaving the compound eyes exposed. Under these conditions activity rhythms free-run (Truman, 1976). The use of Drosophila mutants has further eliminated the possibility of a role of retinal photoreceptors in the entrainment of the endopterygote circadian system. Mutations in the no-receptor-potential-A gene, which abolishes photoreception in the eyes and ocelli of Drosophila, have no effect on entrainment (Konopka, 1980; Dushay et al., 1989). Similarly sine oculis and ocelliless mutants respond normally to photic entrainment (Konopka, 1980; Helfrich, 1986). The exact location of these extraretinal photoreceptors still remains a mystery, although a putative photoreceptor-like organ has been located in adult
Drosophila between the retina and the lamina (Hofbauer and Buchner, 1989 reviewed in Jackson (1993)), and an extraoptic photoreceptor has also been localised to a position between the lamina and the medulla in the tenebrionid beetle Zophobas morio (Fleissner et al., 1993).

The output from the locomotor clock in cockroaches and crickets appears to be neurally based. Cutting the optic tract or the circumoesophageal commissures causes arrhythmia, which would not be expected if the output were humoral (Brady, 1969). Locomotor clock output in silk moths also appears to be neural (Saunders, 1982). Despite the fact that most of the evidence supports neural output pathways in insects, Handler and Konopka (1979) proved the output pathway to involve humoral links in a series of experiments in which they transplanted brains from short period (per<sup>-</sup>) clock mutants of D. melanogaster into the abdomens of arrhythmic per<sup>0</sup> flies, and found that some of the recipients developed a short free-running period (Handler and Konopka, 1979).

Analysis of the arborealisation patterns of the lateral neurons (LNs) in the Drosophila brain (which comprise the locomotor pacemaker) using PDH-immunohistochemistry has resolved these seemingly contradictory results. The processes of the LNs extend not only into the optic lobes but also into the midbrain (Helfrich-Förster, 1996; Helfrich-Förster et al., 1998). The termination site of the central brain processes is in the lateral superior protocerebrum. This in turn is linked to most other brain regions and to the neurosecretory system of the pars intercerebralis and pars lateralis (Helfrich-Förster, 1996). Transplantation of the entire brain may therefore be transferring both neural and humoral links.
1.5 Evidence for the Involvement of Protein Synthesis and the Role of Membranes

Before the use of molecular genetic techniques in the dissection of clock function, experiments relied mainly on the chemical and metabolic manipulation of circadian rhythms in free-running conditions. The rationale behind this type of approach is that the cellular target of a drug may be a part of the clock, or at least intimately associated with it (Edmunds, 1988). In this type of work it is of paramount importance to discriminate between treatments which affect the clock itself and those which merely affect the 'hands of the clock' i.e. the subtle difference between masking and a real clock effect must be determined (Saunders, 1977). These types of chemical manipulation experiments were the main focus of clock function work until the mid 1970s, and showed transcription and translation to be involved in the generation of the circadian rhythm.

In *Gonyaulax polyedra*, treatment with actinomycin D obliterates the circadian rhythm (Karakashian and Hastings, 1962). Protein synthesis was shown to be important in the generation of the circadian rhythm in *Euglena gracilis*, as treatment with cyclohexamide caused a concentration dependent lengthening of the free-running period (Feldman, 1967). In the isolated eye of the sea hare, *Aplysia*, the protein synthesis inhibitor anisomycin was found to damp circadian rhythms in compound action potentials (Jacklet, 1980). An interesting paradox in the marine algae *Acetabularia* must be mentioned here. Dactinomycin abolished circadian rhythms in oxygen evolution in nucleate cells, but in anucleate cells circadian rhythms continued which were unaffected by administration of the drug. The role of the nucleus in generation of the circadian rhythm (in algae at least) was therefore cast in doubt (Edmunds, 1988). It must be reiterated however, that these drugs are non-specific inhibitors of protein synthesis and therefore provide only a crude approach to the analysis of the role of transcription and translation.

In addition to these studies, several other agents were found to alter the phase of the clock, including deuterium, ethanol, and lithium salts (the effects of which include the
ability to influence ionic balances across plasma membranes by affecting either membrane diffusion or permeability). These studies led to the proposal of a membrane model which hypothesised that the generation of a biological rhythm involved the diffusion of an unknown factor ‘X’ across a plasma membrane (Njus et al., 1974; Schweiger et al., 1986).

The combination of the data from the chemical and membrane experiments in *Acetabularia* led to proposal of a coupled translation-membrane model for the generation of circadian rhythms in this species (Schweiger et al., 1986). This model postulated the existence of a polypeptide synthesised on the 80 S ribosomes and integrated into the plasma membrane. The integrated polypeptide was proposed to affect the function of the membrane which resulted in the inhibition of polypeptide production. Degradation of the membrane bound peptide over time was envisaged to release the inhibition and promote polypeptide synthesis (Schweiger et al., 1986). The importance of this model is now largely historical as we know the transcription and translation of specific genes and their autoregulation to generate circadian rhythms in a wide range of organisms. However, it must be remembered that Schweiger’s model was the first to propose a detailed molecular feedback system for the generation of circadian rhythmicity.

### 1.6 The Genetic Basis of Circadian Rhythms

The underlying genetic basis of circadian rhythms was first demonstrated in the bean *Phaseolus* by Bünning in 1935. The circadian rhythm in stem and leaf movement was found to be different in two different strains which exhibited periodicities of 23 and 27 h respectively. Hybridisations between the two strains produced plants with hybrid circadian characteristics and an intermediate period of approximately 25 h (Edmunds, 1988).

According to McClung (1993) ‘The confluence of genetics and molecular biology offers a powerful approach to the study of complex processes such as biological
timekeeping’. This view is one espoused by an increasing number of circadian biologists, and has lead to the wholesale molecular genetic analysis of a number of different organisms in the hope of elucidating the mechanisms by which biological clocks work. Two of the fundamental questions about biological rhythms ask how organisms measure time and how this information is used to regulate overtly rhythmic processes. Both of these questions can be addressed by genetic means, as the isolation of the genes involved in the generation of circadian rhythms provides insight into the molecular mechanisms of circadian regulation (McClung, 1993).

Analysis of mutations in genes which control circadian rhythms provided the single most important modern discovery in the analysis of circadian rhythms, namely the isolation of the *period* (*per*) gene in *Drosophila*. This type of approach has also resulted in the isolation of mutants in a number of different organisms. Among others, these include the *tau* mutant in the hamster, *clock* in the mouse, *frequency* in the fungus, *perl-4* *wc* and *90*, in *Chlamydomonas*, *Ily* in *Arabidopsis*, *ary* in the sheep blow fly *Lucilia cuprina*, and *timeless, dClock, dBmll* and *doubletime* in *Drosophila*.

### 1.7 Circadian Clock Mutants

#### 1.7.1 *Drosophila melanogaster* Mutants

In the introduction to their now famous 1971 paper, Konopka and Benzer summarised the state of understanding of clock function by the following remark: “Many experiments have attempted to probe the mechanism [underlying circadian rhythms], but the nature of the underlying oscillation remains unknown. Perturbations by inhibitors of RNA or protein synthesis suggest that such molecules are involved. Biochemical systems that oscillate with much shorter periods have been demonstrated both *in vivo* and *in vitro* but their relation to circadian rhythms is not clear.” (Konopka and Benzer, 1971). The authors went on from this introduction to describe the first isolation of a biological clock gene (*period*) in *Drosophila melanogaster*, the discovery
of which is still hailed as the most remarkable accomplishment in metazoan clock genetics. The isolation of the three semidominant period mutants; \( \text{per}^+ \) (with a short 19h free-running period), \( \text{per}^l \) (with a long 27h free-running period) and \( \text{per}^0 \) (displaying arrhythmic behavioural patterns) was conducted by large scale chemical mutagenesis and 'brute force' testing of behavioural rhythmicity (Kyriacou, 1994). The mutations described were found to effect the free-running period of both eclosion (emergence of the adult fly from the puparium) and adult locomotor activity rhythms. \( \text{Per}^+ \) and \( \text{per}^l \) mutations not only alter period length but also have allele-specific effects on the photosensitivity and temperature response of the circadian oscillator. Thus period length changes reciprocally in the two mutants in response to changes in environmental light regimes (LL to DD, DD to LL) and alterations in ambient temperature (Konopka et al., 1989).

Two other X-linked mutations have effects on the period of the circadian rhythms in \( D. \ melanogaster. \) These semidominant mutations, clock and andante, shorten and lengthen the free-running period respectively (Dushay et al., 1990; Konopka et al., 1991). Clock was identified in a genetic screen for X-chromosome mutations that affect the free running period, and was mapped to a position 0.015 map units proximal to the \( \text{per} \) gene. Andante was identified in a behavioural screen designed to isolate temperature sensitive mutations which affect the period and or phase of eclosion rhythms, and is the only clock mutation in \( Drosophila \) which is also associated with a morphological marker (Konopka et al., 1991).

In addition to the mutants which affect the period of the circadian rhythm there are also mutants which alter the phase of the rhythm. These mutants include \( \psi \text{-}2 \) and \( \psi \text{-}3 \), which map to the second and third chromosomes, and which result in an advance of eclosion gating by 2 and 3h respectively (Jackson, 1983). A third phase mutant, gate, displays an erratic eclosion rhythm in LD cycles which becomes completely arrhythmic after 2 cycles in DD (Jackson, 1983). The more recently isolated lark mutation is the fourth mutant which has been shown to alter the timing of eclosion. lark function has also been shown to be essential for the maintenance of the eclosion rhythm (Newby and Jackson, 1993; McNeil et al., 1998).
Other genes including *timeless, doubletime, dClock*, and *dBmal1* which play an essential role in the generation of the circadian rhythm in *D. melanogaster* have been isolated using molecular genetic techniques. These will be dealt with in a subsequent section (Section 1.9).

**1.7.2 The *tau* Mutation in the Golden Hamster**

The *tau* mutation in the golden hamster *Mesocricetus auratus* was isolated when a male hamster from a biological supplier was found to have a free-running period of 22 h compared with the 24 h wild-type (Ralph and Menaker, 1988). This mutant was heterozygous for a single autosomal mutant gene *tau*. The *tau* allele was subsequently found to be codominant, and the homozygote to have a phenotypic free-running period of 20 h. This mutation also drastically affects the phase response curve of the animals, and alters the photoperiodic response of breeding such that mutants require only 1 h of light per day to maintain reproductive status compared with the 12 h minimum in wild type hamsters (Menaker and Refinetti, 1993).

**1.7.3 Neurospora crassa Circadian Clock Mutations**

The haploid ascomycete fungus *Neurospora crassa* has long been a model system for the molecular genetic analysis of circadian rhythms along with the fruit fly *D. melanogaster*. In addition to the recently identified *white collar* gene, seven other genetic loci have been identified as being involved in the control of the circadian rhythm of conidiation (sporulation) in this species. Six of these genes; *chr, cla-l* and *prd 1-6* are identified by single alleles. The seventh, *frequency* (*frq*), is identified by 10 alleles. The *frq* locus has been the focus of the largest amount of research. It produces both short and long period mutants some of which have lost the ability of temperature compensation (reviewed in Dunlap et al., 1993). Of particular note is the *prd-6* mutant isolated from *N. crassa* in 1997. This mutant has two features novel to
clock mutations. Firstly, the mutation is temperature sensitive. At restrictive temperatures (above 21°C) the mutation shortens the free-running period, and at permissive temperatures (below 21°C) the mutant has a 20.5 h period close to the length of the wild-type (Morgan and Feldman, 1997). Secondly, prd-6 is epistatic to the prd-2 mutation, and in the double prd-2/prd-6 mutant a temperature compensated 18 h period is detected (Morgan and Feldman, 1997).

The circadian feedback loop in Neurospora is proposed to rely on the actions of frequency and two other genes; white collar-1 and white collar-2 (Crosthwai te et al., 1997). The roles of these two central players is summarised in Fig. 1.3 (Section 1.10). The exact roles of the prd, chr and cla genes are as yet unknown.

### 1.7.4 Clock Mutants of the Alga Chlamydomonas

Following the discovery of a rhythm of phototaxis in the marine alga Chlamydomonas, mutants displaying periods shorter and longer than the wild type were isolated (Bruce, 1972). Analysis of naturally occurring mutants resulted in the discovery of two short period mutants (w/c and 90') and a long period mutant (Lo104). Nitrosoguanidine mutagenesis also produced four long period mutants (per1-4) with periods ranging from 26 to 28 h. Unlike the D. melanogaster and N. crassa mutants, the long period characteristics of these per mutants are unlinked and are controlled by several single genes at different loci. Thus, crosses between single or multiple mutants result in the generation of extra long periods (up to 40 h in the quadruple mutant) (Edmunds, 1988). The function of these clock related genes remains unknown in this species, although there are data which suggest the involvement of a changed metabolic rate (Mergenhagen, 1980).
1.7.5 The Late elongated hypocotyl Mutant of Arabidopsis thaliana

Mutants with disrupted circadian rhythms and photoperiodic responses such as toc and elf have been identified in *A. thaliana* (Millar *et al.*, 1995; Hicks *et al.*, 1996). However, the mutant which has most clearly been shown to be involved in the functioning of the circadian pacemaker of *Arabidopsis* is *Ihy* (Schaffer *et al.*, 1998). This dominant mutation results in a day-length insensitive late flowering phenotype with an elongated hypocotyl and reduced chlorophyll content. It also results in arrhythmic leaf movements and the arrhythmic expression of clock regulated genes (Schaffer *et al.*, 1998).

1.7.6 The psi Mutation in Aedes krombeini

Among the dipteran clock mutants which have been isolated is the *psi* mutant in the tropical mosquito *Aedes krombeini* (Joshi, 1996). The isolation of the *psi* mutant was conducted as part of an ongoing study of various aspects of the circadian physiology of this mosquito. The expression of an altered oviposition rhythm is the phenotypic trait which is altered in the *psi* mutants. Not only is the phase of the oviposition rhythm advanced by approximately 7 h in light cycles, but the free-running period is shortened by approximately 4 h and the photic sensitivity of the adults to phase delaying light pulses is greatly enhanced. This *psi* mutation is proposed to be homologous to the *psi* mutants of *D. melanogaster* (Section 1.7.1), and in the heterozygous state mutants express an intermediate phase angle between the wild type and the homozygous mutant (Joshi, 1996).

1.7.7 The ary Mutant in Lucilia cuprina

The analysis of eclosion in laboratory cultures of the sheep blow fly *Lucilia cuprina* resulted in the discovery of a strain in which eclosion was arrhythmic in constant conditions (DD) (Smith, 1987). Further analysis of adult flight activity showed this
behaviour to be arrhythmic in addition to eclosion. Arrhythmicity of eclosion in one colony was found to result from the homozygosis of a recessive allele *arrhythmic (ary)* on chromosome 5 (Smith, 1987). This allele was proposed to be homologous to the XR arrhythmic allele in *D. pseudoobscura* (Smith, 1987). One note of caution mentioned by Smith (1987) was that all of the behavioural recordings conducted on the *ary* mutant were carried out at the population level. It is thus difficult to distinguish whether *ary* causes a *bona fide* arrhythmia, or whether it merely causes desynchronisation of the population. Further analysis of this mutant was discontinued in the late 1980s and the mutant stocks discarded (*pers. comm.* Smith, 1998).

1.8 The *period* Gene of *Drosophila melanogaster*

1.8.1 Isolation of the *period* Gene

Formal genetic studies of circadian behavioural rhythms began with the discovery of the *per* mutations in *Drosophila*. Earlier work had provided evidence for the heritability of period length, but single-gene mutations with strong measurable effects on rhythmicity were lacking (Baylies *et al.*, 1993). The three behavioural mutants generated by the ethyl methane sulphonate (EMS) mutagenesis of *D. melanogaster* undertaken by Konopka and Benzer (1971) produced periods which were reproducible and heritable. Circadian rhythmicity is altered in both population (eclosion) rhythms, and in individual (adult locomotor) activity patterns, indicating beyond doubt that arrhythmic individuals are not merely desynchronised from other flies but are truly arrhythmic (Konopka and Benzer, 1971).

In addition to altering circadian rhythms, *per* also appears to affect the ultradian courtship song of *D. melanogaster*. The initial findings of Kyriacou and Hall (1980) that periodicity was changed from 54 s in the wild type to 41.5 s in the *per* mutant and 82 s in the *per* mutant (Kyriacou and Hall, 1980), were found to be severely compromised by poor experimental design and subjective analysis methodology.
The proposed effect of \textit{per} mutations on ultradian rhythms thus became a subject of hot debate for many years (Crossley, 1988; Logan and Rosenberg, 1989; Bennet-Clark, 1990). Recent re-analysis (Kyriacou \textit{et al.}, 1993; Konopka \textit{et al.}, 1996; Alt \textit{et al.}, 1998) using less subjective analytical techniques has presented evidence to support the effect of \textit{per} on ultradian rhythmicity. These findings are, however, rather difficult to reconcile with the present understanding of the role of \textit{per} in the generation of circadian rhythms, as the basis of \textit{per} function appears to be strictly circadian and not ultradian (see Sections 1.8.3 and 1.9).

Since chemical mutagenesis proved so valuable a technique in the generation of the three initial \textit{Drosophila per} mutants, it was used repeatedly over subsequent years in research which led to the discovery of five more \textit{per} mutants. To provide room for expansion, the three original mutants (\textit{per}^+ \textit{per}^- and \textit{per}^0) were renamed \textit{per}^+ \textit{per}^{1-1} and \textit{per}^{01}. The first new \textit{per} mutant to be induced and isolated was \textit{per}^{1-2} (Konopka, 1987). This mutant is to all intents and purposes identical to \textit{per}^{1-1} although it exhibits slightly weaker activity rhythms (Hall and Kyriacou, 1990). \textit{per}^\textast\textit{var}, the second new generation \textit{per} mutant isolated (Konopka, 1988), is somewhat unusual (Hall and Kyriacou, 1990). The mutation, when hemizygous in males or homozygous in females, results in apparent arrhythmic behaviour. However, when the \textit{per}^\textast\textit{var} mutation is uncovered by deletions which remove the normal allele (\textit{per}^+) but have one chromosomal breakpoint very near the gene, an anomalous phenotype occurs (Hall and Kyriacou, 1990). These \textit{per}^\textast\textit{var}/\textit{Df} females are usually arrhythmic, but in 5-10\% of cases they are rhythmic with very long periodicities of 30-35 h (Konopka, 1988). Rhythmnicity in \textit{per}^\textast\textit{var} is therefore said to be variegate.

Another new \textit{per} mutation, known as \textit{per}^{04}, is classed in the ‘zero’ series. The jump from \textit{per}^{01} to \textit{per}^{04} is a consequence of \textit{per}^{02} and \textit{03} turning out to be caused by the same nonsense mutation responsible for \textit{per}^{01} (Hamblen-Coyle \textit{et al.}, 1989). The \textit{per}^{04} mutant results not only in arrhythmicity but in hyperactivity. Thus, as in \textit{per}^+, the \textit{per}^{04} mutants display a doubling of activity amplitude (\textit{\alpha}). \textit{per}^{04} also produces an anomalously truncated mRNA in addition to a full-length mRNA (Hamblen-Coyle \textit{et al.}, 1989).
The next new \textit{per} mutant to be described was \textit{per}^{Ch} which reduces the free-running periods of both eclosion and adult locomotor activity from 24 h to 22.5 h, but leaves the ultradian courtship song rhythms unaffected (Dushay \textit{et al.}, 1990). The dissociation of the effect of \textit{per} on circadian and ultradian rhythms in this mutant further confuses the issue of how \textit{per} might affect ultradian rhythms when the very nature of its feedback mechanism is undeniably circadian.

The most recent addition to the \textit{per} family occurred in 1994 when Konopka \textit{et al.} (1994) isolated an ultrashort \textit{per} mutant dubbed \textit{per}^{T}. Heterozygous flies carrying a single \textit{per}^{T} allele exhibit an intermediate (20 h) period between the 16 h homozygote and the 24 h wild-type, and both hetero- and homozygous forms demonstrate a lack of temperature compensation.

In addition to these \textit{in vivo} mutagenesis studies, a particularly elegant series of experiments conducted by Baylies \textit{et al.} (1992) used \textit{in vitro} mutagenesis and P-element mediated transformation to show the existence of a ‘short period mutable’ domain of approximately 20 amino acids (585-601) in \textit{D. melanogaster}. A wide variety of missense mutations in this domain, which lies within the C2 conserved region of PER, predominantly result in short period mutants (Baylies \textit{et al.}, 1992).

To locate the mutant genes responsible for the \textit{per} phenotypes, Konopka and Benzer (1971) measured recombination with respect to morphological markers. This resulted in the location of \textit{per} to the left hand side of the X chromosome. As the three original mutations were mapped to the same area, complementation experiments were conducted to assess whether the mutations represented changes in the same gene. Females with a different rhythm mutation on each of the X chromosomes allowed all of the combinations of mutants to be analysed. Heterozygotes with wild type/arrhythmic and wild type/long period combinations exhibited rhythmic behavioural patterns with a period close to 24 h. The short period/wild type combination produced an intermediate free-running period, and the short period/long period combination produced a mutant with a period close to the wild-type. These data
indicated that the mutations were partially dominant, and it was subsequently concluded that they result from changes in the same gene (Konopka and Benzer, 1971). Further analyses involving synthetic overlapping chromosomal deletions of the per region showed per not to be necessary for viability and the null phenotype to be arrhythmicity (Young and Judd, 1978). Complementation tests were also performed using deficiencies in the X chromosome, and the position of the gene was more finely mapped to the 3B region of the X chromosome between cytological bands 3A6 and 3C2 (Young and Judd, 1978; Smith and Konopka, 1982).

The chromosomal region containing per was independently cloned by two research groups in 1984. Bargiello and Young (1984) isolated the per region DNA by physically mapping the chromosomal breakpoint of a translocation which altered per activity, while Reddy et al. (1984) used microexcision techniques.

Although some splicing heterogeneity was initially thought to occur (Citri et al., 1987), the major biologically active transcript produced from the per locus was found be a 4.5 Kb polyA+ RNA encoding a 1218 amino acid protein (Reddy et al., 1984). Conclusions derived from the original cloning and mapping of per were confirmed by genetic transformation studies. Plasmids carrying the sequence which encoded the 4.5 Kb transcript were found to restore rhythmicity in per\(^\circ\) and null per mutants. Not only did transgenic flies show rhythmic eclosion and adult activity patterns, but the ultradian courtship song periodicity was also recovered (Bargiello et al., 1984; Zehringer et al., 1984).

In their 1971 paper Konopka and Benzer also suggested that the different phenotypes of the three per mutants might be explained if the long and short mutants contained missense mutations producing changes in the quantity or quality of a gene product, whereas the arrhythmic mutant lacked the substance altogether. This hypothesis was borne out when the per mutations were mapped on the DNA sequence. All three mutations involve single base substitutions in protein coding regions of the gene (Baylies et al., 1987; Yu et al., 1987). per\(^\circ\) introduces a stop codon in the N-terminal third of the coding sequence through nucleotide substitution (a cytosine to thiamine...
transition). A thiamine to adenine transversion changes a valine to an aspartic acid in the \(\text{per}^{l}\) mutant, and a transition from a guanine to an adenine in \(\text{per}^{s}\) changes a serine to an asparagine (Baylies \textit{et al.}, 1987; Yu \textit{et al.}, 1987).

1.8.2 Biochemical Characterisation of PER

The overall characteristics of the PER protein are those expected of a soluble protein, despite the predominance of uncharged polar and non-polar residues (Baylies \textit{et al.}, 1987). The two salient characteristics of PER are its PAS domain, which consists of two imperfect repeats and which mediates protein dimerization, and its threonine glycine (TG) repeat region. PER has long been known by researchers in the field as 'the protein from hell' as the assignment of any particular biochemical function has proved extremely difficult (pers. \textit{comm.} Kyriacou). The sequence gave no immediate clues as to action of the protein, but the conspicuous TG repeat was suggested to give PER proteoglycan-like properties.

1.8.2.1 The TG Repeat

Sequence homologies were initially reported between the protein backbone of well-known proteoglycans and PER (Shin \textit{et al.}, 1985; Jackson \textit{et al.}, 1986). Preliminary investigations suggested it may have the characteristics of a heparin-sulphate proteoglycan (Reddy \textit{et al.}, 1986), and that it might form part of the extracellular matrix at the cell boundaries. The TG repeat was proposed to provide sites for \(O\)-linked glycosylation given its similarity to Ser-Gly repeats in mammalian proteoglycans (Yu \textit{et al.}, 1987b). Following a suggestion that proteoglycans affect coupling of cells, the role of PER in gap junctional communication was investigated. Measurements of dye permeability and electrical coupling by Bargiello \textit{et al.} (1987) indicated the protein to be central in intercellular communication. PER was therefore proposed to affect behavioural rhythms by altering gap junctional communication in the CNS of \textit{Drosophila}, acting as a coupling agent rather than as a pacemaker.
(Bargiello et al., 1987). However, these data could not be replicated in later studies (Saez et al., 1992).

The finding that deletion of the entire TG repeat did not affect circadian cycling did not discourage researchers from thinking PER was a proteoglycan. This possibility was finally laid to rest when it was shown that PER is not modified by carbohydrates but is in fact phosphorylated following translation (Edery et al., 1994; Kyriacou, 1994).

So what is the significance of the Thr-Gly repeat? One of the striking features of the repeat is its homology to similar regions of clock genes in *Neurospora* and mammals. The TG repeat and its flanking sequences have been implicated in the control of species-specific song cycles in *Drosophila*, as the phenotypes of transformed flies lacking the entire repeat show a normal circadian rhythm but a significantly shorter ultradian courtship song (Yu et al., 1987). The repeat is also polymorphic in both laboratory and field conditions. In the field the most common variants are 17, 20 and 23 repeat forms. All of these polymorphisms can be related by the insertion or deletion of an 18 nucleotide repeat unit (Yu et al., 1987; Costa et al., 1992). This repeat length has been implicated in conferring thermostability to the circadian phenotype (Costa et al., 1992; Sawyer et al., 1997; Kyriacou, 1997). Evidence for this postulate comes from the analysis of arrhythmic *per* flies which have been rescued with a *per* gene lacking a TG repeat. These flies exhibit rhythmic behaviour, but the ability for temperature compensation is lost (Ewer et al., 1990). These data are further supported by the observation of a structured latitudinal cline in repeat length in *Drosophila* throughout Europe (Costa et al., 1992). Populations of *Drosophila* from Southern Europe show a higher frequency of the 17 repeat form than those collected from Northern Europe, and it is suggested that the different TG alleles may confer on their carriers different adaptive circadian responses to latitudinal temperature variation (Costa et al., 1992). In the laboratory, flies with the 17 repeat form produce a free-running period approximating 24 h in warm temperatures which is decreased at colder temperatures. The converse is true in 20 repeat flies which show a lengthening of free-running period at lower temperatures (Sawyer et al., 1997). Despite the implication of the PAS region of PER as the main player in temperature compensation, it may be
possible that the TG repeat fine-tunes this temperature compensation (Chalmers and Kyriacou, 1993).

1.8.2.2 The PAS Domain

PAS domains were named after the three proteins in which this structural motif was first identified: Drosophila PER, mammalian Arnt, and Drosophila Sim (the product of the single-minded gene). In general, members of the PAS family are transcriptional regulators which control a variety of developmental and physiological events including neurogenesis, toxin metabolism and hypoxia responses (Crews, 1998). These functions are conferred by the ability of the PAS domain to mediate protein-protein interactions.

Once the idea of a negative feedback and the autoregulatory function of per had been hypothesised (see Section 1.8.3), the search began for a region of the PER protein which could indicate this autoregulatory transcriptional function. Consistent with the hypothesis that per was a transcriptional factor was the predominantly nuclear localisation of the protein (Liu et al., 1992). The key to how per performs such a role lay in the PAS domain (Kyriacou, 1994).

PER has been shown to mediate both homo- and heterotypic protein interactions (Huang et al., 1993), but unlike other PAS proteins it lacks a basic helix-loop-helix region. PER PAS can however bind to itself and to other PAS containing proteins (reviewed in Price, 1997). In order for PER to be part of a transcriptional network, it was suggested that it needed a heterodimeric partner. Feedback evidence also pointed to the need of a partner, and in 1995 this heterodimeric partner, Timeless (TIM), was isolated in a yeast two hybrid screen (Gekakis et al., 1995). The gene (timeless) was subsequently cloned (Myers et al., 1995) and shown to encode a central element in the Drosophila circadian system. Mutations in the PAS domain of PER, such as the per\(^{l}\) mutation, have been shown to alter the period and the temperature compensation of the rhythm by altering the ability of this domain to facilitate protein interactions with Timeless (Gekakis, 1995; Price, 1997). PAS is now recognised as a signature motif of
clock proteins (Kay, 1997; Sassone-Corsi, 1997b) and its sequence may provide evidence as to the evolutionary origin of clocks and clock genes (Kay, 1997).

1.8.3 Spatiotemporal Analysis of \textit{per} Expression in \textit{D. melanogaster}

Initial sequence analysis of the \textit{per} gene was disappointing in that a related protein with a defined biochemical function was not found. In an attempt to understand the role of \textit{per} in the generation of circadian rhythms, researchers analysed both the expression patterns of the mRNA and protein, and attempted to localise \textit{per} expression to specific anatomical sites.

The results of expression analyses proved intriguing. Initial analysis relied on the generation of \textit{Drosophila} gynandromorphs carrying a short period (\textit{per}’) circadian clock mutation. This approach localised the locomotor pacemaker to the head (Konopka \textit{et al.}, 1983) and the ultradian courtship song oscillator to the thorax (Konopka \textit{et al.}, 1983; Ewer \textit{et al.}, 1992; Konopka \textit{et al.}, 1996). Further analysis of the locomotor pacemaker implicated a group of neurosecretory cells in the lateral brain (the lateral neurons) in the control of locomotor rhythms (Helfrich-Förster, 1996). Expression of \textit{per} in these cells is not only necessary, but is sufficient for the generation of locomotor activity rhythms (Frisch \textit{et al.}, 1994; Helfrich-Förster \textit{et al.}, 1998).

Histochemical observations, antipeptide antibodies (Siwicki \textit{et al.}, 1988), \textit{per-\beta}-galactosidase fusion genes (Liu \textit{et al.}, 1988) and Northern analysis were also used to examine expression in different tissues and life cycle stages. These studies revealed \textit{per} expression in the embryonic nervous system and salivary glands, in the adult brain and visual systems, the thoracic nervous system, and the ovaries and testes (Siwicki \textit{et al.}, 1988; Saez and Young, 1988). Transcription is first detected early in embryogenesis (Young \textit{et al.}, 1985). Very low levels are detected in larval stages (Bargiello \textit{et al.}, 1987), but during pupal development \textit{per} RNA and protein expression
is detected in the brain, ring gland complex, thoracic ganglia, ovaries and testes (Liu et al., 1988; Saez and Young, 1988; Hardin, 1994).

With an increase in the number of anatomical locations in which *per* expression was examined, came a corresponding increase in the number of sites of detection (Hall, 1995). Recent analysis of *per* expression patterns using *per*-GFP and *per*-luciferase constructs has indicated that *per* is expressed in almost all tissues (including sensory bristles in the wing and leg) (Plautz et al., 1997).

The original finding of Siwicki et al. (1988) that PER-immunoreactivity in the *D. melanogaster* adult visual system fluctuated, provided the impetus for other researchers to analyse the temporal rather than spatial expression of *per*. Shortly thereafter, circadian fluctuations in PER-immunoreactivity were shown to occur in the adult CNS in addition to the visual system, and were shown to exhibit a single peak each day (Zerr et al., 1990). These oscillations in PER protein were subsequently shown to be attributable, at least in part, to the circadian control of *per* transcription, which peaks approximately 6 h before the peak in PER-immunoreactivity (Hardin et al., 1990).

The data of Hardin et al. (1990) were particularly significant in that they presented the first evidence that the cycling of *per* mRNA and protein levels could result from a negative feedback system (Hardin et al., 1990, 1992). This feedback system was further substantiated by the illustration that the period of mRNA and protein oscillations in the different *per* mutants was identical to their behavioural periods, and that the arrhythmic behaviour of the *per*\(^9\) mutants was accompanied by the arrhythmic expression of *per*. Accordingly, the first molecular feedback model involving *per* was proposed which suggested that high PER protein levels inhibited transcription of *per* mRNA (Hardin et al., 1990). All of the current models explaining the molecular control of circadian rhythms in *D. melanogaster* are based on Hardin’s original model.

Building on this solid base, Hardin (1994) showed the rhythmic expression of *per* to occur in many different adult tissues with the exception of the ovary. Recent re-
analysis of these expression patterns using powerful reporter gene technology has made an even more astounding discovery; that almost all tissues not only express per rhythmically, but that these rhythms maintain their photoreceptive properties in explanted tissue (Plautz et al., 1997). In light of these findings, the suggestion was made that a central pacemaker does not exist in Drosophila, however this suggestion does not take into account the very clear demonstration that specific regions of the CNS are directly involved in the generation of rhythmic behaviour (Section 1.3).

1.9 The Missing Molecular Elements of the Drosophila Feedback Jigsaw

The isolation (Sehgal et al., 1994; Gekakis et al., 1995) and subsequent cloning (Myers et al., 1995) of timeless, whose protein product is the heterodimeric partner of PER, advanced the knowledge of clock functioning significantly. PER’s theoretical partner had finally been revealed and was shown to produce circadian oscillations in both mRNA and protein levels (Sehgal et al., 1995). These oscillations occur in phase with those from the per locus, and were found to be acutely altered by the administration of light (Sehgal et al., 1995; Myers et al., 1996). The correct functioning of the circadian system, and the nuclear entry of the PER protein at specific times of the circadian cycle were shown to rely on the action of tim (Sehgal et al., 1994). The attributes of this new player were used to explain previously inexplicable properties of the circadian system such as photic entrainment (Myers et al., 1996; Lee et al., 1996) and temperature compensation (Price, 1997; Sidote et al., 1998). The initial isolation of Timeless relied on its ability to dimerise with PER through the PAS dimerisation domain. More intricate analysis of PER/TIM binding revealed that TIM also binds to a putative cytoplasmic localisation signal on the PER protein and that this, in combination with the fact that there are nuclear localisation signals on both PER and TIM, results in the nuclear entry of the complex (Fig. 1.1)
Nuclear entry of the PER/TIM complex, which occurs only after a specific circadian time (between Ct 19-20) places the complex in the nucleus (a necessary function for the inhibition of transcription). This nuclear entry also helps to explain the phase advancing and delaying effects of light pulses at different circadian times (Myers et al., 1996; Lee et al., 1996). Light pulses resulting in a phase delay degrade TIM while it is building up in the cytoplasm and thus delay nuclear entry and negative feedback. Those pulses falling at advancing times are seen to degrade nuclear TIM and therefore release the repression of per and tim transcription early, causing a phase advance (Myers et al., 1996; Lee et al., 1996; Hunter-Ensor et al., 1996).

Hopes that the sequence analysis of Timeless would reveal a DNA binding domain were dashed when these analyses failed to detect such a region, and the exact function of the PER/TIM dimer in the nucleus remained obscure. One proposed function of the dimer, based on nothing more than pure conjecture, was that it preferentially bound other transcriptional activators which otherwise promote transcription from the per and tim genes. The whole circadian loop was recently closed when this hypothetical transcriptional activator was isolated by two groups in the USA (Allada et al., 1998; Darlington et al., 1998; Rutila et al., 1998) (reviewed in Schibler, 1998). The activator consists of a dimer of the protein products of dClock and dBmall (two Drosophila homologues of mammalian clock genes). The dClock/dBmall dimer (synonymous with Clk/Cyc) binds to E-box sequences in the promotor regions of per and tim.
PER and TIM proteins block dClock’s ability to transactivate their promoters via the E-box (Darlington et al., 1998; Rutila et al., 1998).

Within a month of the publication of these stimulating results, the discovery of a further element in the feedback loop was described. Using EMS mutagenesis Price et al. (1998) isolated three alleles of a mutant which they named doubletime, whose phenotypes were short (dbf) and long (dbf') behavioural periods, and (dbf') which eliminates larval per and tim cycling and which causes pupal lethality (Price et al., 1998). Due to the fact that PER proteins constitutively accumulate, remain hyperphosphorylated and no longer depend on TIM for their accumulation in the dbf' mutant, Doubletime protein is proposed to reduce the stability of PER and therefore the level of cytoplasmic accumulation of monomeric PER proteins (Price et al., 1998). The significant delay between the peak in per and tim transcription and PER/TIM complex formation, which is an essential element of the D. melanogaster feedback loop, can now been seen to be attributable in part to the action of Doubletime (Price et al., 1998).

The molecular feedback model which summarises the interaction of these genes in the generation of the feedback loop is represented below in a diagrammatic form (Fig. 1.2).
Rhythmicity can be seen to result from the action of a per and tim feedback loop. per and tim mRNA are transcribed and translated. Transcription from these genes is driven by the binding of the dClock/dBmal1 dimer to the E-box of per and tim regulatory elements (Darlington et al., 1998; Rutila et al., 1998). The formation of the PER/TIM dimer in the cytoplasm is delayed by the binding of PER by Doubletime (Price et al., 1998). A light pulse at this time results in the rapid degradation of TIM and a subsequent lag in nuclear entry (Lee et al., 1996; Myers et al., 1996; Hunter-Ensor et al., 1996). When the heterodimer does form, it undergoes nuclear entry during a specific time window (Ct 19-20) (Lee et al., 1996). The nuclear complex then binds to dClock/dBmal1 thereby removing the transcriptional activation of per and tim (Darlington et al., 1998; Rutila et al., 1998). In the absence of photic stimulation during this time, the PER/TIM dimer degrades and the transcription of per and tim is resumed. In the presence of a light pulse, the nuclear degradation is expedited and therefore the phase of the circadian cycle is advanced (Lee et al., 1996; Myers et al., 1996; Hunter-Ensor et al., 1996). This particularly elegant feedback loop can be seen
to generate time-delayed negative feedback and therefore circadian oscillations in \textit{period} and \textit{timeless} mRNA and protein levels. If the titre of cytoplasmic TIM protein, for example, is seen to affect the transcription of downstream genes, then it becomes easy to envisage how circadian oscillations of hormonal and neuronal firing can be produced which give rise to rhythmicity at behavioural and physiological levels.

1.10 The Same...But Different: Molecular Circadian Organisation in Other Species

Isolation of full-length \textit{per} homologues from a number of different species including the giant silk moth \textit{Antheraea pernyi}, (Reppert \textit{et al.}, 1994), and more recently mice (Tei \textit{et al.}, 1997; Sun \textit{et al.}, 1997; Zylka \textit{et al.}, 1998a), rats (Sakamoto \textit{et al.}, 1998), and humans (Tei \textit{et al.}, 1997), along with the isolation of partial \textit{per} fragments from a number of insect species (Reppert \textit{et al.}, 1994; Nielsen \textit{et al.}, 1994; Regier \textit{et al.}, 1998) has illustrated the wide degree of preservation of this central clock component. In addition to the detection of \textit{per} homologues in other species, homologues of mammalian clock genes such as \textit{Clock} and its heterodimeric partner \textit{Bmal1} have been isolated from \textit{Drosophila} (Darlington \textit{et al.}, 1998; Rutila \textit{et al.}, 1998). A very recent publication by Sangoram \textit{et al.} (1998) has outlined the isolation of mouse (\textit{mtim}) and human (\textit{htim}) \textit{timeless} homologues, and has shown the hTim protein to dimerise with \textit{Drosophila} and mouse \textit{PER}, both \textit{in vitro} and \textit{in Drosophila} S2 cells, and to feed back on the Clock/Bmal1 complex (Sangoram \textit{et al.}, 1998). This finding provides further proof of clock conservation in a wide range of organisms. Members of the \textit{Neurospora} circadian loop \textit{frq} and \textit{wc-1} and \textit{wc-2} also show a limited amount of homology to other clock genes in that they contain PAS domains (Kay, 1997).

Despite the preservation of these homologues, \textit{per} has diverged extremely rapidly at the primary sequence level (Regier \textit{et al.}, 1998). The subtle functioning of these homologues is also amazing diversely diverse, even within the insects. While in the fruit fly cycling of \textit{per} and \textit{tim} mRNAs and proteins, and the nuclear entry of the PER/TIM
dimer are essential for the generation of a circadian rhythm, in Lepidoptera oscillations of sense and antisense mRNA circumvent the need for nuclear entry in the generation of the circadian rhythm (Sauman and Reppert, 1996). In the house fly, *Musca domestica*, neither *per* mRNA nor protein levels appear to cycle, and to date there has been no evidence of nuclear entry (pers. comm. Kyriacou, 1997). The mechanistic nature of the house fly feedback loop thus remains elusive. The functional diversity of circadian clocks is further illustrated by the murine system. Mice not only possess three *per* homologues which respond differentially to light at particular times of the circadian cycle (Shearman et al., 1997; Zylka et al., 1998a), but mTim levels in the SCN and retina do not oscillate (Sangoram et al., 1998) and are not acutely altered by light during the subjective night (Zylka et al., 1998b). To add yet another twist to the murine story, mTim protein does not interact with mPer (Zylka et al., 1998b).

The parallels between the different clock systems cannot be overlooked however as there are striking similarities. A review by Dunlap (1998) proposes an archetypal circadian clock and the diversification of the *Neurospora, Drosophila* and mammalian clocks from it. This figure is reproduced below (Fig. 1.3) and shows the common elements of these three major systems and the generation of a feedback loop in all of them.

Fig. 1.3. Archetypal circadian clock and its diversification into the *Neurospora, Drosophila* and mammalian systems. (excluding mTim). Grey indicates hypothesised elements. (after Dunlap, 1998).
As attractive as these models appear, there are what Dunlap (1998) calls 'discomforting facts' which hint that the system is vastly more complex than is presently believed. PER protein cycling has been found to continue weakly in the absence of mRNA cycling (Cheng and Hardin, 1998) and levels of per mRNA begin to decline before the PER/TIM dimer is predominantly nuclear (So and Rosbash, 1997). Both of these findings suggest additional regulatory loops. It must also be remembered that there are exceptions to the rule in the moth Antheraea pernyi and the house fly Musca domestica. The existence of antisense (potential gene silencing) strands in both the moth and the fungus Neurospora is suggestive of further regulatory loops (Sauman and Reppert, 1996; Dunlap et al., 1995). It is thus more than likely that the circadian clock consists of several different interacting autoregulatory loops.

1.11 The Molecular Basis of Entrainment

One of the missing links in the molecular characterisation of the circadian clock has been the molecular basis of entrainment. Both light (Lee et al., 1996; Hunter-Ensor et al., 1996; Zeng et al., 1996) and temperature (Sidote et al., 1998) have been shown to degrade TIM and PER and to have a phase-shifting effect on the clock. An understanding of how state variables are modulated by external stimuli is proposed to provide insights into the mechanisms underlying entrainment (Sidote et al., 1998). Exactly how these environmental signals are perceived by the clock remains to a large extent unknown. Until recently, the only clue as to how entraining signals are mediated was provided by the analysis of immediate early gene (IEG) expression in the mammalian SCN. However, the publication of several recent papers has outlined the potential roles of cryptochromes and novel retinal opsins in entrainment, and is very literally shedding more light on the situation.
1.11.1 The Role of IEGs in Entrainment

The initial discovery that light induced the expression of the IEG c-fos in the ventrolateral portion of the SCN was made in rodents (Aronin et al., 1990; Kornhauser et al., 1990). These experiments showed the light induction of Fos to result from an increased expression of the c-fos mRNA. Initial experiments such as these were rapidly built upon, and it was not long before a second startling discovery was made; that c-fos expression was increased only in response to those light pulses which phase shifted overt behavioural rhythms (such as wheel-running activity) (Rusak et al., 1990). The expression of c-fos was therefore proposed to be intimately linked with circadian clock entrainment. This expression pattern of c-fos is paralleled by a second IEG, jun-B (Kornhauser et al., 1992), and as Fos and Jun are known to form heterodimeric AP-1 complexes, it is thought that the combinatorial action of these two proteins is important in entrainment of the circadian clock.

As IEGs are signal transducers coupling short term cell stimuli into longer term transcriptional regulatory changes (Morgan and Curran, 1989) their expression is transient. After a light pulse at the appropriate circadian time, expression of c-fos and jun-B is increased. This increase is rapid and transient, peaking 30 min after the onset of light and returning to basal levels as soon as 2 h after initial induction (Kornhauser et al., 1992, 1993).

There has been much debate in the past about whether the expression of c-fos and Jun-B is involved in the entrainment pathway or whether it is merely a result of entrainment (ie. whether IEG expression is a cause or an effect). This debate is due mainly to the fact that IEGs are expressed in response to diverse stimuli, including drug induced seizure, epilepsy, heat shock and photic and olfactory stimulation (Calamandrei and Keverne, 1994). Conclusive evidence for the role of IEGs in the entrainment pathway came in 1995 when Wollnik et al. (1995) demonstrated that administration of antisense constructs specifically directed against c-fos and Jun-B prevented the phase shifting effects of light pulses at specific circadian times. IEG
expression thus seems inextricably linked to the entrainment of the circadian pacemaker at least in mammals.

The binding of AP-1 to downstream genes can be seen to affect the pacemaker by entraining the free-running rhythm to that of the environmental cycle. However, as the induction of c-fos in response to light only occurs at certain circadian times (Kornhauser et al., 1990; Rusak et al., 1990), and as there is no circadian fluctuation of c-fos under constant conditions, the pacemaker must exert an upstream gating effect on the expression of the c-fos gene. This effect may act at many different levels:

1. At the light input pathway: the oscillator may have an effect on the retinal ganglion cells, the RHT or in the SCN cells of mammals by either changing the electrical excitability of the neurons or by altering synaptic transmission.
2. The signal transduction pathway may be altered ie. second messenger molecules (protein kinases) could be changed/modulated.
3. The pacemaker could act directly on Fos through positive/negative regulatory transducing factors (eg. oxidation of the cistine residues on any IEG results in the protein being unable to bind DNA). Thus, the post-translational modification of the Fos protein necessary to render Fos in an active state, may only occur at appropriate circadian phases (Morgan and Curran, 1989).

The IEGs provide a tantalising but frustrating insight into the molecular mechanisms of entrainment. The expression patterns of c-fos and jun-B seem inseparably linked to the entrainment of the circadian clock and yet the target gene to which the AP-1 complex binds remains elusive.

1.11.2 Cryptochromes and Opsins: the Entrainment Panacea?

The search for further molecules involved in photoentrainment was launched from two different directions, one based on the analysis of photosensitive pigments known as photolyases and cryptochromes, the other focussed around the isolation of novel vitamin A based retinal opsins.
An analysis of the role of photopigments related to the cryptochromes of *Arabidopsis* (Mathews and Sharrock, 1997; Quail *et al*., 1995) in the control of circadian photoreception became possible when homologues were isolated in rodents (Todo *et al*., 1996), *Drosophila* (Emery *et al*., 1998) and humans (Hsu *et al*., 1996). The functional analysis of these cryptochromes has revealed an interesting but as yet poorly understood tangle of data. In *Arabidopsis*, a range of phytochromes and cryptochromes appear to be involved in the entrainment of the circadian oscillator (Somers *et al*., 1998). Mice possess two cryptochromes (CRY1 and CRY2) and mutants lacking CRY2 display a reduced sensitivity to acute light induction of mPer1 in the SCN, a free-running period 1 h longer than wild-type mice, and high amplitude phase shifts in response to light at Ct 17 (Thresher *et al*., 1998). The phenotype of the *D. melanogaster* null cryptochrome mutant (cry3) is one of poor synchronisation to light dark cycles, and the absence of behavioural responses to brief light pulses (Stanewsky *et al*., 1998). Levels of *Drosophila* CRY protein are drastically altered by light exposure, and overexpression of cry causes heightened photic sensitivity (Emery *et al*., 1998). The entrainment role of the cryptochromes (at least in mammals) is cast in doubt, however, by the fact that the known action spectra for circadian phase shifts do not confer with those of the cryptochromes. For this reason it has been suggested that cryptochromes do not form the basis of the primary photoreceptors mediating circadian photoreception (Lucas and Foster, in press).

Justification for the hypothesis that photoreceptors mediating mammalian circadian responses differ from the classical photoreceptors of the visual system came from the observation that degeneration of rod and cone photoreceptors is not associated with a reduction in sensitivity of circadian responses to light (Foster *et al*., 1991; Provencio *et al*., 1994). Mice completely lacking both rod and cone photoreceptors exhibit entrainment and light induced suppression of melatonin (Lucas and Foster, in press). Cloning of two novel opsins; VA opsin (Soni and Foster, 1997; Soni *et al*., 1998) and melanopsin (Provencio *et al*., 1998) has led to the suggestion that these molecules are involved in the clock entrainment pathway (Foster, 1998; Lucas and Foster, in press). Support for this thesis comes from the fact that action spectra indicate a vitamin A-
based pigment is involved in circadian photoentrainment and the novel opsins are vitamin A-based (Lucas and Foster, in press). The ubiquity of the role of these vitamin A-based opsins for entrainment is, however, brought into question by the indication that in Drosophila complete vitamin A depletion has no effect on light mediated entrainment (Zimmerman and Goldsmith, 1971).

Despite this new wave of photoentrainment research, the jury is still out on how the photic entrainment signal is mediated. Although action spectra for phase shifting appear to be consistent with a single photopigment there may be redundancy in the entrainment pathway (Van Gelder, 1998). The gap still remains therefore between zeitgeber and the clock, but with the speed of recent progress this may not be the case much longer.

1.12 Molecular Connectives to the Hands of the Clock

Although the anatomical analysis of output pathways from the central pacemaker to driven processes has progressed well (with the analysis of neural and humoral links both in vertebrate and invertebrate systems), little is known about the molecular mechanisms required for the transmission of temporal information from the clock to the physiological and behavioural processes it regulates. It is generally assumed that PER or TIM control the circadian expression of other genes (Van Gelder and Krasnow, 1996), but candidates for this control have only recently come to light. In a variety of species clock controlled mRNAs and proteins have been characterised (Green and Besharse, 1996; Van Gelder and Krasnow, 1996), but the output function of most of these genes is uncertain (McNeil et al., 1998). A mammalian exception is the transcription factor DBP (Wuarin and Schibler, 1990; Lavery and Schibler, 1993; Lopez-Molina et al., 1997) whose rhythmic expression is clock controlled and is reset in tissue culture by serum shock (Balsalobre et al., 1998). DBP is thought to be involved in readying the body to combat potential toxins present in food (pers. comm. Schibler, 1998). A Drosophila clock-controlled gene whose function has been demonstrated is lark (Newby and Jackson, 1996; McNeil et al., 1998). Lark protein
undergoes circadian changes in abundance in per wild-type flies, but in arrhythmic per
mutants the circadian nature of these fluctuations is eliminated (McNeil et al., 1998).
The role of lark appears to be in the control of eclosion gating, as increased lark gene
dosage leads to a late-eclosing phenotype (Newby and Jackson, 1996). Furthermore
null lark alleles have dominant effects on daily timing of adult eclosion but do not
affect the circadian rhythm of adult locomotor activity (Newby and Jackson, 1996).

Many of the humoral aspects of clock output which have been demonstrated in
mammalian systems have also been inferred in insects. Serotonin has been implicated
in the regulation of locomotor activity and phase shifting in crickets (Cymborowski,
1970), cockroaches (Page, 1987), praying mantis (Germ, 1997) and blowflies
(Cymborowski, 1998). However, the genes which encode these hormones have not yet
been isolated.

1.13 Quantitative Modelling of Biological Oscillators

The production of models to explain biological processes is fundamental to the
understanding of complex biological phenomena. The ultimate goal of any biologist is
to produce models of one type or another to explain the ways in which cells, organisms
or communities function. A successful model summarises available experimental
evidence and is able to predict the behaviour of the system under new circumstances
(Pavlidis, 1978a).

If the aim of biology is the development of models, then the aim of chronobiology is to
develop models of clock systems using current data which will account for rhythmic
behaviour and reflect the nature of the timing system (Lewis, 1994). The first insights
into clock function were provided by a series of models which inferred the clock
mechanics by observing the effects of environmental manipulations on overt processes
driven by the oscillator. With the aid of molecular genetic techniques the concrete
nature of the underlying molecular clockwork is being dissected, and the models used
to describe these clock-workings are becoming increasingly complex.
Despite the increased insight into the very heart of the clock provided by molecular biology, the quantitative modelling of circadian oscillators has not lost any of its import. The use of mathematical computer models allow us to determine whether molecular ‘word’ models can actually produce a functional circadian system (Lewis et al., 1997). By producing a model which contains all of the elements described by molecular researchers, one can assess whether these elements can interact to generate a functional feedback loop and therefore produce a circadian rhythm in the way envisaged by the molecular biologists.

Circadian clock models have evolved from the relatively simple notion that circadian rhythms result from the non-sinusoidal oscillation of a ‘chemical’ which passes through a threshold twice a day. Activity was considered to be induced only when the concentration of the ‘chemical’ exceeded this threshold (Aschoff et al., 1971). From these humble beginnings the ideas of negative feedback and time delay were introduced as central elements of the oscillator (Sweeny, 1976; Njus et al., 1974; Gander and Lewis, 1979) which was viewed as a membrane bound entity producing a chemical (X). This compound was seen to diffuse passively across a membrane (time delay), to a sensor which confers feedback (Gander and Lewis, 1979). Temperature compensation in this system was achieved through lipid adaptation (Njus et al., 1974). These models grew in complexity and in predictive value to the point where they were able to predict the complex labilities exhibited by the circadian system such as spontaneous changes in free-running period, scalloping and shattering (Lewis et al., 1991).

In keeping with Pavlidis’s dogma, models have now been produced which summarise the molecular data available and predict the responses of the circadian clock at the behavioural and molecular level to environmental perturbations (Lewis et al., 1997; Leloup and Goldbeter 1998). These models are also able to predict the different responses of clock mutants to environmental regimes (Lewis et al., 1997; Leloup and Goldbeter, 1998).
1.14 The Sheep Blow Fly *Lucilia cuprina*

The study organism in the present work is the Australian sheep blow fly *Lucilia cuprina*. *L. cuprina* is a calliphorid dipteran which was first described by Wiedemann in 1830 under the genus *Musca* (Waterhouse and Paramanov, 1950; Zumpt, 1965). Differentiation of *L. cuprina* from the closely related *L. sericata* relies mainly on the microscopic examination of the size, number, and arrangement of setae in the head region. The one salient macroscopic character is fore-leg colour, which is iridescent green in *L. cuprina* and black in *L. sericata* (Waterhouse and Paramanov, 1950; Holloway, 1991; Bishop, 1991). A subspecies level classification of *L. cuprina* was made in the taxonomic research of Waterhouse and Paramanov, who proposed a subdivision of *L. cuprina* into *L. cuprina dorsalis* and *L. cuprina cuprina* (Waterhouse and Paramanov, 1950). This classification was not widely embraced when first presented (Zumpt, 1965), but it has grown in popularity and the existence of two subspecies which can be classified on their habitat preferences and anatomical features is now widely accepted (Norris, 1990). *L. cuprina cuprina* colonises carcasses more frequently than live hosts, and is a less voracious pest than *L. cuprina dorsalis* which is almost exclusively restricted to live hosts in Australasia (Waterhouse, 1947; Norris, 1990).

Of the two subspecies, *L. cuprina dorsalis* has been documented as occurring in Africa, Australia, India and New Zealand, and *L. cuprina cuprina* has been collected in the New World, Asia, Indonesia, and Australia (Norris, 1990). As *L. cuprina dorsalis* has been the only subspecies of *L. cuprina* described in New Zealand, use of the specific name *L. cuprina* will be in reference to *L. cuprina dorsalis*. 
1.15 The Life Cycle of *Lucilia cuprina*

A member of the saprophagous evolutionary root of dipterans, *L. cuprina* is defined as a facultative parasite inhabiting carcasses, rotting vegetable matter and dung in addition to live vertebrate hosts (Zumpt, 1965). However, in Australasia *L. cuprina* is almost entirely restricted to live hosts, competing poorly with other species in carcasses and other potential habitats (Dymock and Forgie, 1993). A detailed description of the life-cycle of *L. cuprina* has been documented elsewhere (Warman, 1995) so for the sake of brevity a life cycle is represented below in graphical form (Fig. 1.4)

![Diagram of the life cycle of *Lucilia cuprina*.](image)

Fig. 1.4 Diagrammatic representation of the life cycle of *L. cuprina*. Stages are indicated in upper case and events in lower case. Those events which are known to be clock controlled are italicised. The number of days after hatching that each of the events occur (at 25°C) is indicated. (After Warman, 1995).

1.16 Flystrike and *Lucilia cuprina*

The most useful definition of flystrike is that provided by Zumpt (1965) who states it to be: ‘The infestations of live human or vertebrate animals with dipterous larvae, which, at least for a certain period, feed on the host’s dead or living tissue, liquid body-substances or ingested food’ (Zumpt, 1965).
'Flystrike' or 'fly-blow' is the condition of ovine cutaneous myiasis (Zumpt, 1965). In this case eggs are laid on the live host, first instar larvae irritate the skin to form an oedematous lesion, and feed on the resultant exudate (Bowles et al., 1988). Second and third instar larvae feed invasively using high activity proteases and tearing mouth-hooks to digest the dermal and subdermal layers of the host (Greenberg, 1973; Bowles et al., 1988). If sustained strikes occur, large amounts of musculature may be infected, and larvae may even invade the peritoneal cavity (Zumpt, 1965; Heath, 1986). The pathological effects on sheep are numerous and have again been reviewed elsewhere (Warman, 1995), but in addition to mechanical and secondary infection problems, behavioural abnormalities including anorexia cause severe problems to the infested sheep.

The first recorded case of flystrike in New Zealand was in 1896 (Heath, 1986). By the 1900s flystrike flies were well established throughout the country. Until the 1980s, however, flystrike was a seasonally occurring problem of temporary concern to farmers. From 1982 onwards there was a significant increase in the prevalence and severity of flystrike in New Zealand (pers. comm. Heath, 1995), and in 1988 L. cuprina was identified in collections of larvae from feral goats at Paparoa (36° 06’S, 174° 13’E) (Holloway, 1991). Re-examination of samples collected from 1984-87 have confirmed the wide distribution of this species in the North Island by the mid 1980s (Bishop, 1993). From this evidence it is suggested that L. cuprina has been present in New Zealand since the mid 1970s (Holloway, 1991; Bishop, 1993), with the introduction of this pest most probably occurring from Australia in infected stock or farming equipment (pers. comm. Heath, 1995). The arrival of L. cuprina in New Zealand has coincided with an apparent doubling in flystrike prevalence (Heath, 1990), and this species has now been recorded as far south as Rakaia (43° 45’S 172° 01’E) (pers. comm. Heath). L. cuprina is a far more persistent pest than the other species of blow fly in New Zealand, surviving almost solely on live hosts (Dymock and Forgie, 1993), and has most probably been the main contributor to an estimated $37 million annual loss to the New Zealand sheep farming industry (Gleeson et al., 1993).
1.17 Pesticide Resistance and *Lucilia cuprina*

The profligate use of the organochlorines in the 1940s and 1950s induced resistance development in many insects (Carson, 1962; van Emden, 1989) including the sheep blow fly *L. cuprina*, and by the mid 1960s the use of these pesticides in the control of *L. cuprina* had been abandoned (Shanahan, 1966).

The second class of insecticides used in an attempt to control flystrike were the organophosphates, with the most commonly used being diazanon. The first recorded case of field strains of *L. cuprina* developing resistance to organophosphates was in 1965, and monogenic and polygenic resistance has now been described in *L. cuprina* collected throughout Australasia (Hughes and Raftos, 1985; Parker et al., 1991).

Two types of organophosphorous resistance are associated with a reduction in ‘ali-esterase’ (E3 isozyme) activity in *L. cuprina* (Campbell et al., 1998). Diazanon resistance is attributable to an amino acid substitution in the enzyme’s active site which increases the rate of dephosphorylation yielding organophosphate turnover, and which abolishes ali-esterase activity (Newcomb et al., 1997). Malathion resistance results from a different amino acid substitution in the same E3 gene which also results in decreased E3 activity (Campbell et al., 1998).

An increase in control problems, particularly with diazanon usage in New Zealand, prompted an investigation of the resistance status of *L. cuprina* in New Zealand using both biochemical investigations of E3, and toxicological means (Gleeson et al., 1993). This research has indicated a high level of resistance to diazanon throughout New Zealand (Gleeson et al., 1993).

The chemical control of flystrike is now almost exclusively restricted to the use of synthetic pyrethroids, synthetic insect growth regulators and *Bacillus thuringiensis*. The reduction of control options is a strong incentive to investigate an integrated pest management (IPM) approach to the control of this parasite, and should inspire the
frugal use of current chemical control methods to ensure an increase in their effective lifetime.

1.18 The Circadian Clock of *Lucilia cuprina*

Not only are different clocks proposed to exist in individual organisms to control seasonal and daily patterns, but multiple circadian clocks are also thought to exist at different developmental stages to control the timing of crucial events in the life cycle. The existence of multiple light entrained oscillators controlling the timing of behaviours at different life cycle stages was dramatically demonstrated in the flesh-fly *Sarcophaga argyrostoma* by Saunders (1986). The presence of multiple oscillators in *Sarcophaga* prompted an examination of rhythmic behaviours in the life cycle of *L. cuprina* (Warman, 1995). In total five different behaviours have been shown to be rhythmic and under circadian clock control. During larval development there are at least three different rhythmic events; post-feeding larval exodus, larval wandering and larval burrowing (Warman, 1995; Warman and Lewis, 1997, Smith *et al.*, 1981) which appear to be under the control of at least two distinct endogenous circadian clocks (Warman, 1995). The restriction of these behaviours to a specific phase of the day-night cycle (around dawn) is proposed to reduce the exposure of the vulnerable larvae to desiccation and predation while they are in a relatively unprotected environment (Warman, 1995). Eclosion is the only pupal rhythm which has been investigated in *L. cuprina*. It is highly gated (Warman, 1995; Smith, 1985) and under the sole control of an endogenous clock with a free-running period of 24 h. The locomotor activity of both individual adults (Warman, 1995) and populations (Smith, 1983) is also clock controlled, and the 22.5 h free-running period exhibited is similar to that of other blowflies (Saunders, 1982; Hong and Saunders, 1994).

The common theme among all of the behaviours analysed is that they all result from the action of endogenous pacemakers which maintain, with the exception of eclosion, a free-running period deviating from 24 h. In order for the behaviours to be expressed at the correct phase of the day-night cycle, the oscillators are entrained on a daily basis by
light (Warman, 1995). The basic properties of circadian oscillators, namely free-run and entrainment, have thus been demonstrated to exist and play an important role in the timing of vital behavioural events in the life cycle of this fly.

1.19 Research Aims

The combination of the fact that *L. cuprina* has been well characterised at a behavioural level, and that a naturally occurring arrhythmic mutant has been isolated makes it an ideal choice of study animal for the comparative analysis of circadian function in dipterans. Its phylogenetic relationships to other species which are being investigated further increases its attractiveness as a subject.

The aim of the present work was to develop and test a dynamic model of the circadian system of *L. cuprina* in order to provide a comparative approach to the analysis of fly circadian clocks. To these ends a simulation model of the *L. cuprina* circadian clock (based on *D. melanogaster* molecular data and *L. cuprina* behavioural data) was developed, and a *L. cuprina* *per* cDNA was cloned and its mRNA and protein expression levels quantified. Molecular and behavioural analyses were used to test the simulation model and to exemplify the similarities and differences between the blow fly clock and the fruit fly clock. The model was also used to highlight the areas of molecular research which require immediate attention.

An increased understanding of the molecular processes underpinning behavioural rhythmicity furnished by the model provide the basis from which a biological clock-based pest control strategy may be built. Finally, this thesis illustrates that the amalgamation of concepts from 'classical' and 'molecular' chronobiology provides an invaluable tool in the analysis of circadian rhythms, and that without the use of both the understanding of biological clocks is severely compromised.
CHAPTER TWO: DEVELOPMENT OF A MOLECULAR SIMULATION MODEL FOR THE CIRCADIAN CLOCK OF L. CUPRINA

2.1 An Introduction to Circadian Modelling

2.1.1 Models Defined

In establishing the usefulness of quantitative circadian models, an acceptable definition of a model must first be established. The most appropriate definition of a model is that provided by Pavlidis (1973), which states 'Basically a model is a hypothesis about how a physical system works. In general it must have the following two essential properties: (1) It must summarise the available experimental evidence so that the description of the physical system through the model must be more concise than the description through a table of experimental results. (2) It must predict the behaviour of the system under new circumstances'. Pavlidis (1973) also added that a model need not say anything about the 'deep' structure of the system. However, as knowledge of the 'deep structure' (or molecular basis) of circadian clocks has become more refined, the circadian models which are developed must account for these data. Inevitably then, they begin to explore the depths of the basic structure of circadian clocks while still accounting for overtly rhythmic behaviour (Goldbeter, 1996; Lewis et al., 1997).

The purpose of this chapter is to describe the development of a comprehensive simulation model of the circadian system of L. cuprina, which simulates rhythmic behaviour and its underlying biochemical basis. This model is then tested in subsequent chapters at a molecular and behavioural level.
Chapter Two: Molecular Simulation Modelling of the *L. cuprina* Clock

2.1.2 The *per* Feedback System: A Modelling Approach

The most recent static model for the molecular generation of circadian rhythms in *Drosophila melanogaster* (which can be found in one form or another in numerous publications eg. Darlington *et al.*, 1998; Schibler, 1998; Dunlap, 1998; Sassone-Corsi, 1997a) is represented diagrammatically in Fig. 1.2 (Chapter One). As stated previously, the feedback loop relies on the negative feedback of the *period* and *timeless* gene products on their own genes transcription through the interaction with a transcriptional activator (dClock/dBmal1).

One can see that at its most basic level this system relies on two pivotal elements to generate circadian rhythms: negative feedback and time delay. These two central components have facilitated the development of many static models such as the ones described above, whose purpose is to concisely explain how a rhythm may be generated.

Whilst these models are important in that they identify the essential components of the system, they are static and do not convey any information about the interactions between the elements which lead to the living clock. In producing accurate models, one needs to breathe life into the clock by viewing it as a vital system and modelling it accordingly in a dynamic quantitative fashion.

In order to begin to understand how quantitative dynamic models can be produced to explain the molecular generation of circadian rhythms, it is helpful to first focus on the *per* side of the story and illustrate how a simulation model might be formulated. Once the parallels between the model and the real data have been established the usefulness of clock modelling will be highlighted by reviewing the development of quantitative models of circadian clocks in the past. Having stressed the importance of circadian modelling, molecular modelling will be addressed and the development of a comprehensive molecular model to explain circadian function in *L. cuprina* will be described.
The concept of the per feedback loop as an essential element in the generation of circadian rhythms began with the static model of Hardin et al. (1990). This model built on the theoretical gene regulation studies of Goodwin (1963, 1965) and Jacob and Monod (1961) and provided a conclusive link between high PER protein levels and the inhibition of per transcription (Hardin et al., 1990). An autoregulatory loop had thereby been identified. Since feedback alone leads to steady-state output, it seemed likely that time-delay was a central element of the per feedback loop. The demonstration that oscillations in PER protein levels lag 4-6 hours behind per mRNA levels (Siwicki, 1988; Hardin et al., 1990, 1992) provided evidence for the involvement of this time-delay. Negative feedback and time delay therefore became concrete elements of the circadian system.

The most basic version of the Hardin et al. (1990) model is illustrated below (Fig. 2.1A), and can easily be represented as a control systems diagram (Fig. 2.1B). The benefit of representing it in this way is that the control systems representation concentrates on the flow of information between the components of the system rather than the physical aspects of the system itself. This immediately facilitates the modelling of the system in a quantitative, dynamic fashion, allowing the comprehension of what is happening at every moment of the circadian cycle. Additional levels of autoregulation suggested by Hardin et al. (1990) can equally be represented in control systems format.
The parallels between the control systems diagram and the real data are best appreciated in tabular form. For this reason a table indicating the molecular elements of the feedback loop and their control-systems counterparts has been constructed (Table 2.1).
### Table 2.1 Tabulated parallels between elements of the per feedback loop and its control systems representation.

<table>
<thead>
<tr>
<th>Control-Systems Element</th>
<th>Molecular Element</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference ($c_{rel}$)</td>
<td>transcription threshold</td>
</tr>
<tr>
<td>Trans</td>
<td>transcription/translation</td>
</tr>
<tr>
<td>Time delay</td>
<td>phosphorylation and 'gated' nuclear entry</td>
</tr>
<tr>
<td>Feedback</td>
<td>suppression of transcription by PER protein</td>
</tr>
<tr>
<td>Ct</td>
<td>cytosolic concentration of PER protein</td>
</tr>
<tr>
<td>Ct' (time delayed)</td>
<td>nuclear concentration of PER protein</td>
</tr>
<tr>
<td>Loss</td>
<td>degradation of PER protein</td>
</tr>
</tbody>
</table>

#### 2.1.3 Modelling the Basic Properties of Circadian Rhythms

A useful way of demonstrating how models aid in the comprehension of molecular clock function is to illustrate how they have assisted in the understanding of behavioural rhythms. Even though detailed information of the underlying mechanics of circadian rhythms has only recently become available, models have been used since the 1960s to investigate the properties of circadian clocks (Goldbeter, 1996).

Over the years the concepts of feedback, thresholds, protein synthesis and time delay had been incorporated into a number of published models for circadian clocks (Sweeny, 1974; Njus et al., 1974; Schweiger et al., 1986; Benson and Jacklet, 1977). Pittendrigh’s empirical model in *Drosophila* (Pittendrigh and Minis, 1964; Pittendrigh, 1966) was one of the first, and was rapidly generalised to other systems (Pavlidis, 1967a,b; Pavlidis and Kauzmann, 1969). One of the earliest quantitative models, however, was that of Johnsson and Karlsson who proposed a model to account for rhythmic petal movement rhythms in *Kalanchoë blossfeldiana* (Karlsson and Johnsson, 1972; Johnsson and Karlsson, 1972; Johnsson et al., 1973). The following section is concerned with the historical development of quantitative models which have facilitated the prediction of increasingly complex behavioural phenomena, and which
have paved the way for the development of quantitative molecular models to help explain the biochemical basis of circadian clocks.

The two most basic properties of circadian clocks are the generation of endogenous rhythmicity and the entrainment of this rhythmicity. Despite the array of different single oscillator models available to explain these properties (Wever, 1965a; Winfree, 1970, 1980; Pavlidis, 1973), the most physiologically explicit is the single oscillator model of Gander and Lewis (1979) which is based on the Kalanchoë model of Karlsson and Johnsson (1972). This systems approach contrasts the use of Van der Pol-type oscillators (e.g. Kronauer et al., 1982) in that each component of the model describes an element of the real system.

In the single oscillator model of Gander and Lewis (1979), the oscillation of an activity promoting chemical (c) is postulated to control the timing of the active phase of a rhythm as its concentration passes through a threshold value (Gander and Lewis, 1979; Christensen et al., 1984). The time-delayed concentration of c is compared with a reference (c_{ref}) to generate an error signal. If positive, this error activates the synthesis of c. The synthesis function is temperature sensitive with a Q_{10} of 2. The loss rate of c is temperature insensitive, but concentration dependent. An endogenous oscillation is produced as a result of the time-delayed negative feedback. The time-delayed concentration (c_{i}), which is compared with c_{ref}, represents the concentration of c which was in the system at a fixed time interval earlier. The oscillator is responsive to light, as light destroys c at a rate proportional to intensity. This model is illustrated as a control systems diagram in Fig. 2.2 below.

![Control systems diagram of the single oscillator model for the circadian system of the weta Hemidiena thoracica. After Gander and Lewis (1979) and Christensen et al. (1984).](image-url)
When the control systems model is formalised in computer code with a fixed reference value and loss rate, it exhibits self-sustained temperature compensated oscillations (around the reference value) which mimic the known behaviours of the circadian clock (illustrated by the wave forms in Fig. 2.3) (Gander and Lewis, 1979; Christensen et al., 1984). If activity is represented as the time when c is above threshold, a simulated actogram can be generated which exhibits a free-running pattern (Fig. 2.3A,B). A light pulse administered during the falling stage of the oscillation (Fig. 2.3A) phase advances the rhythm, one falling during the rising stage results in a phase delay. When a 24 h light cycle is imposed on the system the oscillations are retarded daily and the free-running period is entrained (Fig. 2.3B).

2.3 Simulated actograms using the single oscillator model of Gander and Lewis (1979) and Christensen et al. (1984). Each asterisk indicates a 20 min time interval in which activity occurred. The boxes represent ‘lights on’. The simulated wave form indicates the underlying oscillation of chemical X which gives rise to the rhythm. A. Simulation of free-run and phase advance B. Simulation of entrainment C. Simulation of Aschoff’s rule D. Simulation of constant bright light.
In addition to free-run and entrainment, the single oscillator model predicts the response of the clock to other environmental perturbations, simulating Aschoff’s rule (lengthening \( \tau \) in dim LL) (Fig. 2.4C) and bright light effects (Fig. 2.4D) (Christensen et al., 1984; Lewis, 1990). All of these simulations have been supported by behavioural experiments, thus testifying to the predictive value of the model.

As early as 1960 Pittendrigh suggested that circadian time was organised by the interaction of different oscillators within an individual, and although single oscillator models explained many simple clock responses they left unexplained a class of behaviours known as free-run period lability (Pavlidis, 1978b; Pittendrigh, 1974). The explanation of these behaviours relied on the development of population oscillator models.

### 2.1.4 Modelling More Complex Clock Behaviour: Coupled/Population Oscillators

A curious behaviour of circadian clocks is that of rhythm shattering (or desynchronisation) which can occur spontaneously, or in response to a light pulse of a specific length positioned at a specific circadian time. Two explanations can be proffered to explain forced shattering or desynchronisation. The first is the singularity concept (Winfree, 1971, 1980) which proposes rhythmicity to result from the action of a single oscillator, and the shattering effect of a light pulse at a specific time (\( T^* \)) and of a specific duration (\( S^* \)) to send the oscillator into a singularity which causes the apparent cessation of rhythmicity (Winfree, 1971, 1980, 1986). Similar theories have been proposed to explain the induction of arrhythmicity in *Gonyaulax* and *Kalanchoë* (Taylor et al., 1982; Engelmann and Johnsson, 1978).

The second explanation is based on the desynchronisation of a population of oscillators which together comprise the clock (Christensen, 1978; Christensen and Lewis, 1982; Lewis, 1990). This model proposes that biological oscillations result from the output of a population of mutually coupled oscillators, and that shattering results from the differential effect of light on individual oscillators, some of which are phase advanced.
and some of which are delayed. The outcome of this is a desynchronisation of the oscillators which then oscillate at different phases and have insufficient power to drive an overt locomotor rhythm. Resynchrony is attained in this model when increasing numbers of individual oscillators couple and eventually create a nucleus for mutual entrainment (Christensen and Lewis, 1982; Lewis et al., 1991; Lewis, 1990, 1994; Enright, 1980).

Further support for the involvement of coupled or population oscillators in the generation of the circadian rhythm came from the ability of these types of models to simulate a wide range of free-run labilities. These behaviours include rhythm splitting, day-skipping (Clopton, 1984), spontaneous changes in free-running period, and after-effects of a treatment (Pittendrigh, 1974). Spontaneous changes in free-running period are normal in a large range of animals (Pittendrigh, 1974) and the period changes can be random (Christensen and Lewis, 1982), or show a recognisable shortening or lengthening effect (Lewis, 1994). After-effects are also common and occur particularly in response to single pulses of light, dim light, and entrainment (Pittendrigh, 1974).

Many models involving the coupling of more than one oscillator have been proposed to explain free-running lability. Some of the better known of these include Enright's coupled stochastic population model (Enright, 1980), and Wever's circadian multi-oscillator for man, which relied on the use of two coupled van der Pol oscillators (Wever, 1965a). Pavlidis's inhibitory coupling model proposed circadian rhythmicity to result from the inhibitory coupling of ultradian oscillators (Pavlidis, 1969). Pittendrigh's A and B model (Pittendrigh and Bruce, 1959; Pittendrigh, 1981) was formulated to explain the occurrence of transients in the Drosophila eclosion rhythm following a light perturbation. Kronauer's mathematical model of the human circadian system incorporated two mutually interacting oscillators (X and Y) to account for desynchronisation (Kronauer et al., 1982), and the action of zeitgeber and partial entrainment (Gander et al., 1984a,b; Kronauer, 1990; Jewett and Kronauer, 1998). Carpenter and Grossberg (1985) also formulated a coupled approach to explain the behaviours of splitting, after-effects and motivational interactions, as did Christensen
and Lewis (1982) and Lewis et al. (1991) to elucidate the mechanisms of splitting, day-skipping, shattering and scalloping in the nocturnal insect *Hemidiena thoracica*.

Hard evidence to support the existence of population oscillators as opposed to single unit oscillators came initially from experiments such as that of Jacklet and Geronimo (1971) who reported a shortening of the $\tau$ of the compound action potential rhythm in the *Aplysia* eye in response to a reduction in the retinal population. More conclusive evidence for population oscillators has come recently from physiological and biochemical studies of pacemaker tissue *in vitro*. Individual SCN cells, for example, not only exhibit circadian rhythms in firing rate (Green and Gillette, 1982), but when maintained in cell culture they exhibit an independently phased firing rhythm (Welsh et al., 1995; Shinohara et al., 1995). Recent studies using reporter gene technology have demonstrated that the circadian expression of *per* and *tim* mRNAs in individual insect cells continues for several days in cell culture (Emery et al., 1997; Plautz et al., 1997).

The development of coupled/population models culminated in the X-Y type model (Kronauer, 1982; Lewis et al., 1991). In these models the clock is seen to be comprised of two distinct populations of oscillators (X and Y) which are linked by mutual feedback. In the Lewis et al. (1991) model, each of the two populations are comprised of 15 sub-oscillators (Fig. 2.4). The X population is light insensitive and is not directly involved in the generation of locomotor activity, while the Y population is involved in the generation of the locomotor rhythm and is light sensitive. Coupling of the unit oscillators is achieved by sharing feedback information and is weak.
Fig. 2.4 Control systems diagram of the X-Y model for the simulation of circadian rhythms in *Hemidiena thoracica*. Each individual oscillator is coupled to other oscillators within the same population and is coupled to the other population. After Lewis *et al.* (1991).

The mean free-running periods of the two populations is different as are their coupling strengths. Locomotor activity in this model is triggered as any Y oscillator rises through a concentration band. Each oscillator therefore contributes to the active phase for a short time. The active phase is consequently made up of a series of bursts of activity as each Y oscillator passes through the critical concentration band (Lewis *et al.*, 1991). The length and coherence of the active phase is governed by the degree of synchrony of the Y population, and the output of the X system influences activity indirectly via the coupling factor (Lewis *et al.*, 1991).

The incorporation of the dual (X-Y) population concept into the system helps explain another level of complexity of the real behavioural data, and ‘realistic’ rhythms are achieved which show free-running lability (eg. Fig. 2.5).
It is evident from the background presented here that behavioural models have progressed considerably since their first conception. Not only is it possible to produce a quantitative model of circadian rhythmicity, but an insight into the intricate nature of free-running lability is provided by these models and predictions can be made about the response of the clock to different situations.

These models fulfil Pavlidis's modelling requirements at many levels in that they account for behavioural data, explaining it more concisely than in tabular form and they have predictive value. However, with the advancement in our knowledge of circadian clock function at a molecular level, it has become necessary to produce models which explain the underlying molecular mechanics of the clock in addition to the behavioural rhythms.

2.1.5 Development of Clock Models Incorporating Molecular Processes

By the mid 1990s the stage was set for the development of a more comprehensive level of circadian modelling incorporating the concepts of the molecular feedback loop. By this time data concerning the nature of the molecular loop had grown to the point
where modelling not only became possible but vital. Initial models were necessarily cautious and focussed only on the per side of the feedback loop. The first of these was that of Goldbeter (1995) who produced a model capable of simulating sustained oscillations of per mRNA and protein levels, which were presented as wave forms and limit cycles. The successive phosphorylative steps shown to occur to the PER protein by Edery et al. (1994) were used as the basis for the essential element of time-delay. In typical Goldbeter fashion the loop was characterised by numerous mathematical equations; the rate of synthesis of PER, the rate of phosphorylation, and the rate of nuclear entry were all characterised by first order rate constants, and degradation was described by a Michaelis constant. This model not only showed how negative feedback exerted by PER protein could give rise to sustained oscillations in per gene expression, but also how changes in the degradation rates of PER could alter the period of oscillations or suppress rhythmicity entirely (Goldbeter, 1995, 1996). Once developed, the model was used to predict the way in which the per mutations in D. melanogaster result in altered clock behaviour (Goldbeter, 1995). Leloup and Goldbeter (1998) extended the original Goldbeter (1995) model with the incorporation of Timeless, and were able to generate phase response curves matching those obtained experimentally for the wild-type and per^Drosophila mutants.

Another approach to the modelling of the per feedback loop is that provided by Lewis et al. (1997). Rather than the description of the loop in mathematical terms, the per model of Lewis et al. (1997) is less abstract in that each element of the real data is related to an element of the model. In this model the essential elements of the molecular system are supplied and the system is observed over numerous iterations to see whether it can actually produce rhythmic oscillations in per mRNA and protein, and therefore rhythmic behaviour.

Just as in the previous behavioural models (eg. Christensen and Lewis, 1982; Lewis et al., 1991), the Lewis et al. (1997) molecular model presents behavioural simulations in addition to the underlying wave-form giving rise to these rhythms. The important difference between this and the previous models is that the wave form is a true representation of PER protein levels based on molecular observations (Lewis et al.,
1997). A direct correlation between the level of PER protein and the behavioural response is therefore readily apparent, and the response of the system at both the molecular and behavioural levels to environmental manipulation is easily established. This model succeeded not only in simulating free-run, phase shifting and entrainment, but was also able to simulate the different responses of all three Drosophila per mutants to light (as described by their phase response curves). The simulated PRCs corresponded well to the real data, and therefore provided insight into the elements of the feedback loop which may be altered in the mutants (Lewis et al., 1997).

In the present work, a single oscillator model similar to that of Lewis et al. (1997) has been developed which is even more biochemically explicit, as it incorporates all of the known elements of the feedback loop and provides the underlying wave forms for both per and tim mRNA and protein levels. This model was based on D. melanogaster data and was subsequently modified to account for the shorter free-running period of L. cuprina by introducing a shorter time delay between mRNA oscillations and protein oscillations. The relevance of this model to both the Drosophila and Lucilia systems is discussed, and its assumptions investigated and predictive value tested in subsequent chapters. The incorporation of classical ideas of coupled and population oscillators into molecular modelling to explain previously irreconcilable differences between molecular and behavioural data is also discussed.

### 2.2 Methods of Simulation Modelling

As stated above, the current molecular feedback model was formulated initially to explain the D. melanogaster data and therefore it is based on D. melanogaster experimental results. The assumption was made here that the L. cuprina circadian feedback loop functions in a similar manner to that of D. melanogaster. Modifications of the model to account for the behaviour of L. cuprina were relatively minor, involving a change in the time delay values of the model to produce the characteristic 22 h free-running period, as opposed to the 24 h free-running period of
D. melanogaster. The basic qualities of the model are similar to those presented in Lewis et al. (1997), and are representations of the molecular evidence presented by other researchers.

2.2.1 The Control Systems Representation

All of the elements of the feedback loop were initially represented as a control systems diagram (Fig. 2.6) to allow the programming of the model.

![Control systems representation of the PER-TIM negative feedback loop in L. cuprina. The top part represents the timeless portion of the loop while the bottom half represents the period portion. Light can be seen to affect nuclear and cytoplasmic TIM and (to a small extent) per mRNA.](image)

The model was programmed using BBC Basic Version V and an Acorn Archimedes computer. The listing for the program is shown in Appendix One. Each iteration of the feedback loop represents one 20 min interval of real time. The program carries out 1440 iterations (20 days), and plots the wave forms of the levels of cytoplasmic and nuclear PER and TIM protein, and per and tim mRNAs in addition to representing light levels. The overt behaviour derived from these oscillations (as determined by the threshold) is also plotted. The simulated effect of light perturbations can thus be observed both at the behavioural level and at the level of the underlying molecular oscillations.
2.2.2 Negative Feedback

Negative feedback in the model is seen to result from the binding of the PER/TIM heterodimer to the transcriptional activator (the dClock/dBmal1 heterodimer) in the nucleus (Darlington et al., 1998; Allada et al., 1998; Rutila et al., 1998). The binding of PER/TIM to dClock/dBmal1 switches off the transcription of per and tim mRNAs. In the simulation model this autoregulation is represented by the following code:

\[
\text{diff} = 30 - \text{nper(iter\%)} \\
\text{IF syn} > 2.6 \ \text{THEN syn} = 2.6 \\
\text{IF syn} < 0 \ \text{THEN syn} = 0 \\
\text{and} \\
\text{diff} = 30 - \text{ntim(iter\%)} \\
\text{IF syn} > 2.6 \ \text{THEN syn} = 2.6 \\
\text{IF syn} < 0 \ \text{THEN syn} = 0
\]

Where 30 represents the threshold concentration of dClock/dBmal1 above which nuclear PER or TIM levels must rise to turn off transcription.

Nuclear entry of the PER/TIM dimer occurs rapidly after cytosolic levels have exceeded a specific threshold at a specific circadian time (Ct19-20) (Lee et al., 1996).

This rapid nuclear entry is represented as:

\[
\text{DEF PROCentry} \\
\text{IF phosper(iter\%)} > 40 \ \text{AND phostim(iter\%)} > 40 \ \text{THEN losstus\%=4} \\
\text{losstus\%=losstus\%-1} \\
\text{nper(iter\%)} = \text{nper(iter\%)} + 13 \\
\text{phosper(iter\%)} = \text{phosper(iter\%)} - 13 \\
\text{IF phosper(iter\%)} < 0 \ \text{THEN phosper(iter\%)} = 0 \\
\text{ntim(iter\%)} = \text{ntim(iter\%)} + 13 \\
\text{IF ntim(iter\%)} < 0 \ \text{THEN ntim(iter\%)} = 0.1 \\
\text{phostim(iter\%)} = \text{phostim(iter\%)} - 13 \\
\text{IF phostim(iter\%)} < 0 \ \text{THEN phostim(iter\%)} = 0.
\]

Where 40 represents the threshold level above which PER and TIM dimerise and undergo nuclear entry (within 1 h).
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2.2.3 Time-Delay

Time delay results from the time required for levels of monomeric PER and TIM to build up to sufficient levels to allow dimerisation and nuclear entry of the dimer. This time delay is due, at least in part, to the destabilising effect of the Doubletime protein on cytoplasmic PER protein (Price et al., 1998), and in part on the time taken for nuclear PER/TIM to degrade and release the transcriptional inhibition of per and tim. In the code this is represented by the statements:

\[
\begin{align*}
\text{perRNA(iter)} & = \text{perRNA(iter-1)} + \text{syn} - (0.087 \times \text{perRNA(iter-1)}) \\
\text{IF iter} & < 12 \text{ THEN pertranslation} = 0 \text{ ELSE pertranslation} = ((\text{perRNA(iter-12)}) - 5) \times 0.04 \\
\text{and} \\
\text{timRNA(iter)} & = \text{timRNA(iter-1)} + \text{syn} - (0.12 \times \text{timRNA(iter-1)}) \\
\text{IF iter} & < 12 \text{ THEN timtranslation} = 0 \text{ ELSE timtranslation} = ((\text{timRNA(iter-12)}) - 5) \times 0.04
\end{align*}
\]

2.2.4 Degradation of PER and TIM

Degradation of TIM protein in the model occurs at a rate proportional to the concentration of protein present. The rate of degradation of PER in both the nucleus and the cytoplasm is inversely proportional to the amount of TIM protein present. This concurs with the evidence that PER protein is stabilised by the presence of TIM (Lee et al., 1996; Price et al., 1998), and is modelled by the following statements:

\[
\begin{align*}
\text{timRNA(iter)} & = \text{timRNA(iter-1)} + \text{syn} - (0.12 \times \text{timRNA(iter-1)}) \\
\text{ntim(iter)} & = \text{ntim(iter-1)} - (0.15 \times \text{ntim(iter-1)}) \\
\text{and} \\
\text{phosper(iter)} & = \text{phosper(iter-1)} + \text{pertranslation} - (1 / \text{hostim(iter)}) \times 0.0001 \\
\text{nper(iter)} & = \text{nper(iter-1)} - ((1 / \text{ntim(iter)}) \times 8))
\end{align*}
\]
When nuclear concentrations of PER and TIM are reduced by degradation the dClock/dBmal1 dimer re-activates transcription from the per and tim genes (see Section 2.2.1).

### 2.2.5 The Simulation of Light Effects

As has been shown by a large amount of research (Hunter-Ensor et al., 1996; Lee et al., 1996; Myers et al., 1996) light acutely degrades TIM protein. This degradation causes the PER monomer to be destabilised and hyperphosphorylated by Doubletime (Price, 1998).

Light degrading TIM in the cytoplasm:

\[
\text{phostim(iter\%)} = \text{phostim(iter\%-1)} + \text{timtranslation-light}
\]

can be seen to result in a phase delay, as the build up of the PER and TIM monomers and their subsequent dimersiation and nuclear entry are delayed (Hunter-Ensor et al., 1996; Lee et al., 1996; Myers et al., 1996).

Light degrading TIM in the nucleus:

\[
\text{ntim(iter\%)} = \text{ntim(iter\%-1)} - \text{light} \times (0.15 \times \text{ntim(iter\%-1)})
\]

expedites the drop in nuclear PER levels. Consequently the dClock/dBmal1 dimer activates transcription earlier, phase advancing the clock.

### 2.2.6 The Threshold Concept

In keeping with molecular findings (Siwicki, 1988; Hardin et al., 1990, 1992; Vosshall et al., 1994), PER and TIM protein levels lag approximately 6 h behind their respective mRNA levels, and peak during the subjective night (at ~Ct 20). As Lucilia is day-active, the initiation of locomotor activity is seen to result from sub-threshold levels of TIM (which occur during the subjective day), and is represented thus:
IF phostim(iter%)<30 .....THEN chr=35 (activity)

Where the threshold below which activity is induced is 30.

2.2.7 Phase Response Curves

Simulated phase response curves were generated using an additional section of code similar to that used in Lewis et al. (1997) (See Appendix One). This code calculates the phase shift obtained for 20 min light pulses administered throughout the day and plots the resulting data on a phase response curve (normalised circadian time of the pulse against phase shift in hours).

2.3. Simulation of Free-Running Rhythms

Free-running rhythms were simulated by the model with a period and active phase length similar to the real data (Fig. 2.8). Each figure shows the simulated single-plotted free-running locomotor rhythm, in which each asterisk represents a 20 min time interval during which locomotor activity occurs. The hypothesised wave forms of per and tim mRNAs and nuclear and cytosolic PER and TIM proteins are shown beneath the actogram. Since locomotor activity is hypothesised to be triggered when TIM levels drop beneath the threshold, minimum TIM and PER values occur at the beginning of the subjective day (Fig. 2.7).
These low concentrations of PER and TIM trigger the transcription of the \textit{per} and \textit{tim} genes and the levels of \textit{per} and \textit{tim} mRNA begin to rise (Fig. 2.7). The mRNAs are then translated and the levels of phosphorylated PER and TIM proteins begin to rise towards the end of the subjective day (after a lag caused by Doubletime). When they exceed the threshold, locomotor activity ceases (Fig. 2.7). When cytosolic concentrations of PER and TIM monomers are sufficient, the proteins dimerise and undergo nuclear entry in a short time window (Ct19-20). Once in the nucleus the PER/TIM dimer prevents further transcription from \textit{per} and \textit{tim} by preferentially binding the dClock/dBmal1 dimer. The degradation of TIM in the nucleus towards the end of the subjective night, and the subsequent degradation of PER, reduces their concentrations and the dClock/dBmal1 dimer initiates transcription again (Fig. 2.8).

The fact that the simulated active phases are more stable and regular than their biological counterparts is reflective of the fact that the model only relies on a single oscillator. This important point will be addressed in greater detail later.
2.4 Simulation of Entrainment

When subjected to a lighting regime (LD 5:19) of several days, the simulated activity entrains (Fig. 2.9A). This entrainment can be seen to result from the retardation of the daily cycles of PER and TIM by essentially delaying nuclear entry and negative feedback.
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A. 1. nuclear PER  2. nuclear TIM  3. cytoplasmic TIM
    4. cytoplasmic PER  5. per RNA  6. tim RNA

B. 1. nuclear PER  2. nuclear TIM  3. cytoplasmic PER
    4. cytoplasmic TIM  5. per RNA  6. tim RNA
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Fig. 2.9 Simulation of entrainment of free running period to 24 h with LD cycles. * = activity; # = activity and light. Light is indicated as a histogram below the wave forms. A. Simulation of entrainment with a 6 h light pulse. B. Lack of simulated entrainment in response to a long light pulse (8 h). C. Simulation of entrainment with an 8 h skeleton photoperiod (two 1 h pulses spaced 6 h apart). D. Simulation of entrainment with the same skeleton photoperiod which also accounts for masking.
When subjected to a longer photophase (7 h or more) the simulated rhythm is not entrained (2.9 B). However, when the effect of a light pulse is seen to consist of its skeleton (ie. the clock-effecting parts of the pulse are the hour after the D-L transition and the hour after the L-D transition), entrainment in long photophases is simulated (Fig. 2.9 C). This result is consistent with behavioural data which have shown skeleton photoperiods to entrain clocks as efficiently as full light cycles (Pittendrigh and Minis, 1964; Pittendrigh, 1965, 1966). Further support for the fact that the skeleton photoperiod is the biologically important clock signal comes from data obtained from expression studies of the immediate early genes c-fos and Jun-B. As stated in Chapter One, there is good evidence for the involvement of these genes in the clock entrainment pathway. Expression of c-fos and jun-B is transient (levels peak after approximately 30 min) in response to phase-shifting light perturbations. The light signal which the clock receives can therefore be seen to be transient. The model has thus been modified to take into account the entrainment pathway (Fos/Jun), and a light pulse is seen to comprise two transient (1 h) pulses at either end of the photophase. The portion of the simulated active phases outside the skeleton photophase is proposed to reflect the lack of masking in the model. When the assumption is made that activity after the L-D transition does not occur due to masking, then the simulation mirrors the real data perfectly (Fig. 2.9 D).

2.5 Test of the Differential Effect of Light Pulses Falling at Different Circadian Times

With the addition of the extra portion of code described in the methods section, it became possible to generate a phase response curve (PRC) in order to determine the differential effects of light administered at different circadian times (Fig. 2.10).
Fig. 2.10 Simulated phase response curve for *L. cuprina* showing phase delays and advances in response to 20 min light pulses administered throughout the day.

Brief light pulses (20 min) caused phase shifts which were consistent with experimental data from both *Drosophila* and *Lucilia*. Light pulses administered towards the end of the subjective day resulted in phase delays due to the cytoplasmic degradation of TIM and PER proteins and the subsequent delay in nuclear entry (Fig. 2.11).
Fig. 2.11 Simulation of phase delay and underlying molecular changes associated with it. A drop in cytoplasmic levels of TIM and PER delay build up, nuclear entry and therefore negative feedback. The arrow beneath the simulated wave forms indicates the time of the light pulse.

Light pulses falling during the late subjective night/early subjective day phase advanced the behavioural rhythm due the expedited degradation of nuclear PER and TIM (Fig. 2.12).
Fig. 2.12 Simulation of phase advance and underlying molecular changes. Degradation of nuclear TIM destabilises PER and inhibition of per and tim transcription is removed earlier therefore causing a phase advance. The arrow beneath the simulated wave forms indicates the time of the light pulse.

The simulated *L. cuprina* PRC (a strong or Type 0 PRC) is similar to that obtained from the experimental data (Warman, 1995; Chapter Four, Section 4.4). The only major discrepancy is that the change-over from phase delays to advances occurs at a slightly later Ct than was obtained experimentally. This 'shift' in the simulation may be indicative of a differential nuclear entry time in *L. cuprina* as compared with *D. melanogaster* (discussed in Chapters Four and Five), or it may be related to recent molecular findings which show *per* RNA levels begin to decline before PER and TIM are predominantly nuclear (So and Rosbash, 1997). This finding is suggestive of an additional post-transcriptional regulatory loop (Dunlap, 1998).
There is no doubt that experimentally the phase advancing effects of light are acute (eg. Fig. 2.12). However, as the free-running period of *L. cuprina* is less than 24 h (Smith *et al.*, 1983, 1985; Warman, 1995), light falling in the morning presumably does not naturally phase advance the rhythm as much as light at the end of the subjective day delays it. The biologically important portion of the light pulse may be that portion towards the end of the day, when the build up and nuclear entry of the PER and TIM proteins is delayed. This point is addressed further in Chapters Four and Five.

### 2.6 Prediction of the Clock Response to Constant Light (LL)

Given that the model can account for the basic responses of the clock at behavioural and molecular levels, the most obvious test of its power is to determine whether it can predict the response of the clock to more complex environmental perturbations. Since light is the primary zeitgeber involved in the entrainment of circadian clocks it provides a powerful tool with which to analyse the effects of environmental perturbations and therefore the nature of the clock itself. The analysis of the response of the model to complex photic perturbations thereby provides an ideal test of its limitations.

Besides its synchronising action, light is known to act in other ways. An interesting behaviour to examine is the effect of protracted photophases on the clock. The general maxim for the effect of constant light is that it alters the nature of $\tau$ but allows the continuation of rhythmicity at low intensities, while at higher intensities it causes arrhythmicity.

Aschoff's rule summarises the response of many species to constant light by stating that: 'The period of a free-running biological oscillation ($\tau$) lengthens on transfer from DD to (dim) LL or with an increase in light intensity for dark-active animals, but shortens for light-active animals'. Aschoff's rule does, however, have many exceptions amongst insects (Saunders, 1977). Some diurnal insects, such as *Aëdes*
*aegypti* (Taylor and Jones, 1969) and *Calliphora vicina* (Hong and Saunders, 1994) display a lengthening of $\tau$ after transfer from DD to dim LL.

Continuous bright light can result in complete damping of rhythmicity in a wide range of organisms including plants (Ninnemann, 1979), *Aplysia* (Benson and Jacklet, 1977), mammals (Borbely, 1978), and insects (Winfree, 1974; Konopka et al., 1989). Prolonged bright light has also been shown to damp out the molecular rhythms of *period* and *timeless* in *Drosophila* (Zerr et al., 1990; Qiu and Hardin, 1996a).

When the effects of constant light are simulated by the present model using the Fos/Jun skeleton they do not produce significant changes in the free-running period (as the only two light perturbations which occur are at the beginning and end of the long pulse).

This result agrees with the thoughts of molecular researchers that the clock signal from long light pulses is not mediated through the Fos/Jun pathway (*pers. comm.* Young, 1997; *pers. comm.* Hardin, 1997; *pers. comm.* Rusak, 1997). If the assumption is made that the detection of long light pulses by the clock is not transient, and yet still results in the degradation of TIM (as has been shown by experimental data), then the model simulates the effects of these perturbations well (Fig. 2.13 and 2.14).

Constant dim light results in a lengthening of the simulated free-running period ($\tau$) from 22 h to 23 h (Fig. 2.13) in a clear breach of Aschoff’s rule. When the intensity of this light is increased, simulated activity becomes arrhythmic (constant activity). The simulated molecular response to constant dim light involves a delay in the rate of accumulation of cytoplasmic TIM protein due to degradation by light (Fig. 2.13). This retardation of cytoplasmic accumulation, and therefore nuclear entry, occurs in much the same way as entrainment. At low simulated light intensities, however, the rate of degradation is less than that of synthesis and therefore rhythmic activity continues (Fig. 2.13).
In the case of constant bright light (LL) TIM is degraded to very low levels rapidly and is maintained at these low levels due to the continuation of the pulse (Fig. 2.14). This results in the degradation of PER to low levels by Doubletime and cessation of feedback (as there are insufficient levels of PER and TIM for dimerisation, nuclear entry and feedback) (Fig. 2.14).
Fig. 2.14 Simulation of the effects of constant bright light on behaviour and the molecular oscillations underlying this behaviour. Constant bright light degrades TIM protein therefore destabilising PER and resulting in cessation of feedback. Light is indicated by the square wave beneath the molecular wave forms.

Simulated transcription from the per and tim genes under constant bright light is maximal as there is no repression of the transcriptional activator dClock/dBmal1. When the light is switched off, cytoplasmic TIM begins to accumulate, thus stabilising PER, and the molecular cycles resume (Fig. 2.14). The simulation of the effects of constant bright light mirror the Drosophila data in most respects with one particularly notable exception: per cycling has been found to continue for two to three days in constant bright light before damping out (Marrus et al., 1996; Qiu and Hardin, 1996a). This seemingly irreconcilable difference between behavioural arrhythmicity and the continuation of molecular oscillations is compounded by the fact that these oscillations were found in populations of flies held in LL (Marrus et al., 1996; Qiu and Hardin,
1996a). Presumably, the periodicity of the oscillations in LL would differ between different individuals thereby clouding the real effect at the individual animal level.

The marriage of the present molecular model to the classical X-Y (or A-B) modelling ideas presents a particularly intriguing explanation of these seemingly contradictory results. As explained previously, in the classical models the clock has been viewed as comprising of two mutually coupled populations of oscillators, one of which (Y) is sensitive to light and the other which (X) is not (Section 2.3), and that the light-sensitive population (Y) is the one directly involved in the generation of the activity rhythm.

If the molecular model presented here were developed into a two population (X-Y) model, with the light sensitive (Y) population being coupled weakly and the light insensitive (X) population being coupled more tightly, then one can see how the molecular and behavioural data might be reconciled. Under the conditions of constant bright light, the Y population is desynchronised and damped quickly (due to the degradation of TIM). However, the X population is not damped by light, and as the sub-oscillators are coupled more tightly it continues to oscillate for a number of cycles. The total amount of cycling in each animal is seen to be damped as only the X population is still in synchrony. After 2-3 cycles the synchrony of X is lost (due to the lack of coupling with a synchronised Y population, and presumably the different periods of the X sub-oscillators). Consequently the observable rhythm is also damped. The fact that each individual sub-oscillator (or cell) in the X population is still oscillating could also account for the observation of Marrus et al. (1996) and Qiu and Hardin (1996a) that per mRNA levels are not maximal (as would be expected if all of the feedback had been prevented).

2.7 A Simulation Model for L. cuprina Developed

The L. cuprina simulation model developed here by no means explains the molecular feedback loop in full. The particularly recent publication of Lee et al. (1998) showing
circadian oscillations in dClock protein levels (lagging 4 h behind PER and TIM levels), and a reduction in dClock levels in the absence of PER and TIM, suggests a further twist in the tale. If dClock levels are regulated by PER and TIM, then a double feedback loop exists in which PER and TIM are positively regulating their own transcription through the transcriptional activation of dClock (and possibly dBmal1), and at the same time negatively regulating their own transcription through the binding of PER and TIM to the dClock/dBmal1 dimer (Lee et al., 1998). There is yet more information suggesting further complexities in the feedback loop, including the detection of cycling of PER protein in the absence of per mRNA cycling (Cheng and Hardin, 1998) which will ultimately need to be incorporated into the model.

Despite these recent discoveries, the present model is the most comprehensive model of any circadian system available, and provides useful insight into the functioning of the circadian clock. Not only does it present evidence that the molecular ‘word’ models can act to produce a circadian rhythm, but it indicates the response of the system to environmental perturbations administered at specific times, and facilitates the understanding of what is happening at every moment of the circadian cycle. Furthermore, it allows the prediction of the response of the circadian system to environmental perturbations and provides insight into the molecular alterations which are responsible for behavioural changes. Future development of the model by the incorporation of the coupled population concept, and the newly elucidated role of PER and TIM in the regulation of dClock levels will provide an even more valuable look at the molecular generation of circadian rhythms.

The model presented here provides a strong theoretical framework for the analysis of the molecular and behavioural aspects of the L. cuprina clock. Elements and predictions of the model are tested at a behavioural and molecular level in subsequent chapters. The multifaceted approach of theoretical modelling, molecular analysis and behavioural investigation of the circadian clock is then brought together in Chapter Five to help explain the circadian system of this fly.
CHAPTER THREE: TESTING THE BASIC MOLECULAR ASSUMPTIONS OF THE L. CUPRINA SIMULATION MODEL

3.1 An Introduction to the Structure and Function of per in Insects

When considering the L. cuprina simulation model described in Chapter Two it becomes apparent that per is a central player in the molecular feedback loop. It would therefore be presumptuous to suggest a model for the molecular functioning of the circadian clock of L. cuprina involving per without indicating that it exists in this fly. In order to test the basic molecular assumptions of the L. cuprina simulation model, Chapter Three describes the isolation of a per cDNA homologue from L. cuprina and the subsequent analysis of its mRNA and protein expression levels. By way of an introduction to these molecular analyses, the in-depth knowledge of the nature and function of the period gene in Drosophila and other invertebrate species is described. Information is also given on the technique of PCR to provide the background to its use in cloning strategies and quantitative measurements of per mRNA levels.

3.1.1 The per Red Herrings

Since the birth of per research in 1971 several hundred research papers have been published on the nature and function of the per gene in D. melanogaster. Concomitant with the investigations which have provided useful insight into the functioning of per, have been some dramatic red herrings concerning both the per mutants and the gene defined by these mutations (Hall, 1995). Rather than discuss these spurious results in depth, the foremost errors will be listed briefly before moving on to what we know to be true about per.
The assertion that a small mRNA species transcribed from a gene near to the per locus oscillated in abundance over the course of a day (Reddy et al., 1984) was proved incorrect by Lorenz et al. (1989). The theory that the per gene encoded a proteoglycan (Bargiello et al., 1987; Reddy et al., 1986) was abandoned when PER was shown to undergo post-translational phosphorylation and not glycosylation (Edery et al., 1994). A proposition that three different polypeptides were derived from the per gene by differential splicing (Citri et al., 1987) was disproved by Hardin and Siwicki (1995), and finally, the possible role of per in gap junctional communication (Bargiello et al., 1987) was eliminated in 1992 (Saez et al., 1992).

3.1.2 Isolation of per from a Number of Drosophila Species

Comparison of the complete genomic sequence of per from the two original strains of D. melanogaster, Canton-S (Jackson et al., 1986) and Oregon-R (Citri et al., 1987), in which per was isolated, revealed only 14 nucleotide changes (Citri et al., 1987). Nine of these substitutions were coding, but did not result in any amino acid changes. Four of the changes were intronic, and one was in the 3' untranslated region (Citri et al., 1987). This high degree of conservation within D. melanogaster is contrasted strongly by the situation in neighbouring species within the same genus.

In 1988 Colot et al. cloned and sequenced the coding region of per from D. pseudoobscura and D. virilis and compared the nucleotide and inferred protein sequences to that of D. melanogaster. Surprisingly, they found that sections of the conserved coding sequence were interspersed with large blocks of non-conserved coding sequence up to 100 amino acids long which were so divergent they could not be recognised in the coding DNA of the other two species. Nucleotide alignments between the three species was thus impossible (Colot et al., 1988). The non-conserved portion of the amino acid sequence comprises 33% of the total protein, and includes the TG repeat of D. melanogaster which was found to be absent (or severely truncated) in both D. pseudoobscura and D. virilis (Colot et al., 1988). This high degree of sequence divergence is particularly surprising given that D. pseudoobscura per has
successfully been used to rescue *D. melanogaster* per⁰ mutants (Petersen et al., 1988), although it must be noted that a protracted behavioural period was evident in these flies (Petersen et al., 1988). Due to the presence of alternating regions of high and low conservation, *per* was formally divided into conserved and non-conserved blocks, of which there are 6 and 5 respectively (Colot et al., 1988). Absolute homology in conserved blocks is extensive (up to 40 consecutive amino acids) and overall divergence is low at between 15% and 25%. The conserved blocks have since been found to include three functional domains known to be important in the correct operation of the *D. melanogaster* clock. These domains include the PAS dimerisation domain (in C2), which encodes two highly degenerate 51 amino acid repeats, and which mediates the dimerisation of PER protein to its heterodimeric partner TIM (Huang et al., 1993; Gekakais et al., 1995), the nuclear localisation signal (in C1), which mediates nuclear entry of the PER-TIM complex (Baylies et al., 1993; Vosshall et al., 1994), and the cytoplasmic localisation signal (in C2), involved in the retention of PER in the cytoplasm until such time as nuclear entry is appropriate (Saez and Young, 1996). In addition, the *per¹⁰* and *per⁸* mutations occur within the C2 conserved region.

Sequence analysis of *per* homologues from more than 13 different species of *Drosophila* has subsequently revealed that this gene is highly variable across the genus.

3.1.3 *per* in Flies, Moths, and Cockroaches

Despite the immunocytochemical detection of PER-like antigens in a number of species (Siwicki et al., 1989), a decade lapsed between the isolation of *per* in *Drosophila* (1984) and the isolation of the first *bone fide* *per* homologue in an organism other than *Drosophila*. Responding to this chasm in our knowledge, Reppert et al. (1994) isolated a full-length *per* cDNA homologue from the giant silk moth *Antheraea pernyi* using a PCR-based strategy and 21 sets of redundant primers (Reppert et al., 1994). The choice of a holometabolous insect whose circadian system
had been investigated extensively (Truman and Riddiford, 1970; Truman, 1974) presented an interesting comparison to the *Drosophila* system.

The cDNA of *A. pernyi* *per* encodes a predicted protein of 849 amino acids which shows highest identity (39%) with *D. virilis* *per* (Reppert *et al.*, 1994). The regions of highest homology between giant silk moth and *Drosophila* PER are confined to distinct stretches within the C1-C3 regions. The 77 amino terminal amino acids show 57% homology to *D. virilis*, the PAS domain shows 48% identity, and a region of 50 amino acids spanning the site of the *per* mutation is very highly conserved at 73%.

The amino acid residues mutated in *per* and *per* flies are also conserved in the giant silk moth C3 region. The *Antheraea* sequence shows no significant homology with the C4-C6 regions of *Drosophila* PER, and the TG motif is present but is not repeated (Reppert *et al.*, 1994).

Using the combined sequence information from *Antheraea* and *Drosophila* per, Reppert *et al.* (1994) cloned *per* fragments corresponding to the conserved C2 region from two other moth species and a cockroach. One of the moths, the silk moth *Hylophora cecropia*, from the same family as *Antheraea*, showed 73% identity to *A. pernyi* *per*. The other lepidopteran representative, the tobacco hornworm *Manduca sexta* (Family Sphingidae) showed 70% identity. The cockroach *Periplaneta americana* (Order Blattaria) was 46% and 50% identical to *A. pernyi* and *Drosophila* respectively (Reppert *et al.*, 1994).

Adopting a similar redundant PCR-based strategy, Nielsen *et al.* (1994) isolated and sequenced a region of *per* (corresponding to amino acid positions 543-776 in *D. melanogaster*) from 10 different fly species. Sequence evidence from these species illustrated the extreme stability of a truncated TG repeat outside the *Drosophila* genus and led to the proposal that the repeat region of *per* is based on two different repetitive sequences, one encoding a pentapeptide and the other a dipeptide, which have undergone expansion in the *Drosophilidae* (Nielsen *et al.*, 1994).
The availability of these per sequences on GenBank has facilitated the amplification of per fragments from a number of different species. per has now been isolated from vertebrates as well as invertebrates (Tei et al., 1997; Sun et al., 1997; Zylka et al., 1998a) (Chapter One) and has been proved to be one of the most rapidly evolving coding genes isolated (Regier et al., 1998).

3.1.4 PER the Transcriptional Regulator

In the scramble to assign a biochemical function to PER which occurred in the late '80 and early '90s, one of the all time biggest misses was the failure of researchers such as Reddy et al. (1984) and Young et al. (1985) to observe a circadian fluctuation in the levels of the 4.5 Kb per mRNA which were demonstrated by Hardin et al. (1990, 1992). This oversight is attributed to the analysis of levels in whole bodies as opposed to decapitated heads alone, and to the analysis of samples from DD (in which cycling is less evident than LD (Hardin et al., 1990)). The credit for rekindling an interest in the analysis of temporal expression levels of per should be given to Siwicki et al. (1988) and Zerr et al. (1990) who presented the first clear cut evidence that PER protein levels oscillated in DD with a period equal to the behavioural rhythm (Siwicki et al., 1988; Zerr et al., 1990). The nuclear localisation of PER suggested that this protein may act as a transcriptional regulator (Siwicki et al., 1988). Negative feedback and time delay were also shown to be concrete elements of the PER autoregulatory loop (see Chapters One and Two), and the presence of circadian oscillations became a hallmark of per.

As the period-altering mutants in D. melanogaster effect the phase and period of the molecular oscillations (Hardin et al., 1990; Marrus et al., 1996; Zerr et al., 1990), the reasonable assumption was made that the precision of the circadian period depends on the appropriately timed oscillations of per mRNA and protein. The relative importance and method of regulation of these oscillations has remained to a large extent unresolved (Chen et al., 1998). Analysis of per transgenes driven by heterologous promotors suggests that feedback regulation of per transcription may be dispensable for protein cycling and behavioural rhythmicity (Cheng and Hardin, 1998). While the
transcriptional control of per RNA cycling is well established (Hardin et al., 1992; Hao et al., 1997), recent studies implicate additional posttranslational mechanisms in the control of mRNA (So and Rosbash, 1997; Stanewsky et al., 1997) and protein cycling (Dembinska et al., 1997; Stanewsky et al., 1997). The mechanisms by which these posttranscriptional mechanisms may work are poorly understood, but they may involve delayed (or regulated) translation, and changes in protein stability (Dembinska et al., 1997). The involvement of protein stability and regulated translation has recently been investigated by Chen et al. (1998) who reported the 3' untranslated region (3' UTR) of per to play an important role in the regulation of period length of the rhythm. As the sequences of 3' UTRs are known to alter the half-life of the proteins they encode, a role for this region in the post-transcriptional control of circadian rhythms is feasible. Investigation of the involvement of delayed translation of per RNA failed to reveal existence of any significant translational delay mechanism (Chen et al., 1998).

Despite the isolation of per fragments from in excess of thirty species, there has been an astonishing lack of comparative analysis of per expression and function, especially in different insect species. The one major exception to this rule is the analysis of per in the giant silk moth A. pernyi (Reppert et al., 1994; Sauman and Reppert, 1996; Sauman et al., 1996). Reppert et al. (1994) initially used the presence of circadian fluctuations in per mRNA and PER-like immunoreactivity to support the notion that the gene they had isolated was indeed a per homologue. When per expression was examined in more detail, two distinct systems of PER regulation were demonstrated (Sauman and Reppert, 1996).

The existence of a spatiotemporal expression pattern similar to that of Drosophila in the photoreceptors of the eye notwithstanding, in the central brain (the anatomical location of the moth circadian pacemaker), PER protein oscillations were found to occur in phase with per mRNA oscillations (Sauman and Reppert, 1996). Further analysis of the brain rhythms revealed not only that PER protein was restricted to the cytoplasm (thus precluding the type of feedback present in Drosophila), but also that an antisense per mRNA strand oscillated out of phase with the sense strand. The demonstration of this second type of system in three moth species raises the possibility
that moths employ a gene-silencing approach to the circadian regulation of PER oscillations (Sauman and Reppert, 1996).

3.1.5 An Introduction to the Polymerase Chain Reaction (PCR)

The vital technique which facilitated the isolation of these per homologues from different insect species was the polymerase chain reaction (PCR). PCR is a particularly valuable technique in molecular biology, and is one which was employed heavily in the current work. It has been used here to both isolate and analyse the expression levels of the L. cuprina per gene. As it has provided one of the mainstays of this chapter, an understanding of the technique is essential. An overview of PCR is therefore provided here, and a description of the specific adaptations used to enable isolation and quantitative analysis will be presented in subsequent sections.

PCR allows the in vitro amplification of specific DNA sequences by the simultaneous primer extension of complementary strands of DNA (Taylor, 1994). The use of a thermostable polymerase (an enzyme which synthesises a complementary strand of DNA in a 5' to 3' direction using a single stranded template but starting from a double-stranded region), along with two primers, each complementary to opposite strands of the region of DNA which is to be amplified, results in the amplification of a specific region of DNA between the priming sites. The primers are arranged in such a way that each primer extension reaction directs the synthesis of DNA towards the other. Thus primer a directs the synthesis of a strand of DNA which can then be primed by primer b and vice versa (Fig. 3.1). This results in the de novo synthesis of the region of DNA flanked by the two primers. The reaction requires three distinct thermal steps. The initial step is one of denaturation in which the double stranded DNA is held at 95°C to break the hydrogen bonds between the two polynucleotide chains, and separate the double helix into two strands. The temperature is then lowered to facilitate annealing of the primers to their complementary sequences. Following annealing, primer extension is conducted at a temperature optimal for the polymerase (usually 72°C).
It is evident that if the reaction is repeated with an excess of reagents, then the amount of DNA in the region flanked by the primers will amplify in an exponential fashion. Generally 25-35 cycles is sufficient to produce up to 1μg of DNA of a single copy gene from 50 ng of total starting genomic DNA (Taylor, 1994). The amplification of specific regions of DNA using PCR therefore provides a rapid and simple technique with which to isolate particular regions of a gene of interest from the genome.

Fig. 3.1 Diagrammatic representation of PCR. The initial template is amplified exponentially provided that an excess of polymerase, dNTPs, and primers are present and given that the buffer conditions are appropriate. After Taylor (1994).
3.1.6 Rationale Behind the Analysis of per in L. cuprina

The fascinating differences manifested by the lepidopteran system whetted the appetite of circadian biologists and begged the need for per investigations in other insect species. It seemed likely that other comparative systems could yield interesting information on the nature and conservation of clock systems. This comparative research was hindered initially by the difficulty in identifying per homologues outside the drosophilids (Kyriacou et al., 1996), but analysis finally began with the isolation of per from Musca domestica (Kyriacou et al., 1996). Expression analysis of M. domestica per has indicated a very different system from the Antheraea and Drosophila systems. Neither mRNA nor protein levels appear to oscillate in the house fly (pers. comm. Kyriacou, 1997), and the functional nature of the house fly feedback loop remains elusive.

The phylogenetic position occupied by Lucilia provides an interesting intermediary platform for comparative analysis. Being in the same Sub order (Schizophora) of flies as Drosophila, it remains close to the prominent insect circadian model. At the same time Lucilia is sufficiently distant to provide a potentially attractive standpoint. Significantly, it is also in the same Series (Calyptidae) as Musca, which has proven to possess a very different circadian system from any of the other systems analysed.

The following then, is a description of the isolation of a per cDNA from the sheep blow fly L. cuprina, and the development and use of systems which have allowed the analysis of expression levels of this homologue. The analysis of expression of sense per mRNA and PER-like immunoreactivity indicates whether L. cuprina possesses a house fly or fruit fly-like system, while the investigation of whether an antisense per mRNA strand exists is used to ascertain whether a moth-like gene silencing approach is employed in this fly.
Chapter Three: Testing the Molecular Assumptions of the Simulation Model

3.2 General Methods

3.2.1 Degenerate PCR

Two sets of degenerate oligonucleotide primers (PerF1/PerR2, and PerF3/R4) used for the amplification of a *per* cDNA fragment from *L. cuprina* were designed using consensus data from seven published insect *per* sequences (accession numbers: AF033029; U12769; U12771; U12772; U12773; X13878; X61127), and the unpublished sequence of house fly *per* (courtesy of Dr C.P. Kyriacou, University of Leicester). The sequences of PerF1 (GGN MGN WSN TTY ATH GAY TTY GTN CA) and PerR2 (TTN TCR TTR TAR TTN ARY TGR TTR TA) correspond to amino acid positions 271-279 and 613-701 of the *D. melanogaster* PER protein sequence. The sequences of PerF3 (CAY CAY GAY TAY TAT GA) and PerR4 (CAT YTC RTC RTT RTT RTG YTT) were based on the sequences published in Nielsen *et al.* (1994) and correspond to amino acid positions 598-603 and 771-776 of the *D. melanogaster* PER protein sequence.

Genomic DNA (gDNA) was extracted using the CTAB technique (Appendix Two). Hotstart PCR was used to amplify gDNA using 1 μL of DNA in a 50μL reaction mix containing 1 x PCR buffer (50 mM KCl, 10 mM Tris-Cl (pH 8.3) and 1.5 mM MgCl₂), 50 pmoles of each primer, 100μM dNTP and 1 unit of AmpliTaq DNA polymerase (Perkin Elmer Cetus). Amplification was conducted in a Hybaid Omn-E thermal cycler using the following conditions:

1. PerF1/R2: 94°C 3 min; 30 cycles of 94°C 30 sec, 45°C 60 sec, 72°C 2 min
2. PerF3/R4: 94°C 3 min; 30 cycles of 94°C 30 sec, 50°C 60 sec, 72°C 60 sec.

The last extension (72°C) phase was prolonged for 10 min to ensure fully double-stranded molecules were produced from all nascent products. PCR products were resolved on a 1% agarose 1 x TBE gel and were visualised on a UV transilluminator following staining with ethidium bromide (10μg/mL).
3.2.2 Southern Hybridisation of PCR Products

PCR products were tested for per homology by Southern hybridisation to a radioactively labelled *M. domestica* per probe. Following separation of PCR products on a 1% agarose 1 x TBE gel, the gel was depurinated for 30 min in 0.25 M HCl. DNA was denatured for 30 min in denaturation solution (1.5 M NaCl, 0.5 M NaOH) and then neutralised for 2 x 15 min in 1.5 M NaCl, 0.5 M Tris-Cl (pH 7.2) containing 1 mM EDTA. Southern transfer of DNA onto positively charged nylon membrane (Amersham Hybond N+) was conducted for 15 h at room temperature in 6 x SSC.

The membrane was probed with a $\alpha^{32}$P dCTP radiolabelled fragment of house fly *per* (gift of Dr C.P. Kyriacou, University of Leicester) (using the Life Technologies random prime kit). Prehybridisation and hybridisation were conducted overnight at 45°C in 0.25 M Na$_2$HPO$_4$ (pH 7.2) containing 7% SDS and 1mM EDTA. Membranes were then washed twice in 2 x SSC containing 0.1% SDS (45°C, 30 min) and twice in 1 x SSC containing 0.1% SDS (45°C, 30 min), and were exposed to X-ray film at -80°C for 48 h, after which autoradiographs were developed to visualise *per* reactivity.

3.2.3 Cloning and Sequencing of PCR Products

PCR products purified from 1% agarose 1 x TAE gels using 'GlassMax' (Life Technologies) were ligated into the vector pGEM®-T (Promega). The ligation products were then transformed into JM-109 cells, made competent using the TSS/DMSO method (Appendix Three). Positive transformants were detected by colony PCR using M13 universal forward and reverse primers. Positive colonies were purified using a modified alkaline lysis preparation (Feliciello and Chinali, 1993) and sequenced using a PRISM™ Dye Deoxy Terminator Cycle Sequencing kit and a 373 automated sequencer (Perkin Elmer/Applied Biosystems).
3.2.4 cDNA Library Screening

A 1.2 Kb *per* PCR fragment isolated using the above strategy was radiolabelled (as described in Section 3.2.2) and used to probe a cDNA library constructed in λGT10 from *L. cuprina* adult head mRNA (gift of Dr P. East, CSIRO). The library was pre-screened by PCR using the QPerF1/R2 primer combination (Section 3.2.8) to check for the presence of *per* before a primary screen was conducted of 500,000 plaque forming units (pfu). Overnight cultures of TAP 90 cells in rich LB were used to seed fresh rich LB and grown to mid log phase (approx. 3 h at 37°C). Cells were then harvested by centrifugation at 2000g for 10 min and resuspended in 10 mM MgSO₄ to an OD₆₀₀ of 0.6. 600 μL of these cells were inoculated with 50,000 pfu (in 100 μL of SM buffer) and allowed to incubate for 15 min at 37°C. 10 mL of NZY top agarose (Appendix Four) was added to the incubation and the mixture poured over NZY plates. Plates were left to set and were then incubated overnight at 37°C (further technical details of cDNA library screening are provided in Appendix Four).

Plaque lifts were made using Amersham Hybond N+. Membranes were denatured for 5 min with denaturation solution (0.5 M NaOH, 1.5 M NaCl), neutralised for 15 min (1 M Tris-HCl pH 7.5, 1.5 M NaCl), and equilibrated for 20 min in 2 x SSC.

Membranes were subsequently baked for 2 h at 80°C. Prehybridisation (2 h) and hybridisation (15 h) were conducted at 55°C in 0.25 M Na₂HPO₄ (pH 7.2) containing 5% SDS. After hybridisation, membranes were washed twice in 2 x SSC containing 0.1% SDS (55°C) and twice in 2 x SSC containing 1% SDS to remove non-specific binding of the probe. Membranes were then wrapped in 'glad wrap' and exposed to X-ray film in a hypercassette for 48 h (-80°C). Processed X-ray films showing *per*-reactive colonies were aligned with plates and positive plaques were cored and eluted in 1 mL of SM buffer containing 10 μL of chloroform. Positive recombinant phage clones from the primary screen were subjected to two further rounds of screening. Lambda DNA was extracted from the tertiary positives using the protocol outlined in the Promega Protocols Handbook (1996) and digested with EcoRI to
determine the size of the cDNA inserts. The cDNA fragments were then ligated into pUC 18 for sequencing (Section 3.2.3).

3.2.5 Semi-Degenerate RT-PCR

Semi-degenerate PCR of per cDNA was conducted using one specific primer; PerF4 (CCC TAT GCT GGA GGC CTA TC) designed to the 3' end of the cDNA clone (corresponding to nucleotide positions 2159-2179 of the *L. cuprina* per sequence), and one degenerate primer; DegenR1 (RTC CAT RTG RTC NSW RTT NCC), corresponding to nucleotide positions 2928-2949 of the *L. cuprina* per sequence, which was designed to a conserved region of *per* from eight insect species using the previously described strategy.

3.2.5.1 RNA extraction

Total RNA was extracted from 10 *L. cuprina* heads in 1mL of Trizol® Reagent (Life Technologies), and then precipitated with 0.5 mL of isopropanol. RNA was resuspended in 20μL of DEPC-treated H₂O and residual DNA was removed by digestion with amplification grade DNAsel 1 (Life Technologies) for 15 min at 37°C.

3.2.5.2 RT-PCR

Following denaturation at 70°C for 10 min, 5 μL of RNA was reverse transcribed for 1 h at 42°C in the presence of 10 mM DTT, 1x reverse transcriptase buffer (Life Technologies), 10 units of RNAsin, 10 μM OligodT primer, 0.5 mM dNTPs, and 20 units of M-MLV Reverse Transcriptase (Life Technologies). 1μL of the resulting cDNA was used in a 50 μL reaction (as above) which was conducted using the cycling conditions described for PerF3/R4. PCR products were then purified, cloned and sequenced as described in Section 3.2.3.
3.2.6 3'RACE (Rapid Amplification of cDNA Ends)

RNA isolation and reverse transcription for 3' RACE was conducted as above with the exception that a modified oligoT primer (ATC GAT GGT CGA CGC ATG CGG ATC CAA AGC TTG AAT TCG AGC TCT) which included two specific priming sites, was used for the reverse transcription. PCR was conducted as above using the primer Per3primeF1 (TCC GTT ATG GGT GAC TAT GTA) corresponding to nucleotide positions 2850-2871 of *L. cuprina per*, and Ri (CCG CAT GCG TCG ACC ATC GAT) or Ro (AGA GCT CGA TAA CAA GCT TTG GAT) which are specific for one of the two synthetic priming sites generated on the end of the cDNA by the modified OligoT primer. Thermal cycling was carried out using the following program:

94°C 3 min; 35 cycles of 94°C 15 sec., 60°C 30 sec., 72°C 1 min

Amplicons purified using a 'Qiaquick' PCR purification kit (Qiagen) were cloned (as in Section 3.2.3). Positive transformants initially screened by their restriction profile were then Southern blotted and probed with an overlapping radiolabelled *per* cDNA fragment (the PerF4/DegenR1 fragment). Positive clones were then sequenced and the sequences were aligned using the University of Wisconsin (GCG) programs Gelmerge and Gelassemble (Wisconsin Package version 9.0, Genetics Computer Group, Madison, Wisc.).

3.2.7 Geographic Analysis of the TG Repeat Region

PCR amplification of a 733 bp fragment of *per* was conducted using primers PerF5 (GCC TTC AGA TAC GGT CAA AC) and Per3primeR1 (GAT AGG CCT CCA GCA TAG GG), corresponding to nucleotide positions 1445-1464 and 2159-2178 of *L. cuprina per* respectively. Genomic DNA samples of *L. cuprina* and *L. sericata* collected from a number of different geographic locations (Table 3.1) (donated by Dr R. Newcomb and Dr D. Gleeson, HortResearch, Mt Albert) were analysed.
Chapter Three: Testing the Molecular Assumptions of the Simulation Model

<table>
<thead>
<tr>
<th>Site (L. cuprina)</th>
<th>Latitude</th>
<th>Site (L. sericata)</th>
<th>Latitude</th>
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<tbody>
<tr>
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<td>Vancouver (Canada)</td>
<td>49° 15’ N</td>
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<tr>
<td>Kluang (Malaysia)</td>
<td>3° 0’ N</td>
<td>Bloemfontein (S.A.)</td>
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Table 3.1 Geographic location and latitude of collection of fly samples used for the analysis of TG repeat length

PCR was conducted as outlined previously under the following cycling conditions:

94°C 3 min; 35 cycles of 94°C 15 sec., 55°C 30 sec., 72°C 1 min

Amplicons were purified and sequenced (as in Section 3.2.3) and the sequences were aligned using ‘Sequencher’. Phylogenetic analysis was conducted using PAUP*4.0 (Swofford, Sinauer Associates).

3.2.8 Quantitative Competitive RT-PCR

Primers QPerF1 (TGG AAT ACC AAT AGC CGA ATC ACG C), QPerR2 (CAA TAC CGACAC TGC TGC ACT ACT C), and QComp (TGG AAT ACC AAT AGC CGA ATC ACG CGA TTG ACA TTC CGC GAA GCA CC) corresponding to nucleotide positions 703-728, 1036-1061 and 848-873 of the L. cuprina per sequence respectively (Fig. 3.10), were designed to enable the amplification of a per fragment and a per competitor. PCR amplification was conducted as previously using the cycling conditions shown below. Both of these fragments were cloned into pGEM®-T (as described above) and were sequenced to check their integrity and orientation. The plasmids were then linearised by digestion with NcoI and RsaI respectively, and a sense RNA strand of each fragment was transcribed using a Promega in vitro
transcription kit. RNA was DNase treated (using Promega RQ1 RNase-free DNase1), purified by phenol/chloroform extraction, and ethanol precipitated. Purified RNA was resuspended in DEPC-treated H$_2$O and quantified using a Genequant RNA/DNA Calculator (Pharmacia).

Quantitative competitive RT-PCR was conducted using the standard curve methodology of Tsai and Wiltbank (1996) outlined in Section 3.5. Reverse transcription and PCR were carried out as in Section 3.2.5.1, with the exceptions that 10 pg of competitor RNA was added to the RT mix in addition to 5µg of sample RNA, and that the reverse transcribed cDNA was diluted 1:10 with DEPC-treated water and 10µL of this dilution was added to the PCR (in order to reduce pipetting error). The specific primer QPerR2 was used in reverse transcriptions as opposed to random hexamers to ensure increased specificity of the RNAs which were reverse transcribed. The PCR conditions used were as follows:

94°C 3 min; 35 cycles of 94°C 15 sec., 65°C 30 sec., 72°C 1 min

Gel images were quantified densitometrically using Scion image (NIH) and the data were plotted using Microsoft Excel 7. Experimental samples of 10 fly heads were taken every 2 h for 48 h from flies held in LD 12:12. Total RNA was extracted and quantified, and 5 µg was used in each RT-PCR with 10 pg of competitor RNA. PCR products were visualised and quantified as above. Relative band intensities were converted into pg of per mRNA per µg of total RNA using the standard curve measurements.

3.2.9 Northern Dot Blots

A 291 bp fragment of L. cuprina RP49 cDNA isolated using degenerate PCR and primers RP49F1 (CAC CAG TCG GAT CGN TAT GCC) and RP49R2 (GAC AGC TGC TTG GCN CGN TC) designed to a consensus RP49 sequence from D. acanthoptera, D. subobscura and D. pseudoobscura was cloned, sequenced,
linearized and transcribed as an antisense DIG-labelled probe, using a Promega *in vitro* transcription kit and DIG RNA labelling mix (Boehringer Manheim). This probe, along with an antisense DIG-labelled *per* probe, was used to probe dot blots of 20μg total RNA. Prehybridisation and hybridisation were conducted using DIG-EasyHybe (Boehringer Manheim) at 60°C. Subsequent washes were conducted according to the instructions in the DIG protocols handbook (Boehringer Manheim) (2 x 15 min in 2 x SSC containing 0.1% SDS followed by 2 x 15 min in 0.5 x SSC containing 0.1% SDS) and the reaction was visualised using a chemiluminescent system (Boehringer Manheim).

### 3.2.10 Western Blotting

Samples of 10 fly heads were taken at 3 h intervals from flies held in LD 12:12 and frozen in liquid N₂. Heads were homogenized in 50 mM Tris-Cl (pH 7.5), 10% glycerol, 5 mM MgOAc, 0.2 mM EDTA containing 1mM DTT, 1mM PMSF, 0.1% leupeptin and 0.1% aprotinin (Persichetti *et al.*, 1993). Protein quantification was conducted using the Bradford assay (Bradford, 1976), and 40μg of each sample was resolved on an 8% SDS-acrylamide gel. Following separation and semi-dry Western blotting, the membrane was blocked for 1.5 h in 1 x PBS containing 0.1% Tween 20 (PBS-T) and incubated for 1.5 h with the primary PER antibody (Per #107 (gift of Dr. L. Saez, Rockefeller University)) diluted 1:500 in PBS-T. Incubation with a secondary HRP-linked anti rabbit IgG antibody (Amersham) diluted 1:1000 in PBS-T was conducted for 1 h. Between each of the above steps the following series of washes was conducted: 5 min PBS, 2 x 20 min PBS-T, 5 min PBS. Positive immunoreactivity was visualised using an Enhanced Chemiluminescent system (Amersham).
3.3 *L. cuprina per* Encodes an Inferred Protein of 1037 Amino Acids

The principle step in the molecular analysis of *per* in the sheep blow fly was the extraction of this gene from the genome. The isolation of a 4 Kb *L. cuprina* cDNA was achieved in four steps (Fig. 3.2).

![Diagrammatic representation of the strategy used to isolate per cDNA. Steps 1-4 are chronological representations of the different techniques employed.](image)

The initial isolation of two overlapping fragments from genomic DNA was conducted using a degenerate PCR approach similar to that described by Reppert *et al.* (1994). This was followed by cDNA library screening, semi-redundant PCR and finally 3'RACE.

3.3.1 Degenerate PCR

Primers used in PCR are usually unique oligonucleotides designed from a known DNA sequence. However, for a gene of unknown sequence, PCR may be used as a cloning strategy by designing degenerate primers from the nucleotide sequence of homologous genes (Macpherson *et al.*, 1994). These primers are termed degenerate because at positions where nucleotide differences occur in the consensus sequence 'mixed base' synthesis is employed (ie. if a particular nucleotide is either a G, C, A or T then
addition of all four nucleotides at the coupling step results in 25% of the oligos having a G, 25% having a C, 25% having an A, and 25% having a T).

The two sets of degenerate primers designed to the consensus sequence of conserved regions of *per* from eight insect species resulted in the amplification of products of approximately 1.2 Kb (using PerF1/R2) and 500 bp (using PerF3/R4) as shown in Fig. 3.3 below.

![Fig. 3.3 1% agarose TBE gel of PCR products obtained from the amplification of *L. cuprina* gDNA with *per* primers. Lanes 1 and 10: 1Kb ladder (Gibco/BRL); Lanes 2, 8 and 9: positive control (*L. cuprina* cytochrome oxidase gene); Lane 4: *L. cuprina* PerF1/R2 product; Lane 6: *L. cuprina* PerF3/R4 product; Lane 7: *M. domestica* PerF3/R4 product.](image-url)

Southern blotting and hybridisation of the PCR products to an α³²P-dCTP labelled *Musca domestica per* probe showed these amplicons to contain *per* homology (Fig. 3.4).
Cloning of the fragments into pGEM\textsuperscript{T}, subsequent sequencing of both strands of two separate clones of each fragment, and comparison of the consensus sequences with published \textit{per} sequences (using GCG FastA) validated the identity of these fragments. The PerF1/R2 fragment was a 1142 bp \textit{L. cuprina per} fragment (corresponding to nucleotide positions 813-2103 of \textit{D. melanogaster per}) which spanned two introns. The smaller PerF3/R4 product was solely exonic and 530 bp in length spanning the TG repeat region.

### 3.3.2 cDNA Library Screening

The second step in the isolation of the \textit{L. cuprina per} cDNA involved the screening of cDNA libraries. As the amplified fragments represent a portion of the gene of interest, they can be used as homologous hybridisation probes to screen cDNA libraries for the full-length cDNA.

Screening of four out of five libraries with the radiolabelled 1.2 Kb \textit{per} amplicon isolated by PCR revealed no positives. This low positive rate is not surprising given that most cDNA libraries are not made at the specific circadian times when \textit{per} is expressed. However, PCR pre-screening of an adult head cDNA library (donated by

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**Fig. 3.4** Autoradiograph of PerF1/R2 and PerF3/R4 PCR products hybridised to a \textsuperscript{35}P \textit{M. domestica per} probe. Lanes 1 and 2 \textit{L. cuprina} PerF1/R2 PCR product; Lane 3 \textit{M. domestica} PerF3/R4 PCR product; Lane 5 Radiolabelled 1Kb ladder.
Dr. P. East, CSIRO) with *per*-specific primers, produced products of a size expected from *L. cuprina* *per*. Subsequent screening of this library yielded two distinct *per*-positive plaques (Fig. 3.5).

![Fig. 3.5](image1.png)

Fig. 3.5 Autoradiographs of lifts from the tertiary screen of a *L. cuprina* λGT10 adult head cDNA library probed with the 1.2 Kb *per* PCR product showing two distinct *per*-positive phage clones (B. and J.).

The excision of the cDNA clones from the EcoRI site of λGT10 indicated one of them to be approximately 2.8 Kb in length (B.) and the other to be approximately 600 bp in length (J.) (Fig. 3.6).

![Fig. 3.6](image2.png)

Fig. 3.6 EcoRI digest of phage preparations from the adult head cDNA library screen. Lanes 1 and 4 contain a 1Kb ladder (Gibco/BRL); Lane 2 contains the EcoRI digest of λGT10 clone (B) indicating the presence of an insert of approx. 2.8 Kb; Lane 2 contains the EcoRI digest of λGT10 clone (J) indicating the presence of an insert of approx. 600 bp.
Sequencing of these sub-cloned cDNA fragments by primer walking revealed both to be *per* fragments. The larger fragment extends from a point 425 bp 5' of the *per* initiation codon to nucleotide 2316 (corresponding to nucleotide 2811 in *D. melanogaster per*). The smaller fragment is completely nested within this larger fragment.

### 3.3.3 Semi-Degenerate RT-PCR

Further library screening employed to obtain the remaining 3' sequence of the cDNA proved unfruitful so a semi-degenerate PCR strategy was adopted. Semi-degenerate PCR involves the use of one specific primer designed to a portion of the sequence previously identified, and a second degenerate primer designed to consensus sequence from other species. A 700 bp fragment amplified from cDNA using semi-degenerate PCR (Fig. 3.7) provided a further 600 bp of coding sequence.

![Fig. 3.7 1% agarose TBE gel of semi-redundant PCR using primers PerF4/DegenR1. Lane 1 1Kb ladder (Gibco/BRL); Lane 2 PCR using PerF4/DegenR1; Lane 3 PCR using PerF4/DegenR2; Lane 4 PerF4 single primer control; Lane 5 degenR1 single primer control.](image)

### 3.3.4 3'RACE

The proximity of the 3' portion of the above fragment to the end of the *per* cDNA facilitated the use of 3' RACE to obtain the remaining sequence of the *L. cuprina per* cDNA. This technique involves the initial synthetic addition of priming sites to the PolyT tail of the cDNA at the reverse transcription step. In the subsequent PCR step a
primer designed to this synthetic priming site is used in addition to a specific primer designed to the 3' end of the known sequence.

A product of approximately 700 bp obtained from 3' RACE (Fig. 3.8) was cloned into pGEM-T.

![Fig. 3.8 Products from 3'RACE of L. cuprina per resolved on a 1% agarose TBE gel. Lane 1 1Kb ladder (Gibco/BRL); Lanes 2 and 3 product obtained with Per3primeF1/Ri primer combination; Lane 4 3primeF1 single primer control; Lane 5 Ri single primer control.]

The testing of 17 different clones containing 700 bp inserts by Southern hybridisation to a $^{32}$P labelled overlapping per fragment indicated a single per-reactive clone (Fig. 3.9).

![Fig. 3.9 Autoradiograph of Southern hybridisation of 3'RACE PCR fragments digested out of pGEM®-T and probed with the radiolabelled PerF4/DegenR1 product. Lanes 1-17 contain digests of plasmids with an insert of approximately 700bp. The insert in lane 15 shows per homology. Lanes 18 and 19 contain different dilutions of the original 3'RACE PCR.]

Sequencing of this clone revealed it to contain the remaining 3' portion of the per transcript including the polyadenylation signal (Fig. 3.10).
Chapter Three: Testing the Molecular Assumptions of the Simulation Model

Fig. 3.10 Nucleotide and inferred amino acid sequence of *L. cuprina* per cDNA. Motifs corresponding to *D. melanogaster* functional domains are highlighted in different colours. Green - nuclear localisation signal (AA 65-80). Yellow - PAS A domain (AA 183-232) - PAS B domain (AA 307-396). Blue - cytoplasmic localisation signal (AA 394-454). Pink - sites of *period* mutations in *D. melanogaster*. (I). *Per*<sup>1</sup> mutation (AA 186), (II) *per*<sup>5</sup> mutation & (III) *per*<sup>2</sup> mutation. Green 2. - TG repeat region (AA 634-638). The consensus polyadenylation signal present in the 3' flanking region underlined. Nucleotide positions are numbered from the start of translation.
3.3.5 *L. cuprina per*

The entire *L. cuprina per* cDNA shown in Fig. 3.10 was assembled from the consensus sequence of both strands of *per* clones using 'Gelassemble' and 'Gelmerge' (University of Wisconsin, GCG). Where a PCR based strategy was used, the sequence of at least two separate clones was used to compile the consensus. The CAP site of the mRNA is not present in the sequence, but the initiation codon is evident (nucleotide number 1). The stop codon (beginning at nucleotide 3109) is present, as is the polyadenylation signal (nucleotides 3397-3402). Regions similar to the *D. melanogaster per* protein are also apparent in the sequence. These include the PAS A and B regions, the TG repeat and the cytoplasmic and nuclear localisation signals (Fig. 3.10). The inferred amino acid sequence is shown beneath the coding region. *L. cuprina per* appears to encode a 1037 amino acid protein with a molecular weight of approximately 110 kD.

The overall identity of the inferred protein to *M. domestica*, *D. melanogaster* and *Antheraea pernyi* PER is 84%, 73% and 37% respectively. Within the conserved regions (C1-C6) this homology is increased up to 88%, 80% and 58% respectively (Fig. 3.11). As with *A. pernyi* (Reppert et al., 1994) the regions of highest homology to *D. melanogaster* are confined to distinct stretches within the C2-C3 regions. These regions correspond to the functional domains of *D. melanogaster* PER (Table 3.2).

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Table 3.2. Amino acid identity of the functional domains of *L. cuprina per* with those of *M. domestica* and *D. melanogaster*. NLD-nuclear localisation domain; CLD-cytoplasmic localisation domain; PAS A and PAS B are the two regions of the PAS dimerisation domain.
### Chapter Three: Testing the Molecular Assumptions of the Simulation Model

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3.3.6 What Does the L. cuprina per Sequence Tell Us About the Blow Fly Clock?

per has recently been noted for its extremely rapid rate of evolution (Regier et al., 1998; Schmid and Tautz, 1997). Even within the relatively well-conserved PAS domain, lepidopteran per has evolved 5 to 40 times more rapidly than other lepidopteran nuclear genes (Regier et al., 1998). The presence of particular regions of conservation and variability in the per coding sequence first mentioned by Colot et al. (1988) was evident in L. cuprina (Fig. 3.11), and many of the conserved regions were associated with functional domains (Fig. 3.10, Table 3.1). Despite the reputation of per for rapid evolution, L. cuprina per maintains a relatively high sequence homology to other dipteran per homologs. This high homology in flies cannot be attributed to a
shorter evolutionary time scale as the radiation of the higher flies and the moths both occurred during the Cretaceous period (Beverley and Wilson, 1984).

In the evolution of Diptera, the calypterate and acaleypterate flies (members of the Division Schizophora) are thought to have first diverged 135 million years ago (Beverley and Wilson, 1984). While the house fly *Musca domestica*, and the sheep blow fly *Lucilia cuprina* belong to different families (Muscidae and Calliphoridae respectively) in the same section (Calypterae), *Drosophila* belongs to the Acalypterae section of flies. The divergence of the Drosophilidae from the Calliphoridae is estimated to have occurred 105 million years ago (Everley and Wilson, 1984; Hennig, 1981). Since *Lucilia* and *Musca* are much more closely related to each other than either are to *Drosophila* (*Lucilia* and *Musca* are different families within the same series (Calyptereae)), it is not particularly surprising that the homology between *Lucilia* and *Musca* is the greatest.

Despite the fact that *Lucilia* maintains a 22 h free-running period in constant conditions (Smith, 1983; Warman, 1995) compared to the 24 h period of wild-type *Drosophila*, the amino acids which give rise to changes in period length when altered in *Drosophila* have been conserved in the wild-type form in *Lucilia* (Fig. 3.10). This suggests that the difference in period length is either attributable to the action of a different region of *per*, or to a difference in another functional element of the *L. cuprina* feedback loop. A discussion of the techniques which could be used to clarify this issue is presented in Chapter Five.

The possession of a PAS domain and in many cases a TG repeat are hallmarks of a clock gene. The presence of these regions in *L. cuprina per* supports the contention that the gene isolated is in fact a *per* homologue, if only at a primary sequence level. PAS domains and TG repeats have been found in clock genes from a wide range of species from *Drosophila* through to the bread mould *Neurospora crassa* (Citri, *et al.*, 1987; Kay, 1997), and it is suggested that the analysis of these regions may provide evidence for the evolutionary origins of circadian clocks (Kay, 1997).
Particularly high conservation of the PAS, CLD and NLD domains between *D. melanogaster* and *L. cuprina* is strongly suggestive of similar functional roles in these species, and the reduction of the TG repeat of *L. cuprina* down to a doublet is characteristic of non-drosophilid flies, all of which have shown this stable repeat length (Nielsen *et al.*, 1994).

Using the PerF1/R2 and PerF3/R4 fragments isolated above, *L. cuprina per* has been shown to reside on the *Lucilia* homologue of the *Drosophila* *X* chromosome, between *arista* and *wavy* (*pers. comm.* Batterham, 1998). This position corresponds to that occupied by *per* in the *Drosophila* genome (linkage groups of higher Diptera have remained relatively intact during evolution (Weller and Foster, 1993)). This mapping also indicates that the *ary* mutation, mapped to chromosome 5 (chromosome 2 equivalent in *Drosophila*), which has severe effects on the circadian phenotype (Smith, 1987), is definitely not a *per* mutation. The *ary* mutation is therefore likely to be either a *Lucilia* homologue of another central clock element or a novel mutation affecting the clock.

### 3.4 Geographic Variation in the TG Repeat Region and Flanking Sequences

The TG repeat region was initially proposed to play a role in the thermal stability of the circadian phenotype because the rescue of arrhythmic *per*⁰ flies with a *per* gene missing the TG repeat produced flies lacking temperature compensation (Ewer *et al.*, 1990). These data were supported by the observation of a North-South latitudinal cline in TG repeat length in European *D. melanogaster* (Costa *et al.*, 1992; Piexoto *et al.*, 1993) and by laboratory analysis of thermal stability in the different repeat forms (Sawyer *et al.*, 1997). The observation of a stable repeat length in many different species of non-drosophilid flies (Nielsen *et al.*, 1994) raised the interesting question of how this stable repeat was able to confer the 'fine-tuning' which was proposed to occur in *Drosophila*. The suggestion was made that the co-evolving flanking regions of the
TG repeat might play a role in this thermal stability, which is such a cardinal feature of circadian clocks (Nielsen et al., 1994).

In order to explore this possibility further, amplification and sequencing of a 733 bp gDNA fragment spanning the TG repeat was conducted from \textit{L. cuprina} and its sister species \textit{Lucilia sericata} collected from eight different geographic locations (see Section 3.2.7).

Analysis of these sequences revealed no polymorphism in TG repeat length across any of the samples analysed. All of the flies analysed maintained the two repeat form (TGTGT) shown in Fig. 3.10. Not only was there no variation in the length of the TG repeat, but none of the nucleotide alterations in the flanking sequence caused amino acid changes in the inferred protein. Phylogenetic analysis of the entire sequence (including the repeat region and the flanking sequence) showed a clear clustering of the two species of \textit{Lucilia}, but failed to show any correlation between sequence homology of regions flanking the repeat and latitude of collection of flies (Fig. 3.12). The phylogenetic tree shown in Fig. 3.12 is the consensus tree obtained from an exhaustive PAUP search. The $g_v$ value produced from this analysis was -0.43266, indicating the sequences analysed contain sufficient signal strength to facilitate tree building (Lento, 1995). The consensus tree produced by the exhaustive search is identical to that obtained from a basic parsimony analysis and a neighbour joining (distance) tree. Bootstrap values for the branches are shown in Fig. 3.12. The outgroup used for the tree is the \textit{M. domestica} sequence (courtesy of Dr. C. Kyriacou, University of Leicester).
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Fig. 3.12 Cladogram generated from the consensus of an exhaustive PAUP search. The sequence used to construct the tree was obtained from analysis of a 733 bp gDNA fragment of *L. cuprina* and *L. sericata* spanning the TG repeat region. Bootstrap values are indicated on those branches which have greater than 50% support. Branches with less than 50% support have been collapsed.

Despite the different habitat ranges and upper lethal limits of *L. cuprina* and *L. sericata*, the lack of variation in TG repeat length, both between and within the species, and the lack of correlation of flanking sequences with latitude of strain collection, suggests that these areas do not confer thermal stability of the circadian phenotype in *Lucilia*. This proposition is further bolstered by the fact that samples of *L. cuprina* collected from different latitudes represent members of two different sub-species adapted to different thermal environments (see Chapter One).

The above findings add weight to the argument that temperature compensation results from differential protein interactions (Huang *et al.*, 1995), or another area of the PER protein such as the PAS domain (as Per1 flies exhibit lack of temperature compensation (Curtin *et al.*, 1995)). Alternatively, an as yet uncharacterised system similar to that of
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Neurospora crassa, involving the production of different transcripts in different thermal environments (Liu et al., 1997) may exist to confer the vital ability of temperature compensation.

Analysis of larger samples collected from different latitudes within a single land mass may provide data more comparable to that obtained from D. melanogaster. However, the lack of significant variation in repeat length or flanking sequence at a gross geographic scale does not bode well for the discovery of systematic variation at a more local scale.

3.5 Development of Quantitative Competitive RT-PCR as a Technique for Measuring Circadian Fluctuations in per mRNA Levels

3.5.1 An Introduction to Quantitative Analysis of mRNA levels

A number of techniques are available to measure the expression levels of specific genes. Most of these techniques are, however, at best only semi-quantitative (such as Northern blots). Perhaps the most versatile of the transcript quantification techniques developed to date is competitive RT-PCR (Hayward et al., 1998), which has been used to quantify mRNA expression levels in a diverse range of situations from the expression of cytokines (Gilliland et al., 1990), through to the quantification of viruses such as HIV (Piatak et al., 1993) and the analysis of environmental pollution (Miller, 1997; Wilson, 1997). As all previous analyses of circadian variations in clock gene expression have been conducted using RNAse protection assays (Hardin, 1990, 1992; Reppert, 1994), the present study has focussed on the development of quantitative competitive RT-PCR as a sensitive alternative method for measuring circadian oscillations in per transcript levels.
3.5.2 General Principles of Quantitative PCR

Quantification of RNA or DNA by PCR relies on the assumption that the amount of product obtained is proportional to the amount of starting material. Despite the exquisite sensitivity of PCR, the exponential nature of DNA amplification is prone to load the data with significant error due to the inherent tube-to-tube variations in amplification efficiency (Zachar et al., 1993). Factors such as pipetting error, temperature variation, and the presence of inhibitors can alter the between-tube amplification efficiency. The effect of these differences is magnified by the exponential nature of PCR to produce considerable differences in product yield. Those techniques which rely on the amplification of samples and standards separately (Irving et al., 1993) are therefore wildly inaccurate. The reliable quantification of DNA or RNA templates by PCR thus necessitates the introduction of an internal standard (Wang et al., 1989; Souazé et al., 1996) which acts as a measure of the amount of mRNA amplified.

Non-competitive internal standards, which have a different sequence and require different primers from the target sequence, have been used in quantitative RT-PCR (Chelly et al., 1990; Noonan et al., 1990). In this case, a known amount of an exogenous mRNA transcript is added to the sample RNA (Chelly et al., 1990), or a known amount of ubiquitously expressed transcript (such as GAPDH) is present in the sample RNA (Noonan et al., 1990). These standards are co-reverse transcribed and co-amplified in the PCR (using separate primers), thus removing the inter-tube variability. However, as different sets of primers and different templates are being used, the thermodynamics of the reverse transcription and amplification of the standard may be different from that of the template, and therefore there may be significant differences between the amplification efficiencies (Reischl and Kochanowski, 1995).
3.5.3 Quantitative Competitive RT-PCR (QC-RT-PCR)

The basis of competitive RT-PCR lies in the use of a standard which has the same priming sites as the native template. Since the internal standard (competitor) has the same primer sites as the template, the problems inherent in using two different templates and two sets of primers are eliminated. This technique was dubbed competitive RT-PCR as the internal standard literally competes with the native target for primers and reagents. Thus, increasing amounts of the native product will be formed as the concentration of the competitor is decreased (Becker-André and Hanlbrock, 1989).

Competitive RT-PCR was first developed as a titration assay in which constant amounts of total RNA were co-reverse transcribed and co-amplified with a dilution series of competitor RNA (Becker-André and Hanlbrock, 1989). The titration point at which the ratio of products is 1:1 (the equivalence point), was determined by plotting the log ratio of target to competitor (log (target/comp)) against the log amount of competitor added (log (comp)). At the equivalence point the log (target/comp) = 0, and therefore the value of competitor at the abcissa intercept is equal to the amount of target in the original sample. Given that the amplification efficiencies of the template and competitor are the same, quantification can be conducted in the log or plateau phases of the reaction (Siebert and Larrick, 1992; Zachar et al., 1993; Raeymakers, 1995). The major drawback of using this technique is that quantification relies on each sample being titrated against a dilution series of competitor from which the equivalence point is then determined. At least five RT-PCRs must be conducted on each sample in order to determine the equivalence point. The use of this technique for the analysis of circadian fluctuations in per mRNA levels is thus impractical.

With the development of a standard curve methodology for competitive RT-PCR (Tsai and Wiltbank, 1996) the analysis of multiple samples became feasible. This procedure follows the same general principles as that of Becker-André and Hanlbrock (1989), but involves the comparison of the ratio of amplification of competitor to native template in unknown samples to a single set of standard samples containing known amounts of
in vitro transcribed native template, and known amounts of in vitro transcribed competitor RNA (Tsai and Wiltbank, 1996). The use of standard curve methodology has been shown to produce results statistically similar to those obtained by the titration method, with a correlation of 94% between the two methods (Tsai and Wiltbank, 1996).

3.5.4 Synthesis of the per Competitor and Generation of the Standard Curve

The problem of how to distinguish the amplified competitor from the amplified template has been addressed in two ways. The first involves the use of a competitor with a novel restriction site. A restriction digest of the PCR following amplification can then allow the resolution of the products (Becker-André and Hanlbrock, 1989). This method is no longer regarded as being competitive, as the extra step of a restriction digest is thought to introduce variability (Reischl and Kochanowski, 1995). The second method available involves the use of a competitor of different size from the native template which can be resolved by electrophoresis following amplification.

Several different methods can be employed to construct a competitor of different length from the native template. Among these are the methods of intron inclusion, which creates a competitor larger than the template (Gilliland et al., 1990; McCulloch et al., 1995), the use of restriction sites to excise a portion of the amplicon and produce a smaller fragment (McCulloch et al., 1995), and primer extension (Celi et al., 1993).

The method of choice in the present work was that of primer extension. This technique relies on the PCR-based generation of a synthetic competitor from the native template. A competitor primer containing a synthetic priming site tag identical to the recognition site of one of the quantitative primers used in the QC-RT-PCR, is used in a PCR (Fig. 3.13). Amplification using a forward competitor primer and a reverse quantitative primer produces a product which is (in this case) shorter than the native template but which contains identical recognition sites (Fig. 3.13).
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Fig. 3.13 Diagrammatic representation of the primer extension technique used to synthesise a competitor. A. PCR of native template using QPerF1 and QPerR2 produces a native product containing QPerF1 and QPerR2 recognition sites. B. PCR of native template with competitor primer QCompF1 and QPerR2 produces a synthetic product smaller than the native product but still containing the QPerF1 and QPerF2 recognition sites.

Amplification of *L. cuprina* cDNA with the QPerF1/QPerR2 primer combination produced an amplicon of 358 bp while amplification with QCompF1 and QPerR2 produced an amplicon of 238 bp (Fig. 3.14).

Fig. 3.14 1% agarose TBE gel of PCR conducted to amplify competitor and native template. Lane 1: PCR of *L. cuprina* cDNA using QPerF1/R2 primer combination. Lane 2: PCR of *L. cuprina* cDNA using QCompF1/QPerR2 primer combination.

Competitive RT-PCR is often performed with a DNA competitor, such as the one above, which is added following reverse transcription (Gilliland *et al.*, 1990; Siebert and Larrick, 1992). However, as stated previously, the variability inherent in the reverse transcription step must be accounted for. The *in vitro* transcription of competitor RNA from a DNA template allows the addition of known amounts of RNA to the RT and therefore the control of this step.

In order to generate a standard curve with known amounts of template and competitor RNA, the two amplicons above were cloned into pGEM®-T. Clones were sequenced to determine insert orientation and integrity, and a sense RNA strand of each fragment was transcribed from the T7 or SP6 site (depending on the orientation of the insert in
the vector). Integrity of *in vitro* transcribed RNA was ascertained by digestion with DNAse and RNAse and electrophoresis on a 0.8% agarose 1 x TAE gel (Fig. 3.15).

![Fig. 3.15](image)

*Fig. 3.15* *In vitro* transcription of the *per* competitor resolved on a 0.8% agarose TAE gel. Lane 1: 1Kb ladder (Gibco/BRL); Lane 2: *in vitro* transcribed competitor RNA; Lane 3 *in vitro* transcribed competitor RNA treated with Promega RQ-1 RNAse-free DNAse 1; Lane 4 *in vitro* transcribed competitor RNA treated with DNAse-free RNAse.

If the standard curve is to present a valid estimate of the amount of RNA in the initial sample it must be generated over a range of values appropriate to the data. Therefore the initial step in the generation of the standard curve is to carry out a titration of equal amounts of sample RNA (containing an unknown amount of *per* mRNA) each spiked with a different concentration of competitor RNA. The titration below (Fig. 3.16) was conducted using 5 µg of total head RNA (Ct 3) and serial dilutions of competitor RNA from 1ng down to 0.1pg.

![Fig. 3.16](image)

*Fig. 3.16* Resolution of a competitive RT-PCR containing 5 µg of total fly head RNA and serial dilutions of the *per* competitor (1ng-0.1 pg) on a 1% agarose TBE gel. Lane 1: 5µg total RNA and 1 ng *per* competitor; Lane 2: 5µg total RNA and 100 pg *per* competitor; Lane 3: 5µg total RNA and 10 pg competitor; Lane 4: 5µg total RNA and 1 pg *per* competitor; Lane 5: 5µg total RNA and 0.1 pg *per* competitor.
As the equivalence point occurred between 10 pg and 1 pg of competitor RNA (Fig. 3.16), subsequent construction of a standard curve was conducted using 10 pg of \textit{in vitro} transcribed \textit{per} competitor and serial dilutions of \textit{in vitro} transcribed native template between approximately 65 pg and 0.5 pg (Fig. 3.17).

![Fig. 3.17 1% agarose TBE gel of QC-RT-PCR \textit{per} standard curve. All lanes contain an equal amount of \textit{per} competitor (10 pg) and a differing amount of \textit{in vitro} transcribed native template. Lanes 1-11 contain 65.5 pg, 31.25 pg, 15.63 pg, 7.81 pg, 3.91 pg, 1.95 pg, 0.98 pg, 0.49 pg, 0.24 pg, 0.12 pg, and 0.06 pg of \textit{in vitro} transcribed native template respectively.]

A knowledge of the reproducibility of the QC-RT-PCR is essential in understanding its limitations, therefore four replicates were used in the construction of a mean standard curve (Fig. 3.18). The high degree of replicability of the standard curve is illustrated by an $R^2$ value of 0.995 which indicates that 99.5\% of the total variance in the data can be explained by the fit of the line (Williams, 1993). The observed variation which does occur results largely from unavoidable errors in pipetting.
Possibly the greatest source of variation in QC-RT-PCR lies in the spectrophotometric measurement of RNA concentrations. However, this only affects the absolute quantification of mRNA levels. Given that the same competitor stock is used for the generation of the standard curve and the measurement of the experimental samples, relative quantification is still possible.

An important aspect of the use of the standard curve methodology as opposed to the titration method which is worth mention, is that it does not necessitate equal amplification efficiencies of the target and competitor (Tsai and Wiltbank, 1996). Relative amplification efficiencies of the competitor and target are related to the slope of the curve produced when the log ratio of the two products is plotted as a function of the log input concentration of competitor (Raeymakers et al., 1995; Connolly et al., 1995). If the final ratio of the target to competitor is described by the equation:

\[ \log(\text{target}_{\text{final}}/\text{comp}_{\text{final}}) = \log(\text{target}_{\text{initial}}/\text{comp}_{\text{initial}}) + n \log(1+E_{\text{target}})/(1+E_{\text{comp}}) \]
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(where \( n \) represents the number of cycles and \( E \) the amplification efficiency of the two products), and if the amplification efficiencies of the two products are equal, then the equation describes a straight line with a gradient of \(-1\) which can be represented thus:

\[
\log(\text{target}_{\text{final}}/\text{comp}_{\text{final}}) = \log(\text{target}_{\text{initial}}/\text{comp}_{\text{initial}}).
\]

If \( E_{\text{comp}} \neq E_{\text{target}} \) but remains constant throughout the reaction, then the line is merely transposed along the abcissa. However, if the amplification efficiency of the two products differs during the reaction (ie. the gradient of the line \( \neq -1 \)) then neither relative nor absolute quantification is possible unless the reaction is stopped in the exponential phase.

Standard curve methodology overcomes the problem of unequal amplification efficiency because the absolute amount of competitor RNA is not used in any of the calculated values (Tsai and Wiltbank, 1996). Instead the amount of \textit{in vitro} transcribed native template is used to calculate the amount of template in the unknown sample. Assuming that the target in the unknown sample amplifies with the same efficiency as the \textit{in vitro} transcribed target, the observed ratio of the products in an unknown sample is reflective of the ratio of products in the standard curve, and therefore the initial amount of (in this case \textit{per}) mRNA. The validity of this assumption was maximised in the present study by the use of a specific \textit{per} primer (QPerR2) in the reverse transcription instead of random hexamers.

3.5.5 Controlling for Changes in Overall Gene Expression

The variability inherent in RNA extraction necessitates the spectrophotometric quantification of RNA samples in order to standardise the amount of RNA which is added to each reaction. Results are then expressed as pg of \textit{per} mRNA/\( \mu \)g of total RNA, and therefore reflect only relative changes in gene expression rather than absolute amounts of expression. It is thus possible that an observed increase in
transcription of per mRNA is an artefact of changes in overall gene expression. This possibility must be accounted for by the use of an external standard which is known to be constitutively expressed over the circadian cycle.

Previous studies of circadian variations in per mRNA levels have used the constitutively expressed RP49 gene as a control (Hardin et al., 1990, 1992; Reppert et al., 1994). As no constitutively expressed genes have been isolated in L. cuprina to date, degenerate PCR was used to amplify a 291 bp fragment of RP49 from L. cuprina using primers designed to the consensus sequence of three Drosophila species. Sequencing of the cloned PCR products revealed the fragment to contain 84% identity to the RP49 gene of Drosophila acanthoptera at the nucleotide level. The sequence of the L. cuprina RP49 fragment shown below (Fig. 3.19) was determined by aligning the consensus sequence of two separately cloned PCR products.

1 CACCAGTCGG ATCGGTATGC CAAGTGTGCG CACAAATGGC GTAAACCAAA
51 AGGTATTGAC AACAGAGTAC GTCGTCGCTT CAAGGGTCAA TACTTGATGC
101 CCAACATTGG TTACGGTTCC AACAAACGTA CCCGTCACAT GCTCCCCACC
151 GGCTTCAAGA AGTTCGTTGT CAACAATGT T AAGGAACCTG AAGTTTTGAT
201 GATGCAAACG CGTGTATACT GCGGTGAAAT GACCGGGCCA
251 AGGAAACGCAA GGAAATCGTT GACCCGGCCA AGCAGCTGTC A

Fig. 3.19 Nucleotide sequence of a 291 bp fragment of L. cuprina RP49 cDNA (corresponding to nucleotide positions 65-355 of D. acanthoptera RP49) amplified by degenerate PCR using RP49F1 and RP4R2.

Isolation of this 'housekeeping gene' makes control for variations in overall gene expression not only possible but relatively straightforward. RNA dot blots containing equal amounts of sample RNA can be probed separately with antisense RNA probes produced by in vitro transcription from the cloned per and RP49 fragments. The analysis of samples at specific circadian times when per levels change, can determine whether these changes are attributable to alterations in overall gene expression or are real circadian changes in per expression.
3.5.6 An Alternative Method for Quantitative Analysis of \textit{per} mRNA Levels

Developed

The emphasis given to the development of QC-RT-PCR here may be justified by the frequent misuse of the term ‘quantitative RT-PCR’. Despite publications such as that of Tsai and Wiltbank (1996), many researchers use RT-PCR in a way claimed to be quantitative which in reality is far from it. Unless the variability inherent in the processes of reverse transcription and the polymerase chain reaction are accounted for, the technique is at the very most only semi-quantitative. The measures which have been taken here to ensure sufficient internal and external controls are in place are essential if RT-PCR is to be used to quantify even relative changes in gene expression.

Given that the appropriate controls are used, the application of QC-RT-PCR to the analysis of circadian rhythms in gene expression provides a useful alternative to the traditional method of RNAse protection assays. In addition to providing a non-radioactive analytical system, this technique provides an extremely sensitive method with which it is possible to analyse changes in gene expression from extremely small amounts of tissue. The use of QC-RT-PCR may prove as important as the analysis of real time \textit{per} expression in live animals (Plautz \textit{et al.}, 1997) in resolving some of the confusing results obtained from the analysis of \textit{per} oscillations in populations of individuals. For example, the quantitative analysis of \textit{per} expression in specific brain regions could validate the existence of an X-Y or A-B-type pacemaker system.
3.6 Analysis of *L. cuprina* per mRNA and Protein Expression

3.6.1 Diel Oscillations in *L. cuprina* per mRNA levels

In spite of the very different functional mechanisms, *per* mRNA levels exhibit a prominent circadian variation in the head of both adult *Drosophila* and *Antheraea* (Hardin et al., 1990, 1992; Reppert et al., 1994). Thus, if the *L. cuprina* per homologue were involved in circadian function in this species, *per* mRNA levels would be expected to oscillate. The purpose of this section was therefore to determine whether the products from the *per* locus oscillate and whether there could be a gene-silencing mechanism operating in *L. cuprina*.

QC-RT-PCR of samples (held in LD 12:12) taken at 2 hourly intervals for 48 h clearly indicate that *per* mRNA levels vary over the course of the day (Fig. 3.20).

When the densitometric ratios of competitor to template are converted into pg of *per* mRNA/μg of total RNA by rearrangement of the standard curve equation:

\[
\log(\text{template/competitor}) = 0.4391(\text{sample}) - 0.1074
\]

to:

\[
\log(\text{sample}) = ((\log(\text{template/competitor})) - (-0.1074))/0.4391
\]
the diel oscillations in *per* mRNA levels become astoundingly apparent (Fig. 3.21).

**Quantification of *L. cuprina* permRNA Levels in LD 12:12**

![Graph showing diel oscillations in per mRNA levels](image)

Fig. 3.21 Mean values for the diel oscillations in *per* mRNA. Pg of *per* mRNA/μg of total RNA is plotted as a function of Zeitgeber time. The white bar at the top of the graph indicates the timing of the photophases while the black bar indicates scotophases. Standard errors are displayed on the graph and were obtained from three replicates of the QC-RT-PCR experiment.

The clear diel rhythm of *per* mRNA levels shown in LD 12:12 (Fig. 3.21) exhibits a period of 24 h, a peak phased at Zt 12 (the light-dark transition) and a five fold amplitude of change (0.5 pg *per* mRNA/μg total RNA to approx. 2.5 pg *per* mRNA/μg total RNA). The representation of the amplitude of *per* mRNA oscillations in terms of total RNA as opposed to percentage of maximal levels is reflective of the use of QC-RT-PCR as opposed to RNAsa protection assays. The five fold change in amplitude may not appear as dramatic as the amplitudes displayed in the *Drosophila* data (Hardin *et al.*, 1990, 1992), but it must be remembered that not only do these data show statistically significant diel oscillations, but they are also presented in a far less dramatic (and yet far more informative) way.

Rhythmic expression of *per* mRNA was shown not to result from differential expression of total mRNA over the circadian cycle by Northern analysis. Samples of
total RNA probed using a *per* antisense probe, and an antisense probe to the
constitutively expressed RP49 mRNA detected circadian variations in *per* mRNA
levels, but failed to detect changes in RP49 mRNA levels (Fig. 3.22).

![Zeitgeber Time](image)

Fig. 3.22 Northern dot blot of 20µg RNA samples taken at the indicated Zt and probed with
DIG-labelled antisense RP49 and *per* probes.

Probing of Northern blots with a sense *per* RNA probe failed to detect the expression
of an antisense *per* RNA strand in *L. cuprina* either at Zt 12 (Fig. 3.23) or Zt 3. It
therefore seems unlikely that an antisense *per* mRNA strand is present in adult
*L. cuprina* heads.

![Northern blot](image)

Fig. 3.23 Northern dot blot of total *L. cuprina* RNA (Zt 12) probed with a sense and an antisense
DIG-labelled *per* probe. Lanes 1, 2 and 3 contain 50µg, 5µg and 0.5µg of total RNA probed with an
sense *per*-probe. Lanes 4, 5 and 6 contain the same dilutions of RNA probed with an antisense
*per*-probe.

### 3.6.2 Diel Oscillations in PER-like Immunoreactivity in *L. cuprina*

The expression of a particular mRNA does not necessarily induce a corresponding
increase in the levels of the protein it encodes. The rhythmic expression of PER
protein is however vital for the rhythmic expression of behaviour (Siwicki *et al.*, 1988;
Zerr *et al.*, 1990). In fact, in light of the data suggesting the role of post-transcriptional
control mechanisms in the generation of the circadian rhythm, PER oscillations may be a far more integral part of the Per feedback loop than per mRNA oscillations.

The initial analysis of PER-like immunoreactivity from protein samples of adult *L. cuprina* using a polyclonal *D. melanogaster* per antibody (donated by Dr. L. Saez, Rockefeller University) indicated an immunoreactive band of approximately 110 kD, a size equal to that expected from the inferred protein sequence (Fig. 3.24).

![Fig. 3.24 ECL autoradiograph of immunoreactive bands resulting from binding of *L. cuprina* protein to the Per #107 polyclonal *D. melanogaster* antibody. Lane 1 contains 10 µg *D. melanogaster* protein (Zt 13). Lanes 2 and 3 contain 40µg of *L. cuprina* protein.](image)

Specificity of this immunoreactivity was demonstrated by a control in which only a secondary rabbit polyclonal antibody was used in hybridisations. This negative control did not produce any reactive bands thereby confirming that the band detected above was not due to non-specific antibody binding.

The question of whether PER levels oscillate in *L. cuprina* was addressed by the analysis of PER-like immunoreactivity in protein extracts taken from fly heads at 3 h intervals throughout the day. The rhythm in *per* mRNA expression illustrated in Fig. 3.21 was mirrored by a diel rhythm in PER protein-like immunoreactivity (Fig. 3.25). The levels of a 110 kD PER-immunoreactive band identified in Western blots were found to oscillate in LD 12:12. During the majority of the subjective day (Zt 0-9) levels of PER-like immunoreactivity remain low. However, towards the end of the day (Zt12), levels begin to rise and peak at approximately Zt 15 (3 h after the light-dark transition). When compared with the mRNA oscillations (Fig. 3.21), protein oscillations can be seen to maintain a delayed phase angle of 3 h (Fig. 3.25), which is
approximately 3-4 h less than the delay evident in Drosophila. These data were confirmed by three separate replicates.

![Western Blot](image)

Fig. 3.25 ECL autoradiograph of a Western blot in which 12 µg of L. cuprina head protein taken at different Zts were hybridised to a polyclonal PER antibody (Per #107). Zeitgeber time of samples is indicated above the lanes. A Coomassie stained total protein sample (C) is shown to the left of the autoradiograph along with a protein standard to indicate the size of the PER-immunoreactive band.

### 3.6.3 Similarity Between the Expression Patterns of L. cuprina and D. melanogaster per mRNA and Protein

The expression pattern of per mRNA in L. cuprina is remarkably similar to that of D. melanogaster, with clear diel oscillations evident and with peak levels maintaining the same phase angle in both species. As in D. melanogaster, peak protein levels lag significantly behind mRNA levels. The assumptions made in Chapter Two that the Lucilia and Drosophila circadian systems function in a similar manner at a molecular level are therefore supported at the most fundamental level. The maintenance of a shorter phase angle between per mRNA and PER protein oscillations in Lucilia with respect to the Drosophila (3 h c.f. 6 h), is particularly interesting when discussed in the context of Chapter Two. The assumption was made in the simulation model that a decreased time delay between mRNA peaks and protein peaks was the underlying molecular cause of a shorter free-running behavioural period. This assumption has been upheld by the molecular investigations of per mRNA and protein expression. The molecular evidence presented here thus confirms the value of the simulation model in the prediction of the molecular functioning of the Lucilia clock. The observation of a
shorter lag between mRNA peaks and protein peaks may also have significant implications for the nature of the feedback loop as the kinetics of transcription and translation are such that the 3 h delay observed in Lucilia does not necessitate an independent delay mechanism whereas the 6 h delay observed in Drosophila does require an extra delay component (pers. comm. Schibler, 1998).

Despite the diversity of per expression in the insect species previously investigated, the pattern in Lucilia emulates that of Drosophila closely. This is somewhat counterintuitive given that (1) the Musca system is itself so different from the Drosophila system, (2) that Lucilia is so much more closely related to Musca than Drosophila, and (3) that Lucilia per maintains maximal sequence identity with Musca. The existence of a 'moth-like' system involving the transcription of an antisense per RNA from the per locus which acts to silence the sense strand at specific circadian times is unlikely in Lucilia as no antisense per mRNA has been detected (Fig. 3.23).

The implications of these data for the use of Drosophila as a fly model are significant. As this is the first system which appears to mirror Drosophila closely, the findings enhance the relevance of the Drosophila model. The Drosophila system of molecular rhythmicity may therefore be the rule rather than the exception, at least in flies, and Musca may, in circadian terms, be the odd one out.

3.7 Making Molecular Inroads into the Blow Fly Circadian System

The molecular characterisation of a blow fly circadian model which began with the isolation of a naturally occurring arrhythmic mutant (Smith, 1987) has been continued with the cloning of a 4 Kb per cDNA from L. cuprina, and the demonstration that the functioning of per at its most basic level appears to resemble that of Drosophila. The data presented here are in full support of the molecular model proposed in Chapter Two.
The lack of any correlation between the latitude of collection of *L. cuprina* and the sequence of the TG repeat or its flanking regions discourages one from believing that this motif plays a role in thermal stability of the circadian phenotype and adds weight to argument that other regions of PER such as the PAS domain play a more central role in conferring this ability.

It is suggested that a failure of attempts to isolate a *timeless* homologue in *L. cuprina* in the present work reflects the lack of consensus sequence available for this gene rather than the absence of this central component from the blow fly clock. A logical extension of the present work would be the isolation of *timeless* now that more consensus data are available. The production and use of *L. cuprina* PER antibodies would also allow the determination of whether nuclear entry of the PER protein occurs in this species. The determination of nuclear entry would close the circadian loop and further test the validity of the simulation model.
CHAPTER FOUR: TESTING THE BEHAVIOURAL PREDICTIONS OF THE SIMULATION MODEL

4.1 Introduction to the Analysis of Clock-Controlled Rhythms

4.1.1 Investigation of the Effects of Light on the \textit{L. cuprina} Circadian System

In proposing a simulation model for the molecular functioning of the \textit{L. cuprina} circadian clock which also explains adult behavioural rhythmicity, certain basic elements of \textit{Lucilia} behavioural patterns were accounted for. These included the 22 h period of free-run in constant darkness, and the entrainment of this rhythm to daily light cycles.

In addition to accounting for the basic properties of adult locomotor activity, the model was used to make predictions about the response of the clock to slightly more complex environmental perturbations, such as short light pulses administered at different circadian times and long light pulses of differing intensity. The purpose of Chapter Four is to investigate the validity of these behavioural predictions and to explain the behavioural responses from molecular and ecological standpoints. Results from the behavioural data have potential implications for the molecular functioning of the \textit{L. cuprina} circadian clock, and the molecular model can aid in the understanding of why particular behavioural patterns result from specific environmental perturbations.

4.1.2 Behavioural Rhythms of \textit{L. cuprina}

Analysis of the 'hands of the clock' and inferral of clock function by the observation of the response of these driven rhythms to environmental perturbations has historically provided an investigatory mainstay of chronobiology. The most evident indicator of clock status, and that which is most readily measured, is locomotor activity. Investigation of behavioural rhythmicity provides valuable indirect insight into clock
function, and also indicates what the significance of a particular clock (if any) may be. In the context of the present work, the analysis of locomotor activity in *L. cuprina* is vital, as it provides an index of clock output. While it is undeniably necessary to gain an understanding of the molecular oscillations controlling behavioural rhythms, it is equally important to observe how these molecular rhythms are manifested at a level which is important to the fly.

The circadian system of *L. cuprina* has been the subject of some scrutiny. In addition to the analysis of behavioural rhythmicity conducted in Australia during the 1980s (Smith *et al.*, 1981; Smith, 1983, 1985, 1987), some New Zealand studies have also focussed on the behavioural aspects of circadian rhythms in this fly (Warman, 1995; Warman and Lewis, 1997). Collectively this research has shown several events in the life cycle of *L. cuprina* to result from the action of an endogenous circadian clock which is entrained on a daily basis by light cycles (see Chapter One for more detail). While we know there are multiple events in the life cycle of *L. cuprina* which are clock controlled, the one which is focussed on here is adult locomotor activity.

**4.1.3 The Effects of Light on Behavioural Rhythmicity**

Among the plethora of behavioural experiments which have been conducted in order to gain an increased understanding of circadian clock function, one particularly interesting area of examination is the response of the clock to different lighting regimes, as light appears to be the zeitgeber of primary importance to the entrainment of a majority of circadian clocks. In spite of our relatively primitive knowledge of circadian photoreception, the analysis of the effects of different light regimes has provided great insight into clock function.

**4.1.3.1 Effects of Short Light Pulses**

Because the majority of circadian oscillators do not produce an endogenous period of exactly 24 h, they are entrainable and therefore undergo phase advances and/or delays
each day to adjust their period to 24 h. In the past much work has been carried out on the effects of both short and long light pulses. Administration of short light pulses at different circadian times allows the construction of phase response curves (PRCs) which provide considerable information about the entrainment of the clock to environmental cycles, the limits of this entrainment, and the time course and structure of the circadian clock (Saunders et al., 1994).

For entrainment to be achieved a light pulse must commence in each cycle on the portion of the PRC which generates a phase shift \( (\Delta \phi) \) equal to the difference between the free-running rhythm \( (\tau) \) and the period of entraining cycle \( (T) \). Thus, if \( \tau \) is less than \( T \) an overall phase delay must be obtained in order for entrainment to occur, and if \( \tau \) is greater than \( T \) an overall advance must result. Provided that the phase change is instantaneous, the PRC provides a concise summary of the response of the clock to light pulses (or zeitgeber of any description) and indicates which phases of the day zeitgeber must fall to entrain a rhythm to a particular geophysical cycle.

PRCs have been used in the past to infer features of behavioural alterations in per mutants, as they provide a great deal of information about the photic sensitivity of an individual or population, and one of the major alterations of the clock of per mutants is their response to light (Saunders et al., 1994). The phase delaying portion of the PRCs of all three Drosophila per mutants \( (per^1 \ per^{1-2} \ and \ per^1) \), which corresponds to the late subjective day/early subjective night, is the area of the PRC which is most drastically affected in these mutants. This suggests that per mutations shorten or lengthen the period of the oscillation particularly in the second half of the subjective day (Saunders et al., 1994).

**4.1.3.2 Effects of Constant Light**

The behavioural effect of prolonged photophases depends very much on the intensity of the light administered. Rhythmic behaviour usually persists in LL if it is below a species-specific threshold (Saunders, 1982; Hong and Saunders, 1994). Early investigations of the effect of LL on the eclosion of *D. pseudoobscura* (Pittendrigh and
Bruce, 1957) showed it to cause damping of the rhythm, with the speed of the damping to arrhythmicity depending on the intensity of the light administered (Saunders, 1982). This effect was considered to be an individual effect and not a desynchronisation of a rhythmic population, and was extended by Winfree (1974) who showed a gradual increase in the intensity of low level blue light (between 0.001 and 0.1 erg cm$^2$ sec$^{-1}$) to cause a progressive protraction of the period between eclosion peaks. Above 0.1 erg cm$^2$ sec$^{-1}$ eclosion became arrhythmic and below 0.001 erg cm$^2$ sec$^{-1}$ no observable effect on eclosion rhythmicity was noted.

A trend observed in the response of vertebrate behavioural rhythms to constant dim light led one of the founding members of modern chronobiology, Dr Jürgen Aschoff, to propose a dogma now known as Aschoff’s rule (and its immediate extension ‘the Circadian rule’). As mentioned previously, Aschoff’s rule states that the period of a biological oscillation lengthens on transfer from DD to LL, or an increase in light intensity for dark-active animals, but shortens for light active animals. Although this rule holds for a majority of vertebrate species, the exceptions to Aschoff’s rule appear to include almost all of the insect species which have been examined (Saunders, 1982). Nearly every species whether diurnal or nocturnal shows a lengthening of $\tau$ on transfer to dim LL (Saunders, 1982; Hong and Saunders, 1994).

The behavioural arrhythmicity induced by constant bright light has unearthed another series of questions which chronobiologists strive to answer. Is the clock stopped by constant bright light, or is the underlying cause of behavioural arrhythmicity more superficial?

Arrhythmicity of Drosophila eclosion in LL is induced after a terminal exposure period of 12 h or more (Pittendrigh, 1960, 1966). Upon release into DD the eclosion rhythm restarts at a particular phase (Ct 12). The implication of these results is that photophases of greater than 12 h in duration damp rhythmicity and hold the oscillator in a fixed state (Ct 12) (Pittendrigh, 1974, 1981).
This story became rather more complex, however, when it was found that transfer of *Drosophila* from constant dim light (in which activity is rhythmic) to constant darkness led to resetting of the eclosion pacemaker irrespective of when the LL to DD transition occurred (Saunders, 1982). The possibility is therefore raised that the clock itself is not stopped but the overt rhythm is suppressed by constant bright light (Saunders, 1982; Hong and Saunders, 1994). Further evidence for this proposition came from failure of constant bright light to stop the eclosion clock of *S. argyrostoma* (Peterson and Saunders, 1980), and the fact that transfer of *Calliphora vicina* from constant bright light or constant dim light into DD does not set the pacemaker at a reliable Ct (Hong and Saunders, 1994).

### 4.2 General Methods

#### 4.2.1 Data Logging

Individual animal locomotor activity of three day old adult female *L. cuprina* was monitored in light and temperature controlled cabinets (held at 20°C±1°C) using an infra-red light beam apparatus similar to that described in Warman (1995) and Kenny and Saunders (1991). This system consists of recording boxes each containing four individual fly recording chambers (Fig. 4.1).
The recording chambers were significantly narrower than those used previously (Warman, 1995) to ensure more effective recording of adult locomotor activity patterns (Fig. 4.1). Across the centre of each arena was positioned an infra-red phototransistor (RS 585-2220) and an infra-red photoemitter (RS 585-242) which were wired in series with 330 ohm resistors and connected to a 12 volt DC power source. A modular system was developed in which each set of four channels was connected to a junction box using a network plug. From the junction box, connections were hard-wired to a multiplexer using 50 ply twisted pair telecommunications cable, and then on to a BBC B computer. Power supply was carried through the hard wired cable to the junction box, and hence through to the photoemitters, and recording signals were carried back to the computer from the phototransistors. This modular system allowed the rapid and simple addition or removal of recording channels as needed. Other than the modifications stated above, the recording techniques used were identical to those described in Warman (1995).
The data logging software used was developed by Mr. C Lewis and Dr R. D Lewis (School of Biological Sciences, University of Auckland). This program sums the number of times a single beam is broken in each 10 min time interval and at the end of each day (144 10 min time intervals) records the activity counts to a floppy disc. At the end of a week records were downloaded onto an Acorn Archimedes computer for analysis and display.

4.2.2 Data Analysis and Presentation

Individual channels of data were extracted using the program Recov32, and the square root of activity in each 10 min interval was displayed using LaserPR28 (both programs were developed by Mr. C. Lewis and Dr R. D. Lewis, School of Biological Sciences, University of Auckland). The period of the rhythms was determined using periodogram analysis (based on Enright (1965) and Williams and Naylor, (1978)), and the results from these analyses were tabulated and the mean periods calculated.

The difference between $\tau$ under different lighting regimes was tested statistically using an ANOVA. Two assumptions were satisfied by the data which made the use of an ANOVA appropriate. These assumptions were that the variances of the different samples were approximately equal and that the errors were normally distributed.

Determination of phase changes were made by manual observation of the actograms. A phase response curve (PRC) of normalised circadian time ($Ct$) of the light perturbation versus normalised change in phase ($\Delta\phi$) was then plotted using the results from these measurements. Values of $Ct$ and $\Delta\phi$ were calculated in hours using the midpoint of the active phase $\alpha$ ($Ct_6$) as a reference point (Cymborowski et al., 1993). Normalised circadian time was calculated using the formula:

$$\text{normCt} = \frac{(p \times 24)}{\tau}$$

(where $p$ = circadian time of the light perturbation and $\tau$ = the period of the rhythm).
Actograms and periodograms were created in vector graphic form, exported in IBM format as draw exchange format (dxf) files, and formatted in CorelDraw (Version 6).

4.2.3 Lighting Conditions

With the exception of the dim light experiments, all experiments were conducted using a fluorescent light source 0.50 Wm\(^{-2}\) (≈200 lux) and an industrial control timer. Bright light controls for the dim light experiments were conducted using a 12 volt tungsten light source at 0.075 Wm\(^{-2}\) (≈50 lux). Dim light experiments were conducted by masking this tungsten light source with various shielding devices. Dim light was considered to be an irradiance of 0.001 Wm\(^{-2}\) (≈0.25 lux) and very dim light was an irradiance of 0.0002 Wm\(^{-2}\) (≈0.05 lux).

4.2.4 Lobectomy and Visual Occlusion Using Paint

Lobectomies were conducted using the protocol described in Cymborowski et al. (1994). Briefly, incisions were made in the rear of the head capsule under carbon-dioxide induced anaesthesia and the optic lobes were removed with fine forceps and vanna scissors. Incisions were then sealed with low melting point wax to prevent desiccation. Removal of the optic lobes was confirmed by histological examination of hematoxylin-eosin stained brain sections (Fig. 4.2). Sham-operated flies were used as controls.
As an alternative to lobectomy, the compound eyes of some flies were occluded with opaque black nail polish (Cover Girl). Control flies were painted with clear nail polish of the same brand to ensure the presence of solvents in the polish was not altering the behaviour of the flies. Lobectomised and painted flies were all deprived of photic cues from their ocelli by cauterisation.

### 4.3 Testing of the Newly Developed Recording Equipment

To ensure that the newly developed recording equipment produced results comparable with those obtained in previous research, free-running periods of *L. cuprina* (maintained in DD and 20°C) were recorded over the period of two weeks. Mean free-running periods obtained from the periodogram analysis of 30 free-running flies was 22.5 h (±0.25 h) (Fig. 4.3) which is not significantly different from the data obtained previously (Warman, 1995).
Chapter Four: Testing the Behavioural Predictions of the Simulation Model

![Actogram of adult *L. cuprina* activity patterns in DD (20°C). The fly was maintained in constant conditions for two weeks. Activity is represented by histograms. Time of the day is indicated above the actogram (on the abscissa) and subsequent days are plotted beneath each other (ordinate). Data are double-plotted to increase the clarity of the rhythm.]

The only noticeable difference attributable to the newly developed equipment was that a greater percentage of total flies produced activity records. This is most likely to be due to the decreased distance across which the infra-red beam must project and the confinement of the flies to a smaller area.

4.4 Effect of Short Light Pulses on the Behaviour of *L. cuprina*

The simulation model (Chapter Two) predicted that the effects of short light pulses on the circadian clock depend very much on the time of administration. During the subjective evening and early subjective night, light pulses cause simulated phase delays, whereas late in the subjective night through to early in the subjective day light pulses phase advance simulated activity. The behavioural response of *L. cuprina* to single short light pulses has been investigated previously in populations (Smith, 1983) and individual flies (Warman, 1995), however, the implications of these responses for
the molecular functioning of the clock and the molecular model proposed earlier are of such import that a larger base of data was necessary for analysis.

Phase changes from 15 min light pulses of 30 adult flies recorded over two weeks were used to construct a PRC. After approximately 7 days in DD, flies were subjected to a 15 min light pulse (0.50 Wm$^{-2}$) and were then returned to DD. When summarised by means of a PRC the data clearly indicate a strong (or Type 0) curve (Fig. 4.4). This Type 0 PRC is confirmed by a plot of the relationship between old phase and new phase which approximates a slope of zero. The relationship between old phase and new phase in the present data is described by the equation $y=0.211x + 5.606$.

![Phase Response Curve for L. cuprina](image)

Fig. 4.4 Phase response curve (PRC) of *L. cuprina* to 15 min light pulses, indicating a strong (Type 0) pattern.

The generation of a strong PRC for adult locomotor activity in response to short light pulses is somewhat atypical of the results obtained from similar research in other dipteran species (Saunders, 1982) and from PRCs obtained from population recordings of Australian *L. cuprina* (Smith, 1983). The data are, however, in agreement with those obtained from previous analysis of New Zealand strains of *L. cuprina* (Warman, 1995). A contributing factor in the generation of a Type 0 PRC in New Zealand
populations of *L. cuprina* may be the lower temperature at which the flies were maintained. In the Smith (1983) experiments flies were kept at 27°C whereas in the present study (and that of Warman (1995)) flies were maintained at 20°C. Ambient temperature has been shown previously to alter the photic sensitivity of insect clocks (Saunders, 1982). If one assumes that the molecular oscillations which underlie rhythmicity are of greater amplitude at higher temperature, then at higher temperatures the same ‘amount’ of light would have a lesser effect (ie. it would not degrade levels of TIM protein to sub-threshold as quickly as at lower temperatures).

The Type zero PRC obtained in response to short light pulses in the *per* mutant of *D. melanogaster* (Saunders et al., 1994) is indicative of heightened photic sensitivity. If New Zealand strains of *L. cuprina* do exhibit a similarly increased photic sensitivity, it is definitely not due to a mutation at the *per* site of the *period* gene in *L. cuprina*, as the wild-type amino acid is conserved in this species.

The asymmetric nature of the *L. cuprina* PRC concurs with the previous investigation (Warman, 1995). Phase delays occur over a period of 7 h (between Ct 6-13) and phase advances over a 12 h period (between Ct 14-02) (Fig. 4.4). This observation also agrees with the finding of Pittendrigh (1960) that the area of the phase response curve under the delay section is greater than that under the advance section in nocturnal species, while in diurnal species the area under the advance section is greater (Saunders, 1977). When considered in the context of the underlying molecular oscillations this differential symmetry between nocturnal and diurnal species may reflect differences in the timing of the entry of the PER/TIM heterodimer into the nucleus. In addition, if the nuclear PER/TIM dimer is more stable in diurnal species, then the period over which phase advances occur would be protracted.

Remembering back to the shape of the PRC generated from the molecular simulation model (Fig. 2.10), the range of Cts during which phase delays occurred was far in excess of that expected from the behavioural data (the simulated curve was characteristic of a nocturnal species). The transition from phase delays to phase advances therefore occurred at a much later Ct than is evident in the real data. The late
change from delays to advances and the prolonged period of delays may suggest that nuclear entry of the PER/TIM heterodimer in *L. cuprina* occurs at an earlier Ct than it does in *Drosophila*. An investigation of the timing of nuclear entry of the PER (and TIM) proteins should test the validity of this prediction.

Looking again at the real PRC (Fig. 4.4), the maximal phase delay which can be obtained from a light pulse is much greater than the maximal phase advance (10 h compared with 7 h). This is indicative of the ability of *L. cuprina* to entrain to a maximum T of 32 h but to a minimum T of only 15.5 h. The importance of this finding may be purely theoretical as the free-running period of *L. cuprina* is less than 24 h and thus an overall delay is required to entrain the clock to daily light cycles. It may therefore, only be the delay portion of the PRC that is of biological significance to the fly. This differential amplitude may also have implications for the period of the behavioural rhythm in constant illumination (see Section 4.4.2).

A novel feature of the response of *L. cuprina* to short light pulses was noticed in approximately 15% of individuals. In these flies, phase changes resulted from a shortening of the active phase (Fig. 4.5). If the onsets of activity are measured it would appear that no phase change had occurred, and yet if the offsets are measured then a large phase change would be noted.

![Fig. 4.5 Phase change in *L. cuprina* resulting from a decrease in active phase length in response to a 15 min light perturbation administered at the time indicated by the grey box (05:00 Day 7).](image-url)
Phase shifts of this type have been reported in the past in the golden hamster *Mesocricetus auratus* (Pittendrigh, 1965). If, as suggested by Lewis *et al.* (1991), the active phase is made up of a series of pulses of activity each of which results from the firing of a group of sub-oscillators as they pass through a threshold band, then the shortening of the active phase in response to the phase advancing light pulse (shown in Fig. 4.5) can be seen to result from the phase advancing of those sub-oscillators responsible for the second half of the active phase and the lack of phase resetting of the sub-oscillators responsible for the first portion of the active phase. If the coupling of the sub-oscillators is weak, then it may take some time for the original coupling to reform. As the simulation model proposed in Chapter Two is a single oscillator model rather than a population model, it is not surprising that it does not simulate this type of phase shifting response.

### 4.5 Effects of Constant Light on the Behaviour of *L. cuprina*

A second set of behavioural predictions made by the molecular model concerned the response of the clock to regimes of constant dim and bright light. The apparent slowing down and stopping of the clock on transfer from DD to dim LL and bright LL is evident in many species, and the nature of the response has been a topic of much debate.

#### 4.5.1 Constant Bright Light

The molecular model predicts light induced behavioural arrhythmicity if the assumption is made that bright light is stopping the clock. As light is known to degrade TIM, then constant bright light should hold TIM at very low levels and prevent completion of the molecular feedback loop (Chapter Two). The behavioural effect of this damping depends on the phase of the molecular oscillation during which
activity occurs. If the molecular oscillations which generate rhythmic behaviour in nocturnal and diurnal organisms maintain the same phase relationship, and activity is merely considered to be induced when PER or TIM are below threshold in diurnal organisms and above threshold in nocturnal organisms, then activity should cease in nocturnal animals following transfer into constant bright light and should continue (all of the time) in diurnal organisms.

The analysis of the responses of 40 adult female *L. cuprina* to constant bright light (above 0.075-0.5 Wm\(^{-2}\)) showed that an overwhelming majority (95%) of flies became arrhythmic under these conditions, with constant activity often giving way to arrhythmic bursts after several days in LL (Fig. 4.6).

![Actogram of an adult female *L. cuprina* held in DD for three days and then transferred into constant bright light (0.50 Wm\(^{-2}\)).](image)

In some individuals a tantalising suggestion of ultradian rhythms is evident in constant light (Fig. 4.7). Whether these patterns result from a *bona fide* ultradian clock, or whether they are merely reflective of the need of the fly to cease activity from time to time remains to be resolved.
The major question concerning this arrhythmic behaviour is whether it is a result of a superficial response to light or a true stopping of the clock (Hong and Saunders, 1994). Current opinion sways in favour of the superficial hypothesis due to behavioural data (Hong and Saunders, 1994) and because there seems to be a clear disparity between the behavioural and molecular responses of the clock of *D. melanogaster* to constant bright light (Marrus et al., 1996; Qiu and Hardin, 1996a).

To investigate whether the response of *L. cuprina* to bright light was a photokinesis rather than a clock response, the activity of adult flies deprived of their visual cues was recorded. Three individuals blinded by removal of the optic lobes were used as controls for flies whose sight had been removed by blackening of the compound eyes (n=20). The low survival rate of flies deprived of their optic lobes (1 in 50) was the reason that lobectomy was not used in every experiment. The ocelli of lobectomised and blackened-eyed flies were cauterised to remove the possibility of photoreception from these structures. Histological examination of lobectomised flies confirmed the complete removal of optic lobes (Fig. 4.2). Activity of all experimental flies was indistinguishable from that of controls in both DD and constant bright light (Fig. 4.8 A-D).
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Fig. 4.8 Actograms of flies maintained in DD for three days and then exposed to a constant bright light (0.50 Wm$^{-2}$) for four days. A. Activity of a normal adult fly B. Activity of a fly whose compound eyes had been painted with clear nail polish. C. Activity of a fly whose compound eyes had been occluded with black nail polish and whose ocelli had been cauterised D. Activity of a fly whose optic lobes had been surgically removed and whose ocelli had been cauterised.

These experiments clearly show that photic input from the compound eyes and ocelli does not cause behavioural arrhythmicity in constant bright light. The data do not, however, preclude the action of direct illumination of other regions of the fly in the generation of arrhythmia at a superficial level. Although the results suggest that bright light induces arrhythmicity at a clock level, they are not conclusive. Further evidence comes from the analysis of bright light effects at a molecular level in Drosophila. The damping of molecular rhythms in D. melanogaster exposed to constant bright light (Marrus et al., 1996; Qiu and Hardin, 1996a) indicates that bright light is affecting the clock itself. Indirect support for the contention (in Chapter Two) that the continuation of PER-TIM oscillations for two cycles in LL is due to a dual pacemaker (or X-Y) system comes from the present data which show that arrhythmicity in the first two days of LL is not attributable to photokinesis.
4.5.2 Constant Dim Light

Where Aschoff's rule may have found a stumbling block in predicting the responses of insects to dim LL is in relying on the asymmetrical nature of nocturnal versus diurnal PRCs rather than the amplitude of each portion of the curve. The effect of constant dim light on lengthening or shortening of $\tau$ may not depend so much on the breadth of the advance and delay sections, but more on their relative amplitude.

From the analysis of the relative amplitudes of the phase delaying and phase advancing portions of the PRC of *L. cuprina* it is evident that the delay portion is more sensitive to light than the advance portion. If continuous illumination were of sufficiently low intensity that it did not induce arrhythmia, then the overall effect on the clock would be expected to be a lengthening of $\tau$.

In a clear breach of Aschoff's rule, but in agreement with the prediction made from the amplitude of the phase response curve, the major pattern evident in the data when the light intensity was decreased to 0.001 Wm$^{-2}$ was a lengthening of $\tau$. Not only was a protracted $\tau$ evident in flies held under LL (0.001Wm$^{-2}$) compared to those in DD (Fig. 4.9), but this lengthening was shown to be statistically significant ($p<0.05$). The mean $\tau$ under these conditions was 23.53 h $\pm$ 0.3 h (c.f. 22.51 h $\pm$ 0.25 h in DD). Of the total population, 46% of flies (n=15) showed lengthening (Fig. 4.9), 38% became arrhythmic and the remaining 16% showed no alteration to the free-running period exhibited in DD. From these results it is evident that the photosensitivity of individual flies varies greatly.
Chapter Four: Testing the Behavioural Predictions of the Simulation Model

Fig. 4.9 Actogram of a single adult fly in different lighting conditions. Days 1-6 flies were exposed to very dim constant illumination (0.0002 Wm\(^{-2}\)). Days 7-14 they were maintained in constant darkness. Days 15-21 they were exposed to dim constant illumination (0.001 Wm\(^{-2}\)). The period of the rhythm in each situation is indicated by the periodogram to the right of the actogram.

When the light intensity was stepped down further to 0.0002 Wm\(^{-2}\) 10.5% of flies showed a longer \(\tau\) in LL than in DD, while 78% showed a shorter \(\tau\) (Fig. 4.9, Fig. 4.10), and 10.5% showed no change. Despite the overwhelming percentage of individual flies which exhibited a decrease in \(\tau\), this shortening was not statistically significant (\(P<0.05\)). This is most probably due to the fact that each individual fly exhibited a shortening of \(\tau\) but the overall population effect was masked by the variability of \(\tau\) in different flies. The mean \(\tau\) in LL (0.0002 Wm\(^{-2}\)) was 22.38 h ± 0.3 h (c.f. 22.51 ± 0.25 h in DD).
To ensure that the shortening of $\tau$ in constant dim light (0.0002 Wm$^{-2}$) was not an artefact of the experimental design (such as aging) the experiment was conducted in reverse (DD to LL). The results clearly indicate that the shortening of $\tau$ occurred in response to the light regime (Fig. 4.10).

Fig. 4.10 Response of *L. cuprina* transferred from DD to constant illumination of 0.0002 Wm$^{-2}$ after seven days of free-run. A shortening of $\tau$ is indicated by the periodograms.

When the mean period of $\tau$ is plotted as a function of light intensity at the three different conditions (ie. DD, LL 0.0002 Wm$^{-2}$ and LL 0.001 Wm$^{-2}$) a J-shaped curve results, indicating a non-monotonic response of the circadian system to light (Fig. 4.11).
Chapter Four: Testing the Behavioural Predictions of the Simulation Model

![Graph of Period of Locomotor Activity of L. cuprina as a Function of Light Intensity](image)

Fig. 4.11 Plot of the mean period of locomotor activity of adult L. cuprina as a function of light intensity. n=20 (DD) n=30 (LL (0.0002 Wm$^{-2}$)) n=18 (LL (0.001 Wm$^{-2}$)). Variance in the data is indicated by standard errors.

The increase in $\tau$ at 0.001 Wm$^{-2}$ satisfies not only the predictions made from the real PRC but also the simulated predictions from the molecular model. The mechanisms underlying the lengthening of $\tau$ in constant dim illumination can thus be hypothesised using a combination of molecular and behavioural data. The greater sensitivity of phase delays over phase advances (Fig. 4.4) can be seen to be directly related to the differential stability of the PER/TIM dimer in the cytoplasm and the nucleus. If nuclear PER/TIM is more stable (or less sensitive to light) than cytoplasmic PER/TIM, the overall effect of constant dim light would be a lengthening of $\tau$. It is interesting to note here that the two species which have been shown to have similar functional systems at a molecular level (the fruit fly and the mouse) both exhibit a lengthening of $\tau$ in response to dim LL. Perhaps those species which do not show a lengthening of $\tau$ in dim LL possess slightly different systems of molecular regulation whether they are nocturnal or diurnal. An investigation of the behavioural response of the moth A. pernyi to constant dim light may reveal whether the employment of a different per feedback loop alters the behavioural response to light.
The apparent shortening of $\tau$ in *L. cuprina* at very low light levels (Fig. 4.9, 4.10 and 4.11) makes the story somewhat more perplexing. A limited number of references have been made to the non-monotonic nature of dim light responses in the past (Christensen, 1978; Aschoff and von Saint Paul, 1982; Hoffmann, 1965). The most extensive investigation was that of Christensen, (1978) who demonstrated and modelled this effect in the nocturnal insect *Hemidiena thoracica* (Christensen, 1978). If the response of the circadian clock of *L. cuprina* is similarly described by a non-monotonic curve (Fig. 4.12) and the light intensities used for the bright and dim light experiments are assumed to have fallen in the positions indicated by the arrows A, B and C then the variability of sensitivity of different flies can be seen to generate this differential effect.

![Fig. 4.12 Representation of a non-monotonic response to constant bright light. An initial increase in light intensity from zero to 0.05 lux (C) may shorten the free-running period, and a further increase (up to 0.25 lux (B)) may lengthen it. Light above a threshold (A) (>50 lux) results in arrhythmicity.](image)

Christensen (1978) attributed this J-shaped response to the differential effect of low and very low level illumination on the synthesis and loss functions of the oscillator (Christensen, 1978). At very low intensities $\tau$ was hypothesised to be shortened because the loss rate is expedited while the effect on synthesis is insignificant. At higher intensities the synthesis function was also proposed to be altered and therefore an overall increase in $\tau$ results.
This response in *L. cuprina* could possibly be explained if the sensitivity of nuclear and cytoplasmic PER/TIM to light was not linear over a range of low intensities. Realistically, however, our understanding of the molecular basis of circadian photoreception has not developed to a point where it is possible to reliably model the non-monotonic response. With the recent discovery of the role of cryptochromes and opsins in the entrainment of the circadian clock, this gap in our understanding may soon be filled.

If the single oscillator molecular simulation model developed here (Chapter Two) explained the generation of circadian rhythmicity in full, one would expect an increase in the active phase length ($\alpha$) to accompany an increase in $\tau$ (as the amount of time during which TIM levels are sub-threshold under these conditions is protracted). Despite the fact that $\alpha$ was increased in amplitude in some individuals on transfer from DD to LL (Fig. 4.9), this change was not consistent. The lack of increase in $\alpha$ in LL is evidenced by the mean $\alpha$ in DD and LL which was 37% and 25% of $\tau$ respectively (as measured by the breadth of the form estimate at 50% of the maximal peak height). These data again strengthen the suggestion that the circadian clock is likely to be comprised of a system of interacting oscillators and that each oscillator only contributes to a small portion of the active phase.

### 4.6 Insight Into the *L. cuprina* Clock Provided by Behavioural Analysis

Although the behavioural response of *Lucilia* to short light pulses is somewhat atypical of other dipterans in that the sensitivity is heightened, the response of the clock to prolonged light pulses follows the convention of other flies.

The behavioural predictions of the molecular simulation model which were proposed earlier have been satisfied to a large extent, with the exception that the simulated PRC exhibits a falsely accentuated period of phase delays and that the response of the clock
to dim light appears to be non-monotonic. The failure of the molecular model to produce a simulated PRC identical to that obtained from the behavioural data may suggest that nuclear entry of the PER/TIM dimer in *L. cuprina* occurs at an earlier Ct than in *Drosophila*. Further experiments using antibody staining to resolve this possibility may provide interesting evidence about the molecular basis of the blow fly clock.

Finally, resolution of the burning question as to whether arrhythmic behaviour in constant bright light is a real clock effect or not will rely on an examination of the molecular oscillations under these conditions.
CHAPTER FIVE: GENERAL DISCUSSION

5.1 The Amalgamation of Old and New

‘...a lead editorial in Nature some years ago (231: 97-98, 1971) somewhat plaintively queried why so little was known about the biological clock and suggested it was time to wind it up’ (Edmunds, 1998).

The ambiguity of the phase ‘wind it up’ gives a little leeway to the editorial author, who was proposing an untimely end to the study of biological clocks. The author concerned must now feel a little contrite at this comment, as the field of molecular chronobiology has blossomed. In 1998 alone between 20 and 30 research papers have appeared in leading journals including Nature, Science, Cell and Neuron, outlining the discovery of central molecular elements of circadian clocks.

Behind the fanfare and the excitement which is molecular chronobiology, there lies a veritable gold mine of information which has, to a large extent been left untouched by the molecular researchers. Well before the discovery of the \textit{period} mutants in 1971, which was the ignition spark for the whole field of molecular chronobiology, a vast amount of behavioural and physiological chronobiology had been conducted which still provides great insight into clock function. Ignoring the ideas and discoveries which have come from over 60 years of clocks research is particularly short sighted, and is detrimental to the understanding of circadian pacemakers. If the confluence of molecular and genetic techniques has provided huge insight into clock function, then the confluence of molecular genetic approaches and those of the classical chronobiologists such as Aschoff, Pittendrigh, Büning, Winfree, Wever, and Pavlidis provides an even greater insight into how the circadian clock works.

As one of the few disciplines which breaks out of the normal confines ascribed to other areas of biological investigation, chronobiology requires an integrative analytical approach. The purpose of this chapter is to amalgamate the three different approaches
which have been used in the present research to analyse the circadian system of *L. cuprina*, in order to help describe the functioning of the blow fly clock.

Investigation of the circadian system of *L. cuprina* using theoretical, molecular and behavioural approaches individually, provides useful insight into circadian clock function. However, when these different techniques are used together in a complementary fashion, the power of the combined approach far exceeds the sum of the individual approaches.

### 5.2 The Endogenous Nature of the Biological Clock of *L. cuprina*

In establishing a simulation model to explain the functioning of the biological clock of *L. cuprina* early in the thesis, the framework was provided on which the molecular and behavioural analyses of subsequent chapters were then built. The predictions that *per* existed in *L. cuprina* and that its levels oscillated were supported by the molecular isolation of the *L. cuprina per* cDNA and the analysis of its expression levels. The further assertion that the shorter free-running period of *L. cuprina* resulted from a shorter delay between mRNA and protein peaks was also shown to hold. The conservation of *per* as a central player in the circadian loop has thus been supported in a dipteran genus other than *Drosophila*.

The attenuated free-running period in *L. cuprina* is evidently not a result of a mutation in the *per* site of its PER protein, as this site is identical to that of wild-type *Drosophila*. It remains uncertain, however, as to whether this shorter τ results from an alteration in another region of PER or from a totally separate clock element. An intriguing way of resolving this question would be to rescue arrhythmic *per* flies with the *L. cuprina per* cDNA under the control of the *D. melanogaster per* promotor. If the rescue were to result in *Drosophila* with free-running periods characteristic of *Lucilia*, then the role of PER in the control of this attenuated τ would be substantiated. The specific region of PER involved in the maintenance of this 22 h τ could then be determined by the *in vitro* mutagenesis (or *in vitro* excision) of particular regions of the
L. cuprina per cDNA, and the rescue of arrhythmic Drosophila with these modified forms. Cloning of the per promoter in L. cuprina would provide a logical extension of these experiments, in that it would illustrate whether the per promoter contains an E-box to which the Lucilia homologues of dClock and dBmal1 could bind. The rescue of arrhythmic D. melanogaster with L. cuprina per under the control of the L. cuprina per promoter would also show whether dClock and dBmal1 are functional elements of the blow fly circadian system.

A more immediate experiment and one which would strengthen the certainty of the role of PER in the generation of circadian rhythms, involves the in vitro synthesis of an antisense per mRNA strand and the injection of this antisense strand into the heads of adult flies. This technique has been used in the past to prevent c-fos induced phase shifts in the rat SCN (Wollnik et al., 1995). If injection were timed to occur when per mRNA levels are high, then a phase-shifting effect should result if per is indeed involved in the molecular generation of circadian rhythmicity in L. cuprina.

The cloning of an L. cuprina per homologue, as achieved in this thesis, is the first and most important step in the characterisation of the molecular clockwork of this species. However, in order to close the L. cuprina circadian loop the most evident questions which need to be answered are whether timeless is also involved, and whether the PER and TIM proteins undergo nuclear entry. Both of these questions were addressed in the present study but answers were not forthcoming.

Isolation of a timeless homologue from L. cuprina proved impossible as insufficient consensus data resulted in the failure of degenerate PCR as a cloning strategy. Furthermore, screening of L. cuprina cDNA libraries with D. melanogaster timeless did not reveal any L. cuprina timeless clones. The recent isolation of a mammalian timeless homologue (Sangoram et al., 1998), along with homologues from three Drosophila species (Rosato et al., 1997) should alleviate the problem of insufficient sequence information, and thereby make degenerate PCR a more viable cloning strategy.
The investigation of nuclear entry using a polyclonal \textit{D. melanogaster} PER antibody (PER #107) also proved ineffective. The usefulness of the PER #107 antibody in the analysis of \textit{L. cuprina} PER-like immunoreactivity is thus restricted to Western blotting. In order to resolve whether nuclear entry occurs in \textit{L. cuprina}, a monoclonal \textit{L. cuprina}-specific PER antibody must be raised and used to examine histological sections of blow fly brains.

When applied to the results obtained from molecular genetic investigations, the population oscillator concepts (Chapter Two) which were originally formulated to describe complex free-running labilities, explain many previously unresolved disparities between the molecular and behavioural data. Among these is the apparent dissociation of behavioural rhythms from the molecular oscillations which were first noted by Hardin \textit{et al.} (1990, 1992) (that \textit{per} mRNA oscillations damp in DD while rhythmic behaviour continues). The apparent damping of rhythms in \textit{Drosophila} maintained under DD is most probably a population artefact of the different free-running periods of individuals rather than a true damping of the molecular oscillations. This problem was largely resolved by the analysis of \textit{per} expression in individual flies using reporter gene technology (Plautz \textit{et al.}, 1997). However, even within an individual fly maintained in constant conditions, there may be internal desynchronisation between different oscillators (Hong and Saunders, 1998; Lewis, 1994). Ideally, the labelling of X and Y populations separately would resolve this issue, but as the two populations most probably rely on the same molecular components (ie. PER and TIM) this is not feasible. The use of the highly sensitive analytical technique of QC-RT-PCR, which was developed here to analyse circadian rhythms in \textit{per} expression, provides the ideal opportunity to resolve the question of whether dissociation of X and Y oscillators in constant conditions is the cause of the seemingly irreconcilable differences between behavioural and molecular results. Since this type of analysis can be conducted on relatively small numbers of cells, analysis of \textit{per} levels in specific areas of single brains could prove insightful in untangling the multioscillator nature of the circadian clock.
5.3 The Entrainment of the *L. cuprina* Clock to Light Cycles

To gain an understanding of how circadian clocks function it is of paramount importance to comprehend the endogenous nature of rhythmicity. Equally important however, is an understanding of how they entrain to daily geophysical cycles, as entrainment by zeitgeber is a cardinal feature of circadian clocks.

The *L. cuprina* molecular model simulates entrainment to light cycles by relying on the biologically important ‘skeleton photoperiod’. It is proposed here that the failure of the model to simulate entrainment to long days when the entire photophase is used is reflective of the involvement of the IEGs *c-fos* and *Jun-B* in the entrainment process, and indicates that the clock itself only ‘perceives’ certain parts of the daily light cycle (the first and last 30-60 min). This ‘molecular skeleton photoperiod’ corresponds well with the behavioural ‘skeleton photoperiods’ which Pittendrigh (1965) originally showed to entrain circadian rhythms as effectively as entire photophases.

Until recently, the role of *c-fos* in the entrainment of circadian clocks had only been confirmed in vertebrates (Kornhauser *et al.*, 1993). However, preliminary research conducted over the past three years (Warman, 1995; Cymborowski and King, 1996; Urroz, 1997), has shown the induction of Fos-like immunoreactivity to occur in insect brains in response to phase shifting light pulses. In order to ascertain whether the reactive protein in these insects is indeed Fos, N-terminal sequencing of the purified protein needs to be carried out in addition to the isolation and analysis of insect *c-fos* and *Jun-B* cDNAs.

A more precise relation of the induction of insect IEGs to behavioural PRCs is also vital to the understanding of insect entrainment. The only extensive analysis of IEG induction at different circadian times has been conducted in mammalian systems (Kornhauser *et al.*, 1993). In the golden hamster, phase-shifting light pulses occur from late in the subjective day through to the middle of the subjective night. Phase-shifting pulses do not occur early in the subjective day (around subjective dawn), and
accordingly Fos and Jun expression is not induced at these times. This lack of IEG induction at a time which, in many organisms is considered to produce a phase shift, adds some weight to the argument that only one portion of the skeleton photoperiod is required to entrain an organism to the daily light cycles, and that this is how entrainment occurs in nature.

Stable entrainment does not require a PRC which has the same advance vs. delay topology (Johnson, 1991). In fact, in order for entrainment to occur a circadian oscillator’s PRC need only have a region of negative slope which is less than -2 and a point on the PRC where the phase shift equals $\tau - T$ (Johnson, 1991). It is not even necessary to have a PRC with both delays and advances; if $\tau$ is less than 24 h a curve with only delays will suffice, and if $\tau$ is greater than 24 h, a curve with only advances is sufficient. A specific example of this is Gonyaulax where $\tau$ is 25 h and the PRC consists only of advances (Johnson, 1991). When considering this in molecular terms, it may be that only one part of the PRC involved with the generation of either delays or advances is of particular importance to the specific individual. In an evolutionary context one can imagine that most organisms possess an ‘archetypal’ clock which has the plasticity for advances or delays, but that any particular individual utilises only one of these portions depending on what the free-running period of its clock is. Particularly in burrowing animals such as sand-hoppers there may be reliance on only one daily pulse, as the behaviour of the organism may preclude its exposure to both advancing and delaying pulses. The plasticity of having an advance and a delay section may nevertheless be important for organisms such as sand-hoppers in which some populations have free-running periods which are shorter than 24 h, and others have free-running periods greater than 24 h (Cadenhead, 1995). Each population may thereby rely on a different type of pulse to entrain.

Further evidence that a single short daily light pulse is sufficient for entrainment in some organisms comes from the demonstration that entrainment can occur in response to a LD cycle of 1:23 (Pittendrigh, 1965; Saunders et al., 1994; Hong and Saunders, 1998). The phase relationship which different organisms maintain with short
entraining light pulses may provide evidence as to which pulse is important for entrainment in nature.

The *per* mutant of *D. melanogaster* goes through a series of transients when exposed to LD 1:23, while the rhythm ‘finds’ its stable point on the PRC. At this point the phase relationship between activity and the light pulse is such that light falls at approximately Ct 12 (Saunders *et al.*, 1994). At this point, the phase delay generated is 5 h (corresponding to the delay required to adjust τ from 19 h to 24 h (Saunders *et al.*, 1994)). This point also corresponds to the time during which, in natural conditions, the fly would perceive the last light of the day. In molecular terms this light pulse degrades cytoplasmic TIM to a point which delays the build up of the PER/TIM dimer, nuclear entry of the complex, and negative feedback such that it takes 24 h to complete the feedback loop. The single pulse is therefore sufficient for entrainment.

In the diurnal blow fly *C. vicina* a similar pattern is evident. The phase relationship maintained between locomotor activity and a train of 1 h entraining light pulses is such that the pulse falls at a point on the phase response curve which generates a 2 h phase delay (between Ct 12 and Ct 14 (Hong and Saunders, 1998)). This again corresponds to the delay required to adjust τ to 24 h, and also coincides with the time of the last light pulse this diurnal organism perceives each day.

In both of these species and in the nocturnal weta (York, 1998) the phase relationships between activity and the entraining light pulse correspond to the time during which the last light pulse is seen in nature (at the end of the day). Therefore, these pulses alone are sufficient to entrain the rhythm to 24 h. The absolute necessity for a phase advancing pulse at the start of the subjective day thus becomes somewhat uncertain.

From a molecular standpoint, the comparative analysis of the stability of PER/TIM in the nucleus and the cytoplasm of organisms with different free-running periods should provide information on the importance of different phase altering light pulses. One would expect that those organisms relying on phase advances as the primary cue to
entrain would have more stable nuclear PER and TIM than those which rely on a phase delaying pulse for entrainment.

Both the *L. cuprina* PRC generated by the simulation model (Chapter Two) and that obtained from behavioural data (Chapter Four) produced phase advance and delay portions (Fig. 5.1). Only the phase delaying section of the PRC may be important in *L. cuprina* if the delay obtained at the end of the day approximates 1.5-2 h (τ-T). In *L. cuprina*, the light pulse which produces a delay of the correct size to entrain τ to 24 h falls between approximately Ct 6 and Ct 8. Thus in ‘natural’ conditions a phase advancing light pulse is most probably necessary for entrainment, as the behaviour of these flies is such that they are always exposed to the full photophase and therefore receive light at Ct 12 (a time at which a delay of up to 10 h can occur). The phase advance which occurs at the start of the day (around Ct 0) can then be seen to be dragging the clock back to a period of 24 h.

Where the existence of two phase shifting portions of the PRC may be important in all species is in the adaptation to changing daylengths. If daylight continues past the point on the PRC at which Δφ=τ-T then an excessive delay will occur, and an advance at the start of the subjective day is required to maintain a 24 h period. In molecular terms nuclear entry of the PER/TIM dimer occurs too close to dawn, and therefore nuclear degradation of the PER/TIM complex must be expedited by the photic destruction of TIM in order for the clock to entrain to 24 h.

The disparity between the shape of the simulated and real PRCs illustrated in Fig. 5.1 was suggested in Chapter Four to be indicative of an earlier nuclear entry of the PER/TIM complex in *L. cuprina* than in *D. melanogaster*. If this is the case, then the PER/TIM complex in the nucleus would have to be more stable in *L. cuprina* than it is in *D. melanogaster* in order to obtain a τ of 22.5 h. This heightened nuclear stability is indirectly evidenced by the asymmetry of the PRC obtained from the behavioural data. The wider but lower amplitude of the phase advance section compared with the phase delay section suggests that the PER/TIM complex is more stable in the nucleus than in the cytoplasm.
Comparison of Simulated and Real PRC for *L. cuprina*

![Graph showing comparison of PRCs](image)

Fig. 5.1 Comparison of PRCs generated from real behavioural data (blue) and the simulation model (pink).

The asymmetry of the curve when compared to that of wild-type *D. melanogaster* (Saunders et al., 1994) further supports the contention that nuclear PER/TIM is more stable in *L. cuprina* than in *D. melanogaster*. More conclusive demonstration of this will rely on the immunocytochemical examination of PER and TIM proteins in the blow fly brain using *L. cuprina*-specific antibodies.

### 5.4 Constant Bright Light May Stop the Blow Fly Clock

Despite the fact that constant light is not a biologically relevant phenomenon, an understanding of how and why bright light responses occur provides valuable information about how the circadian clock works. For this reason, the molecular model was used to make predictions about the response of the clock to constant light of varying intensities, and these predictions were tested by behavioural analysis. In
addition to providing vital information about how the clock works, this approach affords an insight into how the clock may be stopped, which could be of benefit to a pest control strategy (see Section 5.6).

The reason for an upper-bound or maximum synthesis rate in the early quantitative models proposed to explain the functioning of biological clocks was to account for the increase in \( \tau \) in dim LL (Christensen, 1978). The incorporation of this concept resulted in the development of relaxation-type oscillators (Wever, 1965b; Christensen, 1978). Now that the involvement of transcription and translation in the generation of molecular oscillations which produce rhythmic behaviour is better understood, the upper bound can be viewed as a concrete element of the system (relating to a maximal rate of transcription and translation). Having developed a molecular model to describe clock function, it can be seen that the lengthening of \( \tau \) in constant dim light occurs in a slightly more complex way than was described in the original models. The *L. cuprina* simulation model predicts that in constant dim light \( \tau \) is lengthened as a result of the heightened sensitivity of cytoplasmic TIM to light compared to nuclear TIM, and that in constant bright light TIM is degraded to such low levels that nuclear entry of the dimer does not occur, thereby preventing feedback and causing behavioural arrhythmicity.

Behavioural data obtained in the present study support these predictions, but more subtle behavioural evidence from other species including *Drosophila* and *Calliphora* suggests that the situation is not quite this straightforward (see Saunders, 1982; Hong and Saunders, 1994). It is proposed here, however, that rather than indicating that the clock continues to oscillate during constant bright light, these data are alluding to the existence of an X-Y population system.

If the concepts of the single molecular oscillator are developed into an X-Y type system it may be possible to explain the behaviours induced by constant lighting regimes in terms of real clock effects. The primary discrepancy between the molecular and behavioural responses in constant light is that molecular oscillations continue for two cycles while the behavioural patterns are arrhythmic (Marrus et al., 1996; Qiu and
Hardin, 1996a). The most obvious explanation for this is that there is a coupled X-Y system similar to that described by Kronauer et al. (1982), Gander et al. (1984a,b) and Lewis et al. (1991), which is involved in the generation of circadian rhythms, and that the damping of the Y oscillator (the oscillator primarily involved in the control of locomotor activity) by light does not destroy the non-light responsive X population immediately. Therefore rhythmic per oscillations continue while locomotor activity is arrhythmic (Fig. 5.2).

![Diagram of X-Y population oscillator model](Figure 5.2)

**Fig. 5.2** Diagrammatic representation of an X-Y population oscillator model proposed to explain the continuation of rhythmic per expression for two cycles following transfer into constant bright light. Y represents the oscillator controlling locomotor rhythms which is light sensitive, X represents the oscillator controlling other rhythmic processes and is light insensitive.

The resetting of the *Drosophila* eclosion clock to Ct 12 on release from constant bright light to DD concurs with the idea that the clock is stopped under these conditions, and is supported by molecular evidence which indicates that constant bright light holds the *Drosophila* circadian oscillator at Zt 12 (Qiu and Hardin, 1996a). However, the resetting of the clock to Ct 12 following release of *Drosophila* pupae from constant
dim light (in which they are still rhythmic) suggests this is not the case. If the clock consists of an X-Y system, containing a large population of Y (light responsive) oscillators which has a strong effect on the X population (through strong coupling), then $\tau$ would be expected to increase after transfer into constant dim light. As TIM is mainly cytoplasmic in wild-type *Drosophila* (nuclear localisation only occurs at Ct 20 and by Ct 0 nuclear concentrations are basal (Lee et al., 1996)), then on release from dim light to DD there would be a surge in cytoplasmic PER/TIM accumulation in the Y population which should rapidly reset to Ct 12. The strong coupling effect of Y on X would then reset X to Ct 12 (Fig. 5.3).

The lack of resetting of the *C. vicina* clock to Ct 12 on release from LL to DD (Hong and Saunders, 1994) may be due to the differential sensitivity to light of the Y populations of different flies (ie. the response of different individual *C. vicina* to light may be less homogeneous than that of a population of *Drosophila*). If TIM is suppressed to a sub-threshold level in constant light, then locomotor activity becomes arrhythmic. However, the absolute value to which these levels are reduced in individual flies may differ. Thus if fly A (Fig. 5.4) has a clock which is particularly sensitive to light, then it would take a number of hours for TIM and PER to build up.
following transfer into DD. In contrast, the levels of TIM in fly B (Fig. 5.4) whose
clock can be seen to be less sensitive to light, are maintained only just below threshold.
Consequently on transfer into DD the clock of fly B would reset to Ct 12 almost
immediately.

Fig. 5.4 Diagrammatic representation of how the C. vicina clock may be stopped in constant light while
the oscillator is not reset to Ct 12 by the L to D transition. A. represents a fly whose circadian system is
particularly sensitive to light while B. represents a fly whose circadian system is less sensitive.

It is clear that the use of population concepts which were originally developed to
explain complex behaviours which occur in response to environmental perturbations
can also provide valuable predictions about the molecular responses of the clock to
these perturbations.

5.5 A Comprehensive Model of Clock Function in L. cuprina

A significant advance has been made in the characterisation of the L. cuprina circadian
clock by the development of a comprehensive molecular simulation model. The single
oscillator model developed here was based on the assumption that the Lucilia and
Drosophila clocks function in a similar manner. This biochemically explicit model has
allowed the prediction of molecular and behavioural responses of the blow fly clock to
different environmental perturbations. The molecular genetic analysis of the *Lucilia* clock provided evidence to support the predictions of the simulation model. The analysis of adult locomotor activity (as a measure of clock output) provided further support for the model. Differences between the behavioural predictions of the model and the real behavioural patterns have helped to refine the focus of future molecular research to determine the precise nature of the molecular clockwork.

The single oscillator model not only explains the basis of free-run and entrainment, but also explains the more complex responses of the clock to bright light. However, as in previous single oscillator models, it does not explain free-run lability either in DD or LL. The use of population models has in the past provided insight into complex behaviours such as rhythm splitting and spontaneous changes in $\tau$. The finer responses of the circadian system, such as the basis of spontaneous changes in $\tau$ and changes in $\alpha$, should be resolved by the further development of the single oscillator molecular model described here into a population model. A molecular population model would also provide a significant advantage over previous population models in that it would not only simulate the complex behaviours which are known to occur both during free-run (Christensen and Lewis, 1982) and constant light regimes (Hoffmann, 1971; Pittendrigh and Daan, 1976; Smietanko and Engelmann, 1989; Hong and Saunders, 1998), but being biochemically explicit it would also simulate the molecular oscillations underlying these responses.

### 5.6 The Prospects For Control of the Sheep Blow Fly Using a Clock-Based Approach

The knowledge of how the endogenous clock is reset and stopped which has come from both behavioural and molecular analyses and from the predictions of the simulation model, may prove useful as part of a pest control strategy for this economically important species.
A light sensitive biological clock is active in *Drosophila* from early embryogenesis, and light treatment of embryos is sufficient to synchronise adult locomotor activity patterns (Sehgal *et al.*, 1992). *per* expression is not only essential for the production of adult circadian rhythmicity, but it also appears to be important for the control of different behaviours throughout development, including eclosion (Qiu and Hardin, 1996b) and egg hatching (Saumann *et al.*, 1996). *per* expression has been detected as early as 5 h after egg laying (Baylies *et al.*, 1993). The role of *per* in the timing of developmental processes raises the possibility that manipulation of *per* could cause aberrant behavioural patterns and therefore increase mortality.

There are multiple ways in which the expression of *per* could be altered, including hormonal and genetic approaches. One particularly appealing approach involves the generation of transgenic flies carrying a copy of *per* under the control of a heat shock inducible promotör. If this transgenic *per* were heritable (i.e. was integrated in the germ line), then offspring from matings of these genetically modified flies with wild-type flies should result in some offspring carrying a copy of the inducible *per*. If *per* were under the control of a heat shock promotör which initiated constitutive *per* expression in temperatures over 36°C (sheep skin temperature), then when eggs were laid on live sheep *per* expression in the embryos would surge and the timing of events such as larval exodus would be perturbed. Current efforts to develop a P-element transformation system for the generation of transgenic *L. cuprina* (at Massey University) may make this possibility feasible in the future.

The present work has illustrated that arrhythmic behaviour can result from damping of the molecular oscillations which are at the basis of clock function. If this concept is turned on its head, it can be seen that overt behaviour could be drastically modified if the underlying molecular oscillations are prevented. The arrhythmic expression of behaviours such as larval exodus and wandering which are normally gated by the clock such that they occur just prior to dawn (Warman, 1995) may cause increased mortality of the larvae, and provide an important non-insecticide based control strategy.
5.7 Closing Comments

A significant step in the comparative analysis of a dipteran circadian systems has begun with the isolation and expression analysis of the *per* homologue in the sheep blow fly, and the development of a comprehensive molecular simulation model for circadian function in this species.

In closing, I should like to reiterate a strong theme of this thesis, which is that as important as it is for ‘classical’ chronobiologists to embrace the new techniques of molecular genetics in the analysis of circadian function, molecular chronobiologists should not lose sight of classical chronobiology concepts and theories, as the use of an integrated approach can provide a very powerful tool in the analysis of biological rhythms.

*Quite often it matters little what your guess is; but it always matters a lot how you test your guess* George Polya, How to Solve It.
APPENDIX ONE: SIMULATION MODELS CODE

L. cuprina Molecular Simulation Model

The following is the code for the molecular simulation model described in Chapter Two. The programming language used was BBC basic version V programming and simulations were run on a Acorn Archimedes computer.

```
ON ERROR PRINT ERL:REPORT:END
DIM phosper(5000)
DIM ntim(5000)
DIM phostim(5000)
DIM nper(5000)
DIM perRNA(5000)
DIM timRNA(5000)
DIM lite(5000)
MODE 25
PROCinit
PROCsetlight
PROCdrawacto
PROCplotnper
PROCplotntim
PROCplotphosper
PROCplotphostim
PROCplotperRNA
PROCplottimRNA
PROCdrawlight
PROCstoreakto
END

DEF PROCinit
VDU 19,128,135,0,0,0,19,1,0,0,0,0
"RAM
name$="acto"
lux=0
cycle%=0
losstus%=0
threshold%=40
ntim(0)=0.1
ENDPROC

DEF PROCsetlight
CLS
INPUT TAB(25,5)"Light intensity",lux
INPUT TAB(25)"Start interval",start%
start%=start%+start%-1
INPUT TAB(25)"Light phase
length",length%
INPUT TAB(25)"Number of cycles",cycles%
```

```
INPUT TAB(25)"Light cycle
period",period%
ENDPROC

DEF PROClight
light=0
IF (iter%>start%+(cycle%*period%))
THEN light=lux
IF (iter%>start%+(cycle%*period%)+length
%)
THEN light=0
IF (iter%<start%)
THEN light=0
IF iter%>start%+(cycle%*cycles%)
THEN
light=0
IF iter%=start%+(cycle%+1)
THEN cycle%=cycle%+1
lite(iter%)=light
ENDPROC

DEF PROCcalc
FOR day%=1 TO 20
FOR i%=1 TO 72
nper(iter%)=nper(iter%-1-
((1/ntim(iter%)*8))
IF nper(iter%)<0 nper(iter%)=0
IF iter%<7 THEN nperdelayed=0 ELSE
nperdelayed=nper(iter%-6)
diff=30-nper(iter%)%1
syn=diff*3
IF syn>2.6 THEN syn=2.6
IF syn<0 syn=0
```
Appendix One: Simulation Models

perRNA(iter%) = perRNA(iter%-1) + syn-(0.087*perRNA(iter%-1))-
(0.66*(ABS(light>1))*perRNA(iter%))
IF perRNA(iter%)<0 perRNA(iter%)=0
ELSE perRNA(iter%)=(perRNA(iter%-%12))/5)*.04
IF pertranslation<0 THEN
pertranslation=0
phosper(iter%)=phosper(iter%-1)+pertranslation-
(1/phostim(iter%)*0.00010)
REM IF phostim(iter%)<0 THEN
phosper(iter%)=(phosper(iter%-1))*.99
IF phosper(iter%)>50 THEN
phosper(iter%)=50
IF phosper(iter%)<0 THEN
phosper(iter%)=0
ENDPROC

DEF PROCcalctim
ntim(iter%)=(ntim(iter%-1)-(light*0.8))-0.15*ntim(iter%)--
IF ntim(iter%)<0.1 ntim(iter%)=0.1
IF iter%<7 THEN ntimdelayed=0 ELSE
ntimdelayed=ntim(iter%-6)
diff=30-ntim(iter%)
syn=diff*3
IF syn>3 THEN syn=3
IF syn<0 syn=0
timRNA(iter%)=timRNA(iter%-1) + syn-(0.12*timRNA(iter%-1))
IF iter%<12 THEN timtranslation=0
ELSE timtranslation=((timRNA(iter%-%12))/5)*.04
IF timtranslation<0 THEN
timtranslation=0
phostim(iter%)=phostim(iter%-1)+timtranslation-light
IF phostim(iter%)>50 THEN
phostim(iter%)=50
IF phostim(iter%)<0.0001
phostim(iter%)=0.0001
phostim(iter%)=phostim(iter%)+
DEF PROCstoreakto
OSCL("ScreenSave"+name$)
ENDPROC

DEF PROCplotperRNA
MOVE 0,400+40
DRAW 2160,400+40
MOVE 0,400
FOR iter%+1 TO 2160
DRAW iter%,nper(iter%)+400
NEXT iter%
ENDPROC

DEF PROCplotphostim
MOVE 0,100+40
DRAW 2160,100+40
MOVE 0,100
FOR iter%+1 TO 2160
DRAW iter%,phostim(iter%)+100
NEXT iter%
ENDPROC

DEF PROCplotnper
MOVE 0,300+40
DRAW 2160,300+40
MOVE 0,300
FOR iter%+1 TO 2160
DRAW iter%,ntim(iter%)+300
NEXT iter%
ENDPROC

DEF PROCplottimRNA
MOVE 0,50
FOR iter%+1 TO 2160
DRAW iter%,perRNA(iter%)+50
NEXT iter%
ENDPROC

DEF PROCplottimRNA
MOVE 0,50
FOR iter%+1 TO 2160
DRAW iter%,perRNA(iter%)+50
NEXT iter%
ENDPROC

DEF PROCstoreakto
OSCL("ScreenSave"+name$)
ENDPROC

DEF PROCplotphosper
MOVE 0,200+40
DRAW 2160,200+40
MOVE 0,200
FOR iter%+1 TO 2160
DRAW iter%,phosper(iter%)+200
NEXT iter%
ENDPROC

DEF PROCdrawacto
CLS
FOR day%=1 TO 20
PRINT
FOR i%=1 TO 72
iter%=((day%-1)*72)+i%

PRINT
Appendix One: Simulation Models

L. cuprina Molecular PRC Simulation Model

In order to generate a simulated phase response curve for 20 min light pulses, the above model was modified by the addition of a section of code similar to that used in Lewis et al. (1997). This extra code simulates the phase shift obtained from light pulses administered at different times throughout the day. These data can then be plotted as a PRC. The code for the above program, including the modifications required to generate the PRC, is shown below.

```
IF phostim(iter%)<15 AND lite(iter%)>0 THEN nper(iter%)=nper(iter%)+13
THEN chr=35 ELSE IF phostim(iter%)<15 AND lite(iter%)=0 THEN phostim(iter%)=0
THEN chr=46 ELSE chr=32
PRINT CHR$(chr);
NEXT %
NEXT day%
ENDPROC

DEF PROCentry
IF phosper(iter%)>40 AND phostim(iter%)>40 THEN losstus%=4
losstus%=losstus%-1

ENDPROC

DEF PROCinit

DEF PROCcalc

END

DEF PROCsetlight

CLS
INPUT TAB(25,5)"Light intensity",lux
INPUT TAB(25)"Start interval", start%:start%=start%-1
INPUT TAB(25)"Light phase length",length%
INPUT TAB(25)"Number of cycles",cycles%
```

ON ERROR CLOSE#0: PRINT
ERL:PRINT REPORT$;END
DIM phosper(5000)
DIM ntim(5000)
DIM phostim(5000)
DIM nper(5000)
DIM perRNA(5000)
DIM timRNA(5000)
DIM lite(5000)
MODE 25
PROCinit
PROCcalc

END

DEF PROCinit
VDU 19,128,135,0,0,0,19,1,0,0,0,0
"RAM
name$="acto"

REM ************PRC INIT
oldstate%=0
oldlight=0
oldslope=1.0
start%=1049
next%=1699
ctcrit=10
phasechange=0
@%=&90A
period=21.7
ldur%=1
REM plotscale=1.0
lux=20
light=0
cycle%=0
losstus%=0
threshold%=40
ntim(0)=0.1
ENDPROC

DEF PROCsetlight

CLS
INPUT TAB(25,5)"Light intensity",lux
INPUT TAB(25)"Start interval", start%:start%=start%-1
INPUT TAB(25)"Light phase length",length%
INPUT TAB(25)"Number of cycles",cycles%
INPUT TAB(25)"Light cycle period",period%
ENDPROC

DEF PROC light
light=0
IF (iter%>=lstart AND (iter%<=((lstart%+ldur%-1))) THEN
light=lux
lite(iter%)=light
ENDPROC

DEF PROC printvalues
@%=131594
PRINT "Phase change :phasechange; CT :circtime; Light intensity :lux;
ENDPROC

DEF PROC calc
REM************PRC CALC
sf=24/period
pulse%0=0
file$=name$
a$="CT Phase-Change Monotonic New-Phase Corr-Phase"
BPUT#a%,a$
FOR lstart%=start% TO start%+(90*(period*3/72)) STEP 3
circtime=pulse%*sf
pulse%=pulse%+1
CLS
PRINT"
...........................................

FOR day%=1 TO 30
FOR i%=1 TO 72
iter%=((day%1)*72)+i%
PROC light
PROC calc tim
PROC calc per
IF (phos per(iter%)>40 AND phost im(iter%))>40 OR losst us%>0 THEN PROC entry
IF phost im(iter%)<15 AND lite(iter%)=0 THEN chr=42 ELSE IF
phost im(iter%)<15 AND lite(iter%)>0 THEN chr=35 ELSE chr=32
IF iter%=next% AND chr=32 THEN
chr=46 ELSE IF iter%=next% AND chr=42 THEN chr=58
PRINT CHR$(chr);

IF iter%>next%-40 AND iter%<next%+40
THEN PROC phase change
NEXT i%
PRINT
NEXT day%
REM************
PROC new phase
@%=&01020206
a$=STR$(circtime)+" +STR$(phase change)+" +STR$(monoton)+" +STR$(new phase)+" +STR$(cor phase)
BPUT#a%,a$
PROC print values
PROC plot it
ner
PROC plot tim
RNA
PROC plot per RNA
PROC plot tim RNA
PROC draw light
ink%=INKEY(8000)
IF INSTR("Pp",CHR$(ink%)) THEN
PROC store ak to
NEXT lstart%
CLOSE#a%
ENDPROC

DEF PROC calc per
nper(iter%)=nper (iter%-1)-
(1/intim(iter%*8))
IF nper(iter%)<0 nper(iter%)=0
IF iter%<7 THEN nper delayed=0 ELSE
diff=30-nperlter%)
syn=diff*3
IF syn>2.6 THEN syn=2.6
IF syn<0 syn=0
per RNA(iter%)=per RNA(iter%-1)+syn-(0.087*per RNA(iter%-1))-
(0.66*(ABS(light>1)))*per RNA(iter%))
IF per RNA(iter%)<0 per RNA(iter%)=0
IF iter%<12 THEN per translation=0
ELSE per translation=((per RNA(iter%-12))-1)*0.04
IF per translation<0 THEN
per translation=0
phos per(iter%)=phos per(iter%-1)+per translation
IF phos per(iter%)>50 THEN phos per(iter%)=50
ENDPROC
DEF PROC calc tim
ntim(iter%) = ntim(iter%-1) - light -
\(0.15 \times ntim(iter%-1)\)
IF ntim(iter%) < 0.1 ntim(iter%) = 0.1
IF iter% < 7 THEN ntimdelayed = 0 ELSE
ntimdelayed = ntim(iter%-6)
diff = 30 - ntim(iter%)
syn = diff * 3
IF syn > 3 THEN syn = 3
IF syn < 0 syn = 0
timRNA(iter%) = timRNA(iter%-1) + syn -
\(0.12 \times timRNA(iter%-1)\)
IF iter% < 12 THEN timtranslation = 0
ELSE timtranslation = \((timRNA(iter%-12)) - 5\) * 0.04
IF timtranslation < 0 THEN
timtranslation = 0
phostim(iter%) = phostim(iter%-1) + timtranslation - light
IF phostim(iter%) > 50 THEN phostim(iter%) = 50
IF phostim(iter%) < 0 phostim(iter%) = 0
ENDPROC
DEF PROC store akt o
PRINT pulse%
OSCLI("SCREENSave +name$+STR$pulse%")
ENDPROC
DEF PROC plot nper
MOVE 0,200+40
DRAW 2160,200+40
MOVE 0,200
FOR iter%=1 TO 2160
DRAW iter%, nper(iter%) + 200
NEXT iter%
ENDPROC
DEF PROC plot RNA
MOVE 0,200+40
DRAW 2160,200+40
MOVE 0,200
FOR iter%=1 TO 2160
DRAW iter%, RNA(iter%) + 300
NEXT iter%
ENDPROC
DEF PROC drawer light
MOVE 0,10
FOR iter%=1 TO 2160
DRAW iter%, light(iter%) + 10
NEXT iter%
ENDPROC
DEF PROC phase change
IF (oldstate% = 0 AND chr = 42) OR
(oldstate% = 0 AND chr = 58) THEN
phase change = ((next% - iter%)/3) * sf
IF (circtime > ct crit AND
phase change > 0) THEN
monoton = phase change - 24 ELSE
monoton = phase change
IF chr = 32 OR chr = 46 THEN
oldstate% = 0 ELSE oldstate% = 1
ENDPROC
DEF PROC new phase
new phase = circtime + phase change
IF new phase > 20 THEN
cor phase = new phase - 24 ELSE
cor phase = new phase
ENDPROC
DEF PROC plot phos per
MOVE 0,300+40
DRAW 2160,300+40
MOVE 0,300
FOR iter%=1 TO 2160
DRAW iter%, phos per(iter%) + 200
NEXT iter%
ENDPROC
DEF PROC plot pho stim
MOVE 0,100+40
DRAW 2160,100+40
MOVE 0,100
FOR iter%=1 TO 2160
DRAW iter%, phos tim(iter%) + 100
NEXT iter%
ENDPROC
DEF PROC plot ntim RNA
MOVE 0,50
FOR iter%=1 TO 2160
DRAW iter%, ntimRNA(iter%) + 50
NEXT iter%
ENDPROC
DEF PROC cent ry
IF phos per(iter%) > 40 AND
phostim(iter%) > 40 THEN losstus% = 4
losstus% = losstus% - 1
nper(iter%) = nper(iter%) + 13
phos per(iter%) = (phos per(iter%) - 13)
IF phosper(iter%)<0 THEN
phosper(iter%)=0

ntim(iter%)=ntim(iter%)+13
IF ntim(iter%)<=0 THEN ntim(iter%)=0.1

phostim(iter%)=(phostim(iter%)-13)
IF phostim(iter%)<0 THEN
phostim(iter%)=0

ENDPROC
APPENDIX TWO: GENOMIC DNA EXTRACTION USING CTAB

*L. cuprina* genomic DNA was extracted using the CTAB technique which is commonly used for samples with high carbohydrate or fat content. This technique was appropriate as quantification of genomic DNA was not necessary. It must be noted however that spectrophotometric quantification of DNA extracted with CTAB is inaccurate as CTAB interferes with spectrophotometry.

**CTAB Buffer:**

10 mL 1 M Tris-Cl (pH 8.0)  
4 mL 0.5 M EDTA (pH 8.0)  
8.18 g NaCl  
2 g CTAB (Hexadecyltrimethylamonium bromide)  

Make up to 100 mL with MilliQ H₂O (Store at room temperature). Immediately prior to use add 2 μL of β-mercaptoethanol.

**Phenol/Chloroform**

Melt 500 g phenol in a water bath and add an equal volume of 1 M Tris-Cl (pH 7.5). Allow to equilibrate over night then remove old Tris-Cl and add fresh Tris-Cl (pH 7.5). repeat procedure until the phenol is equilibrated to a pH 7.5.

Add phenol/ chloroform/isoamyl in a 25:24:1 ratio and use to extract DNA from sample.

**Procedure:**

1. Homogenise 10 fly heads in 500 μL CTAB buffer containing β-mercaptoethanol using a ground glass homogeniser.

2. Incubate the homogenate in a 1.5 mL microcentrifuge tube for 1-2 h at 65°C (rotating).

3. Extract DNA by adding an equal volume (500 μL) of phenol/chloroform/isoamyl to the sample and incubate (agitating) at room temperature for 15 min. Spin tubes at 12,000 g for 10 min at room temperature to separate the phases and remove aqueous phase (containing DNA) into another microcentrifuge tube with a micropipette.

4. Add 500 μL of chloroform/isoamyl (24:1) to this supernatant and incubate and spin as above.
5. Remove the supernatant into a fresh microcentrifuge tube and precipitate DNA with an equal volume of ice cold isopropanol. Spin the samples at 12,000 g for 15 min at 4°C to pellet the DNA. Remove isopropanol and wash with 1 mL of 70% ethanol. Spin again at 12,000 g for 15 min and remove ethanol.

6. Allow samples to air dry then resuspend in 20 μL TE (100 mM Tris-Cl (pH 8.0) 1 mM EDTA).

Note: If the samples are particularly ‘dirty’ addition of extra phenol/chloroform steps may be necessary. Alternatively a phenol step can be used prior to the phenol/chloroform step.
APPENDIX THREE: MAKING COMPETANT CELLS USING THE TSS/DMSO METHOD AND TRANSFORMATION OF BACTERIA

Generation of Competent Cells Using the TSS/DMSO Method (Huynen, 1994)

Competent bacteria were made in order to allow transformation with plasmids containing PCR products or cDNA clones. The method of choice for making competent cells in this thesis was the TSS/DMSO method.

Procedure:

1. Seed 50 mL of luria broth (LB) with 50 µL of an overnight culture of bacterial cells (JM109 or XL1-Blue). Grow cells at 37°C until they reach mid-log phase (OD$_{600}$ 0.6).

2. Harvest cells by centrifugation at 1000 g for 10 min and resuspend in 2 mL of TSS (LB containing 10% PEG 6000 and 50 mM MgCl$_2$ pH 6.5).

3. Add 5% v/v DMSO to the cells on ice and mix gently (mixing must be gentle as cells are fragile following the addition of DMSO).

Transformation of Bacteria With Plasmids

Competent cells were then transformed with the plasmids containing inserts as follows:

Procedure:

1. Add a 100 µL aliquot of competent cells to 5 µL of the ligation product in a 1.5 mL microcentrifuge tube incubate on ice for 30 min.

2. Heat shock transformations at 42°C for 30 sec in a water bath.

3. Add 900 µL of LB (containing 20 µL 10 mg/mL IPTG and 20 µL of 20 mg/mL X-GAL) to the transformation and incubate at 37°C for 45 min to allow the expression of antibiotic resistance.

4. Plate the entire volume (1 mL) onto 100 µg/mL Luria Agar/ampicillin plates and allow to dry in a laminar flow hood.

5. Seal plates with parafilm and grow (inverted) overnight at 37°C. Select positive transformants by blue/white colony selection.
APPENDIX FOUR: cDNA LIBRARY SCREENING

cDNA libraries consist of a collection of bacteriophage which represent all of the mRNA species expressed at a particular stage of development in a particular tissue. Screening of an adult head cDNA library (courtesy of Dr P. East, CSIRO) revealed a per cDNA clone (Chapter Three). The specific technical details of library screening along with the reagents used is given below.

**Media:**

**Luria Broth (rich):**

- NaCl 10 g/L
- Bactotryptone 10 g/L
- Bactoyeast 5 g/L
- Maltose 2 g/L
- MgSO₄ 2.46 g/L
- pH to 7.0 with NaOH and autoclave

**Luria Broth Agar:**

- NaCl 10 g/L
- Bactotryptone 10 g/L
- Bactoyeast 5 g/L
- bactoagar 20 g/L
- pH to 7.0 with NaOH and autoclave

**NZY Agar:**

- NaCl 5 g/L
- MgSO₄·7H₂O 2 g/L
- Bactoyeast 5 g/L
- Casein hydrolysate 10 g/L
- bactoagar 15 g/L
- pH to 7.5 with NaOH and autoclave

**NZY Top Agar:**

- NaCl 5 g/L
- MgSO₄·7H₂O 2 g/L
- Bactoyeast 5 g/L
- Casein hydrolysate 10 g/L
- 0.7% Agarose
- pH to 7.5 with NaOH and autoclave

**NZY Broth:**

- NaCl 5 g/L
- MgSO₄·7H₂O 2 g/L
Solutions:

SM Phage Buffer:
- 100 mM NaCl
- 8 mM MgSO₄
- 50 mM Tris-Cl (pH 7.5)
- 0.01% gelatin

Neutralising Solution:
- 1 M Tris-Cl (pH 7.5)
- 1.5 M NaCl
- 20 x SSC
- 3 M NaCl
- 0.3 M C₆H₃Na₂O₇

Denaturation Solution:
- 0.5 M NaOH
- 1.5 M NaCl

Procedure:

Plate Preparation:
Prepare 10 15 cm plates by adding 80 mL of warm (50°C) NZY agar to each 15 cm bacteriological plate. Allow to set and dry in a laminar flow hood. Store plates at 4°C and pre-warm plates to 37°C before use.

Preparation of Host Bacteria and Phage:
Inoculate 50 mL of rich LB with 50 µL of overnight culture of TAP 90 cells, grow to mid log phase and harvest cells by centrifugation at 1000g for 10 min. Resuspend cells to an OD₆₀₀ of 0.5 in 10 mM MgSO₄. Dilute phage to 50,000 pfu/100 µL.

Inoculation of Bacteria with Phage:
In each of 10 separate tubes add 100 µL of phage dilution to 600 µL of bacterial dilution and incubate at 37°C for 15 min.

Plating of Infected Bacteria:
Add 10 mL of pre-warmed (50°C) NZY top agar to the tube and plate immediately onto NZY plates. Allow to set and grow inverted overnight at 37°C.
Plaques Lifts and Hybridisation:

Details of these procedures are given in chapter Three (Section 3.2.4).

Secondary and Tertiary Screens:

Conduct as above with the exception that 90 mm plates should be used and 100 µL of phage dilution (500 pfu) used to inoculate 200 µL of bacteria. 4 mL of top agarose should be used to pour onto plates.
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References


References


References


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Pofotorno


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