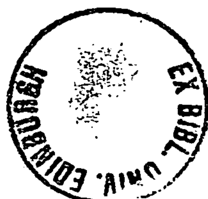
A Comparison of the Circadian Control of Locomotor Activity and Photoperiodism in *Drosophila melanogaster*, Wild-type and *period* Mutants

S.W.Gillanders

Thesis presented for the degree of
Doctor of Philosophy
University of Edinburgh
1993



This Thesis has been composed by myself, and the work described in it is my own.

S.W.Gillanders

Abstract

Mutant *Drosophila melanogaster* flies with drastically altered free-running periods of locomotor activity (*period* mutants) were used to investigate the relationship between circadian control of overt rhythm production and photoperiodic time measurement. Comparison of the free-running rhythms of wild-type, short period and long period mutants revealed that alterations in this periodictity resulted from a decrease in the active phase (short period mutant) or and increase in the inactive phase (long period mutant) when compared with wild-type. Light phase response curves were determined for light pulse of different durations which suggested that the mutants also had an altered sensitivity to light.

Comparisons of the photoperiodic response of these strains revealed a remarkable similarity from which it was concluded that the *period* locus is not essential for PPTM but may be involved in a peripheral role. The light PRC data were used to model the CDL of the photoperiodic response in the wild-type, short period and long period mutants and the modelled results did not agree with the experimental ones. A feedback model for photoperiodic time measurement involving two oscillations, a pacemaker controlling overt rhythmicity and weakly coupled to a slave involved in PPTM was shown to replicate the experimental results.

In conclusion, although the locomotor activity rhythm and the photoperiodic time measuring system are both governed by circadian oscillators, they are believed to be, at best, weakly coupled together so that the oscillator controlling locomotor activity may play a role in photoperiodism.

Contents

Glossary of Symbols and Terms i

Chapter 1: General Introduction 1

Behavioural and physiological circadian rhythms 7

Photoperiodism 21

Circadian rhythms in Drosophilidae 30

Photoperiodism in Drosophilidae 42

Chapter 2: General Materials and Methods 48

Recording the locomotor activity rhythm 51

Analysis of the photoperiodic response 58

Chapter 3: The Locomotor Activity Rhythm in *D.melanogaster* 61

Analysis of the free-running rhythm 66

Production of Phase Response Curves 76

Entrainment to light-dark cycles 98

Summary 113

Chapter 4: The Photoperiodic Response in *D.melanogaster* 114

Photoperiodic response curves in *D.melanogaster* strains 120

Diapause responses of per^{+}, per^{S} and per^{L2} in carefully controlled temperatures 125

Responses to skeleton photoperiods 133

Summary 139

Chapter 5: Modelling the Circadian Photoperiodic Clock in *D.melanogaster* 140

Modellimg the photoperiodic clock using PRC data from the locomotor activity rhythm 147

Modelling the photoperiodic response in *Drosophila melanogaster* 152

Chapter 6: General Discussion 162

References 173

Aknowledgements

Appendices

Glossary of Symbols and Terms

Activity band (α) This is the part of a sleep-wake or activity cycle when an organism is active, and is measured in hours.

Amplitude This refers to the numerical quantity of locomotor activity, and is measured in units of activity.

Circadian rhythm An endogenous oscillation with a natural period (τ) close to, but not necessarily equal to, the period of the environmental day (*i.e.* 24 hours). The endogeneity of the oscillation can only be revealed if it is seen to persist in the absence of environmental cues such as daily light-dark cycles.

Circadian time (ct) Time scale covering one full cycle of an oscillation. The whole endogenous locomotor activity cycle always encompasses a change from ct0 to ct24. The midpoint of the activity band is defined as ct6.

Critical daylength (or **nightlength**) (CDL or CNL respectively) The length of the light (or dark) portion of the light-dark cycle which separates long day effects from short day effects. This is defined, by convention, as the portion which elicits a 50% response.

Diapause A period of arrested growth and development enabling individuals to overwinter, aestivate (summer diapause) or synchronize their developmental cycles to the seasons and other individuals of the same species.

Entrainment The coupling of a self-sustained oscillation to a Zeitgeber (or forcing oscillation) so that the self-sustained oscillator adopts the frequency of the Zeitgeber.

External coincidence model A model for photoperiodic clocks in which light acts to a) entrain the endogenous oscillation and b) control the photoperiodic induction of diapause by coincidence with a photoinducible phase, Φ_i (Pittendrigh & Minis, 1964).

Free-running rhythm A biological rhythm or oscillation which continues with its own 'natural' period (τ) in the absence of entrainment.

Internal coincidence model A model for photoperiodic control of development in which two or more oscillators are independently entrained by the external Zeitgeber. Photoperiodic induction is then a function of the phase relationship between these constituent oscillators (Pittendrigh, 1972).

Period The length of one oscillatory cycle, and therefore the time after which a definite phase of the oscillator recurs.

Phase A particular reference point in the cycle of a rhythm.

Phase response curve (PRC) A plot of the magnitude and direction of phase changes induced by a single perturbation (usually light pulses) at different phases of an oscillator in free-run.

Photoinducible phase (Φ_i) A hypothetical phase point in an oscillator (or driven system) which is light sensitive and an integral part of the external coincidence model.

Photoperiod A given light dark cycle.

Photoperiodic response curve (PPRC) A plot of the response of a population to a range of stationary photoperiods, usually incorporating the critical daylength.

Photophase The light portion of a given light-dark cycle (or photoperiod).

Scotophase The dark portion of a light-dark cycle.

Subjective day (α) The active portion of a locomotor activity cycle.

Subjective night (ρ) The inactive portion of a locomotor activity cycle.

Zeitgeber The forcing or entraining oscillation which entrains a biological rhythm by changing the period of the free-running rhythm to that of the Zeitgeber.

LD Light-dark cycle. LD 8:16 represents a light-dark cycle consisting of an 8 hour light pulse (photophase) followed by a 16 hour dark gap (scotophase).

LL Continuous light.

DD Continuous darkness.

τ The natural period of an endogenous oscillator as demonstrated in constant conditions.

T The period of an entraining Zeitgeber.

Φ_i The photoinducible phase.

α Activity phase.

ρ Inactive phase.

PRC Phase response curve.

PPRC Photoperiodic response curve.

CDL Critical daylength.

CNL Critical nightlength.

ct Circadian time.

CHAPTER 1

General Introduction

An organism's environment varies not only through space but also through time. The variety of different timing systems used to exploit or negate the periodic changes in the environment stretch from the ultradian, less than 24 hours, and including circatidal rhythms of about 12.4 hours, through circadian rhythms of approximately 24 hours, circasemilunar (about 14.7 days), circalunar (about 29.4 days) to circannual rhythms lasting for about one year. These biological rhythms continue in constant conditions, or free-run, and have evolved to approximate the periods of the external environmental rhythms. These external rhythms, or Zeitgebers, are the product of geophysical changes in the environment, the primary causes being the rotation of the earth about its axis, the movement of the moon around the earth and both around the sun. They can entrain the corresponding biological rhythms so that the period of the rhythm exactly equals the period of the environmental cycle. There are also biotic variations in the environment, due to other organisms on different trophic levels, predators, prey or parasites, or on the same trophic level, competitors or mates, which can also entrain rhythms. Far from being constant the environment changes in a regular and predictable way and biological rhythms are a product of the selection pressures produced by such environments.

The evolution of biological rhythms can be traced back to the emergence of eukaryotic life. Whilst the unicellular eukaryotes have generation times in the order of a day prokaryotes tend to have generation times in the order of minutes or hours. Pittendrigh (1981a) argues that whilst an organism needs, in order to express a biological rhythm, a generation time equal to or greater than the period of the rhythm expressed, the evolution of biological rhythms is not a consequence of this increase in

the cell cycle. The extended cell cycle of eukaryotes is not a prerequisite for the evolution of biological rhythms; if prokaryotes could produce and maintain rhythms then they ought to be able to transmit the rhythm across generations as in *Euglena* (Edmunds, 1971). Indeed circadian rhythms in nitrogen fixation have been found in the prokaryotic *Synechococcus* RF-1 (Huang *et al*, 1990; Huang *et al*, 1991). This rhythm has been shown to free-run in constant conditions, its period is temperature compensated between 22°C and 33°C and appears to phase shift when exposed to light-dark cycles.

Pittendrigh (1981a) suggested that light-dark cycles have imposed an exogenous periodicity on cellular reactions and that for a variety of reasons 'metabolic pools' within the cell must have returned to qualitatively different states at different times of the day. In metabolic systems there are plenty of negative feedback loops which contain time constants. All such systems are potential oscillators which may 'lock onto', or entrain to, a stable external cycle that has some input into the metabolic pathway. Any poikilothermic metabolic system which lasts for longer than one day will be forced into a daily cycle of metabolic states due to the heating and cooling effects of the light-dark cycle. In this way temporal order may be imposed upon the metabolites providing a means of co-ordinating metabolic pathways in time, a building block from which natural selection has fashioned endogenous oscillatory systems. The selective advantage of rhythmic control of cell function can only really be guessed at, but most probably is due to the ability of rhythms to time certain events so that they occur at a most appropriate time. An individual is thus able to predict regular events and 'act' in preparation of them instead of reacting to them.

The endogenous circadian rhythmicity which has evolved in eukaryotes is expressed in rhythmic phenomena which can be observed. An individual can express several rhythms simultaneously, all of which may reach their respective peaks at

different times of the external day. For example, in the dinoflagellate, *Gonyaulax polyedra*, rhythms of photosynthesis, spontaneous glow, inducible luminescence and cell division have been observed (Hastings, 1960; Sweeney, 1969). These four rhythms are different expressions of the same underlying oscillating system, or 'clock'. More recently, however, temperature effects upon the two rhythms of bioluminescence (flashing and spontaneous glow) have shown that even in these simple single cell organisms two oscillators may be controlling different aspects of the circadian system (Van der Heyde *et al*, 1992). The rhythms can be considered as 'hands' of the 'clock', the underlying oscillator. The differentiation of the circadian system into rhythms which are the measurable expression of a biological clock and the oscillating system is important. Whilst a multitude of circadian rhythms have been found and described in eukaryotes, and the probable location of the underlying clock found in many animals, the means by which cells produce biological rhythms, the metabolites which rhythmically change and their means of control have not been elucidated. In many cases a 'black-box' approach to an organisms circadian system has had to suffice and whilst many models exist for circadian oscillators very little is known about their actual mechanics.

The circadian system is not simply one circadian oscillator organising a multitude of behavioural rhythms. In animals the multiplicity of circadian rhythms, their different areas of expression and the different times of the day at which they reach a peak reflects the multioscillatory nature of it. Circadian rhythms reflect underlying oscillators but these are arranged into a hierarchical system which has a primary oscillation, or pacemaker, which can entrain to the external Zeitgeber and internalise this signal. This internal signal can then be passed to a number of slave oscillators which can result in the expression of a rhythm, pass the internal signal on to another slave oscillator, or do both. By this method the Zeitgeber which contains the information about the time of day can be used to entrain a plethora of circadian

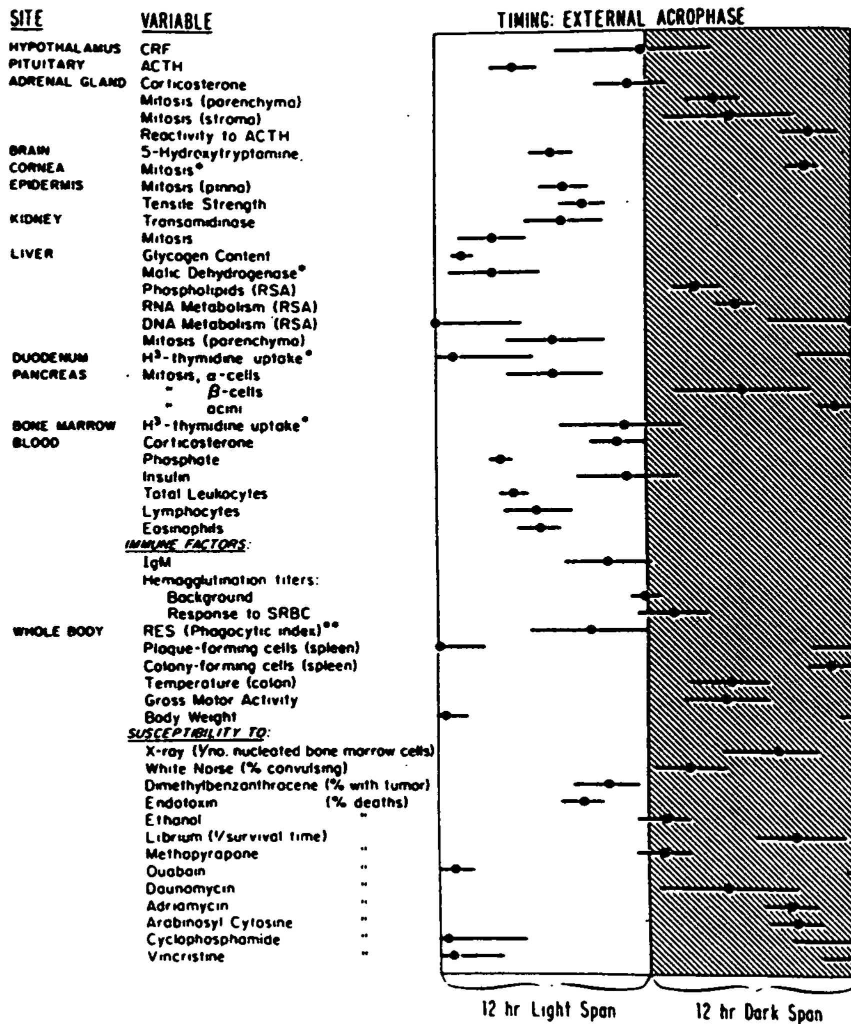


Fig 1.1 A phase map of the circadian system of the mouse (*Mus musculus*) showing the acrophase (the time of the best-fit maximum) and the standard errors of numerous rhythms (From Szabo, Kovats & Halberg, 1978).

rhythms, all or most of which will reach their maxima at distinct and different times of the day (Fig 1.1). The primary difference between a pacemaker and slave oscillation is that there was traditionally only one pacemaker capable of internalising the external temporal information. However, this view may not be strictly true: there may be more than one oscillation capable of responding to the external day. In the beetle *Blaps gigas* each eye contains an autonomous circadian pacemaker, either of which is capable of entraining the circadian system to the external light-dark system (Koehler & Fleissner, 1978) and in humans Aschoff (1969) has shown that the circadian system is controlled by two pacemakers, one controlling the activity-rest cycle and associated rhythms whilst the other produces and maintains a rhythm in body temperature.

Temporal organisation within animals allows particular physiological or behavioural reactions to occur at a most appropriate time of the day. It also, by extension, enables animals to act in preparation of regular variations in the daily environment either to take full advantage of the change or to withstand any disadvantageous factors. Circadian rhythms in animals have been put to several uses where timing of the interaction between individuals and the environment is crucial.

1 Time memory (Zeitgedachtnis) is most fully demonstrated by the honeybee, *Apis mellifera*. Put simply, this is the ability of worker bees to 'remember' not only the location of a food source but also the time of the day at which the food was present so the bees return to food sources at the same time on a number of subsequent days (Beling, 1928; Wahl, 1932). This ensures that food sources can be utilized in the most energy efficient way by the bees.

2 Sun orientation uses the sun's azimuth as a compass to enable animals to maintain a constant compass direction throughout the day. However, the sun's azimuth moves throughout the day from the east, where the sun rises, to the west, where it sets. This sun orientation needs a circadian oscillator to allow individuals to compensate for the apparent movement of the sun (Hoffman, 1960).

3 Behavioural and physiological rhythms are products of the circadian system and ensure an appropriate internal temporal order and phasing of this to the external day. These rhythms are often referred to as the hands of the circadian clock and provide a means of assaying the underlying oscillators.

4 Photoperiodism is a strategy by which individuals can use the changing daylengths to determine what time of year it is. Seasonally appropriate responses can use the daylength as an indicator of the season. Bunning in 1936 first suggested that the circadian system might provide the 'clockwork' which underlies photoperiodism.

Behavioural and physiological circadian rhythms

Both behavioural and physiological circadian rhythms occur in animals. There are a few exceptions which have evolved for life in timeless environments, for example deep cave dwellers like the beetle *Laemostenus navarricus* which exhibits no locomotor activity rhythms (Lamprecht & Weber, 1977). Whilst physiological rhythms can be measured, behavioural observations are often easier to make. An example of a behavioural rhythm which is expressed with a circadian period is given in Fig 1.2 where the locomotor activity rhythm of an individual blowfly (*Calliphora vicina*) is reproduced from Kenny (1989). The locomotor activity of the blowfly has been measured over a large number of days in constant darkness (DD); it is therefore receiving no time cues from the environment. The fly is self-organising its activity into a rhythm with distinct times when the animal is active and others when it is inactive. The active phase of the rhythm begins a little earlier each dav so the rhythm slopes to the left. This actogram plot of the locomotor activity rhythm, where a day's activity is plotted below and to the right of the previous days activity, gives a pictorial representation of the free-running rhythm of locomotor activity. The free-running rhythm has a periodicity called tau (τ), and can be divided into two sections, an active section called alpha (α), and an inactive section, rho (ρ). These two sections are also referred to as the subjective day and subjective night respectively.

There is a second type of circadian rhythm which can be observed in populations but not individuals. An example of one such population rhythm is the eclosion rhythm of *Drosophila pseudoobscura* (Pittendrigh, 1954; 1960) where a mixed age population emerges from their puparia as adults in daily gates. These occur in constant conditions with a regular circadian periodicity. Adults can only emerge within an eclosion gate and if they 'miss' one, because they are not quite developmentally 'ready', they must wait until the beginning of the next eclosion gate the following day.

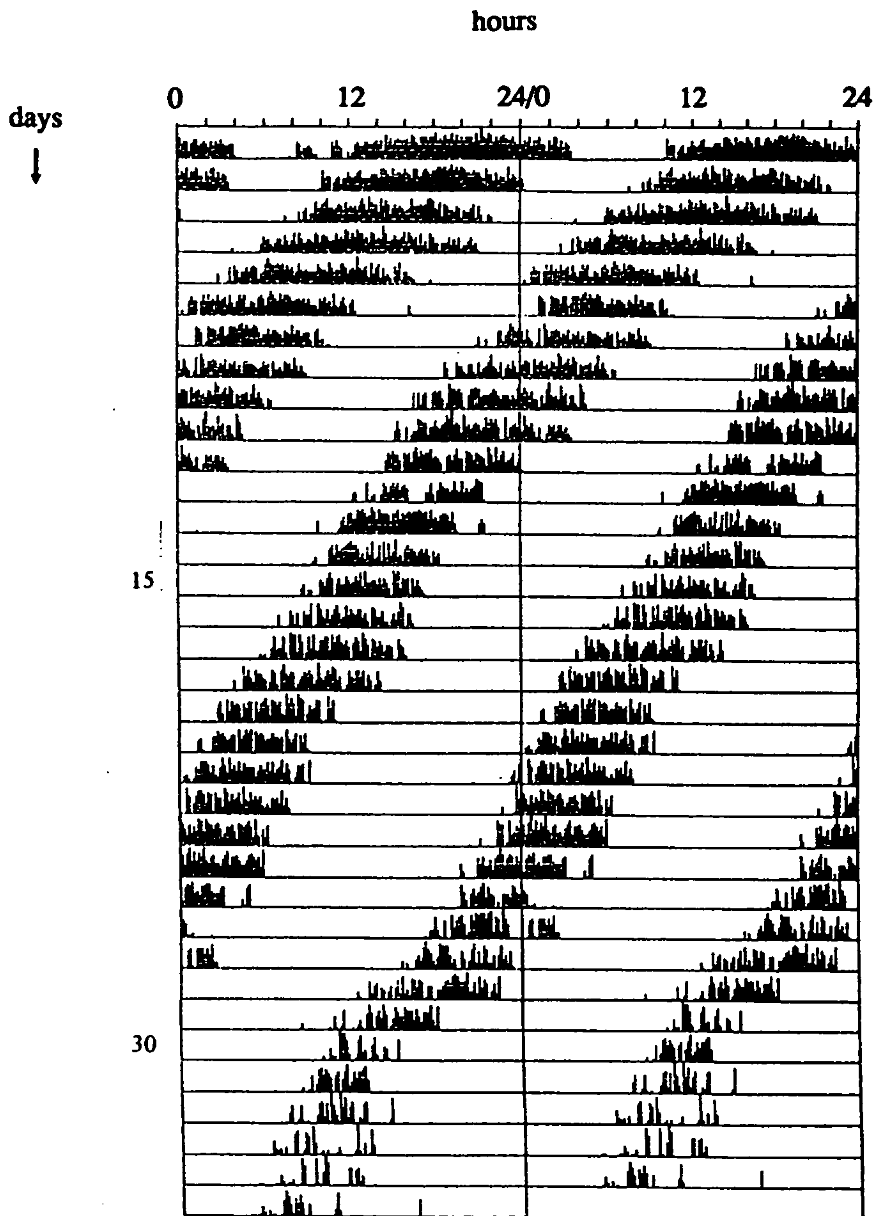


Fig 1.2 A double plotted activity record, or actogram, of an individual blowfly, *Calliphora vicina*, in constant darkness. Subsequent days are plotted below and to the right of each other to produce a clear picture of the time course of the rhythm. The activity of the fly starts earlier each day, demonstrating a free-running rhythm with a period of less than 24 hours (from Kenny, 1989).

These two types of circadian behavioural rhythms, which are expressed in the same organism, although at different times of their life cycle, highlight the multioscillatory nature of the circadian system. The locomotor activity rhythm is believed to be a 'direct' expression of a central pacemaker with a period, tau, which is reflected in the period of the free-running locomotor activity rhythm. The expression of the locomotor activity rhythm is considered to be 'direct' because it is driven directly by the pacemaker unlike the eclosion rhythm which is controlled by two oscillators, the central pacemaker or A-oscillator, reflected in the activity rhythm and a second slave, or B-oscillator, which is entrained by the pacemaker and which controls the emergence of the individual adults from their puparia (Pittendrigh, 1966).

From Fig 1.2 the approximation of the daily activity rhythm to 24 hours can be seen. Natural selection has managed to produce circadian systems which are close to the external day but not exactly 24 hours. At first it seems that natural selection is somehow restricted, never able to produce an exact match of the external Zeitgeber period. The average period of a free-running rhythm, tau, is stable over a great number of days. This does not mean that the duration of each cycle is constant. There is quite a large instability within the system, so that for any endogenous rhythm tau varies about its mean value with a standard deviation of *S*. Pittendrigh (1980) has argued that tau not being equal to T (the period of the external Zeitgeber cycle) is a selected strategy not a tolerated approximation. Entrainment of circadian rhythms by 24 hour Zeitgebers produces a stable relationship between the Zeitgeber cycle and the circadian rhythm, this stable 'phase relationship' (ψ) is necessary if orderly biological change is to be appropriately timed to changes in the environment. However, as tau approaches the value of T then Pittendrigh argues the standard deviation of tau will decrease and the instability of the phase relationship of the Zeitgeber and endogenous rhythm will increase. This is supported by work from several laboratories where it has

been found that the standard deviation of tau is reduced as tau approaches T (Aschoff *et al*, 1971; Pittendrigh & Daan, 1976; Kram, 1980).

Constant darkness (DD) is not the only constant condition which animals can be exposed to; constant light (LL) also gives an animal no temporal information. However, constant light effects circadian rhythms in a different way from DD. The changes which occur in the free-running rhythm of animals when transferred from DD to LL are summarized by 'Aschoff's Rule' (Pittendrigh, 1960) and the 'circadian rule' (Aschoff, 1960). Aschoff's rule states that tau lengthens with an increase in light intensity, or on transfer from DD to LL, for dark active animals but shortens for light active animals. This has been extended in the 'circadian rule' to include the effects of LL upon the ratio of alpha to rho and to the total amount of activity in each circadian cycle. Both of these parameters increase with increasing light intensity in day active animals but decrease in night active ones. These 'rules', whilst predicting the effects of constant light upon many birds and mammals (Hoffmann, 1965), do not hold for several insect species.

Constant light has another effect upon physiological or developmental rhythms like the pupal eclosion rhythm in *Drosophila pseudoobscura*. Whilst these rhythms can be seen to free-run in DD, a change from DD to LL causes them to rapidly 'damp out' apparently becoming arrhythmic. Once damped they appear to be set to a unique phase, from which they then free-run, upon a subsequent return to DD. This difference between behavioural and physiological rhythms is one of the distinguishing features of Truman's Type I and Type II clocks (1971). Type I clocks are associated with developmental or physiological rhythms like pupal eclosion, hatching, or hormone release. Light seems to by-pass the ocular photoreceptor pathways and impinge directly upon the brain, CNS or other tissue. Light sensitivity appears to be high leading to arrhythmia in LL and strong (Type 0) phase response curves (see

later). Type II clocks tend to control behavioural rhythms like locomotor activity. Light is received by 'organized' photoreceptors, like the compound eyes, and the clocks response to light tends to be of the low amplitude variety, Type 1 phase response curves, with free-running rhythms in LL. The division between Type I and Type II clocks has, however, become blurred with several physiological rhythms persisting in LL, pupal eclosion in *Sarcophaga argyrostoma* (Saunders, 1979) for example, and behavioural rhythms which show Type 0 PRCs to light and damping in LL, such as locomotor activity in cockroaches (Wobus, 1966; Brady, 1974; Saunders & Thompson, 1977; Wiedenmann, 1977). Disconnected mutants (*disco*) in *Drosophila melanogaster* with no connection from the compound eye to the optic lobes have a behavioural rhythm, the locomotor activity rhythm, which can be entrained by light-dark cycles (Stellar *et al*, 1987).

The reliable time keeping required of circadian clocks has resulted in the evolution of the unusual feature of temperature compensation of period. Temperature cycles can, however, be used to entrain light-dark cycles. This temperature compensation must then be an active process used to maintain the period of the oscillator. Most metabolic systems do not exhibit this homeostatic control; they have Q_{10} values of around 2-3, which means that for a 10 degree rise in temperature there is at least a doubling in the rate of the metabolic reaction. If a metabolic 'clock' behaved like this, lacking temperature compensation, then the period of the free-running rhythm would half for every 10 degree rise in the ambient temperature and the clock would not keep time accurately and lose the ability to entrain to Zeitgebers. It has been argued that the temperature compensation incorporated into metabolic clocks was a prerequisite for natural selection to produce circadian oscillators. Recently, however, a marine plasmodial rhizopod (*Thalassomyxa australis*) has been described which, at about 22°C has a circadian periodic change between an active and inactive phase. The period length however is strikingly temperature dependent (Silyn-Roberts

et al, 1986) suggesting that temperature compensation of circadian oscillators has been 'acquired' during evolution as the advantages of these clocks became evident.

Although temperature cycles can act as Zeitgebers and entrain oscillators, the most obvious Zeitgeber is the daily change in light intensity associated with the rotation of the earth about its axis. Light signals are used by clocks to regulate their periods so that circadian rhythms have periods of 24 hours, the same as the external day. This entrainment process allows the circadian system to accurately control various events and ensure they occur at appropriate and favourable times of the day. The 'phase relationship' between the light-dark cycle and a specific phase of an entrained circadian rhythm is defined by the circadian system and subtle alterations to this relationship can be brought about through natural selection without having to resort to major changes in the circadian control system. The control of the phase relationship between the external light-dark cycle and the oscillator is due to the properties of the oscillators and the way in which they are entrained by Zeitgebers. Although any entraining agent (including other circadian oscillators) has this effect upon the oscillator it will suffice to describe the effects of light-dark cycles here.

Natural photoperiods (light-dark cycles, LD) generally contain quite long photophases (the light portion of a light-dark cycle; the dark portion is called the scotophase) but it is also possible to entrain a rhythm to a 24 hour external light-dark cycle containing only a minute of light or less. Light can also come in either brief pulses (discrete) or as a sine wave of light intensity (continuous) (Pittendrigh & Minis, 1964). Continuous entrainment could be imagined to effect an alteration in the angular velocity of the oscillator, accelerating the clock at some times during the day and decelerating it at others, whilst discrete entrainment would be the result of light causing abrupt phase shifts in the oscillator's motion either advancing it or delaying it. Experiments involving skeleton photoperiods, where a photophase is replicated by

two discrete pulses, one at the start and the other at the end of a dark period and simulating the 'photophase', indicate that both mechanisms may be involved in natural entrainment (Pittendrigh & Minis, 1964). The experimental approach by Pittendrigh & Minis used the eclosion rhythm of *Drosophila pseudoobscura*. Essentially, skeleton photoperiods can entrain circadian rhythms when the photophases being simulated are less than about 11 hours and the results are indistinguishable from those obtained when (full non-skeleton, or) complete photoperiods are used. However, when the skeleton photophase becomes greater than about 11 hours the entrainment behaviour becomes drastically different from the behaviour under complete photoperiods. The activity is centred around the shorter of the two possible skeleton photoperiods. For example, a skeleton photoperiod of 14:10 is identical to a skeleton photoperiod of 10:14. In this case the activity becomes centred around the shorter possible skeleton photophase, *i.e.* the 10 hour pulse. In order for this to happen, the two light pulses which define the start and end of the skeleton photophase have to exchange roles, dawn becoming dusk and *vice versa*. This exchange of roles is called a phase jump and ensures that the activity occurs in the shortest possible skeleton photophase irrespective of which light pulse, dawn or dusk, illuminates the activity rhythm first. Obviously when complete photoperiods are used to entrain animals this phase jump does not occur, the continuous nature of the photophase allowing animals to tell the difference between light-dark (LD) 10:14 and LD 14:10.

Endogenous circadian rhythms can be described by measuring the duration of their complete cycle and its constituent parts, alpha and rho. Rhythms can also be described using phase response curves (PRCs) which have proved to be very successful in explaining many of the formal properties of oscillating systems, for example, phase jumps during entrainment to skeleton photoperiods and the phase relationship between rhythm and entraining Zeitgeber cycle. To understand these PRCs we first need to discuss what effect single disturbing pulses have upon free-

running rhythms in DD. Light pulses when given as part of a light-dark cycle cause a rhythm to assume the same period as the light-dark cycle and the rhythm becomes entrained. However, a rhythm cannot entrain to a single pulse of light as there is no periodicity to it. But single pulses of light administered to a free-running circadian rhythm in DD cause the rhythm to 'phase shift' and the size and direction of the phase shift depends upon the phase of the free-running rhythm illuminated. This phase shift is produced because the duration of the cycle in which the light pulse occurs is either lengthened or shortened as if the light pulse causes the underlying oscillator to speed up or slow down for one cycle.

Phase response curves (PRCs) show the phase shifts caused by single light pulses falling at different points within one full cycle of the circadian oscillation during its free-run. To ensure that PRCs are comparable between individuals and populations of animals with different endogenous periods the rhythm is divided into 24 circadian hours, where one circadian hour = tau/24 real hours. PRCs show phase shifts in rhythms (generally in real hours) plotted against the phase at which the disturbing pulse falls (in circadian hours). Two examples are given in Fig 1.3 (A & B). One of the characteristics used to distinguish Truman's Type I and Type II clocks was their respective sensitivity to light and their subsequent phase response curves. The two PRCs in Fig 1.3 represent the two types of PRC which are used in Truman's system; Type 1 PRCs (A) and Type 0 PRCs (B) (Winfree, 1980).

The Type 1 PRC in Fig 1.3A can be divided into a section of phase advances ($\Delta\phi$+), a section of phase delays ($\Delta\phi$-) and a 'dead zone' where no phase shifts occur. The dead zone corresponds to the animal's subjective day. Light pulses falling in the late subjective day/early subjective night result in phase delays, and pulses in the late subjective night/early subjective day cause phase advances. The second PRC (Fig 1.3B) is a Type 0; the maximum phase advances and delays are much larger than in

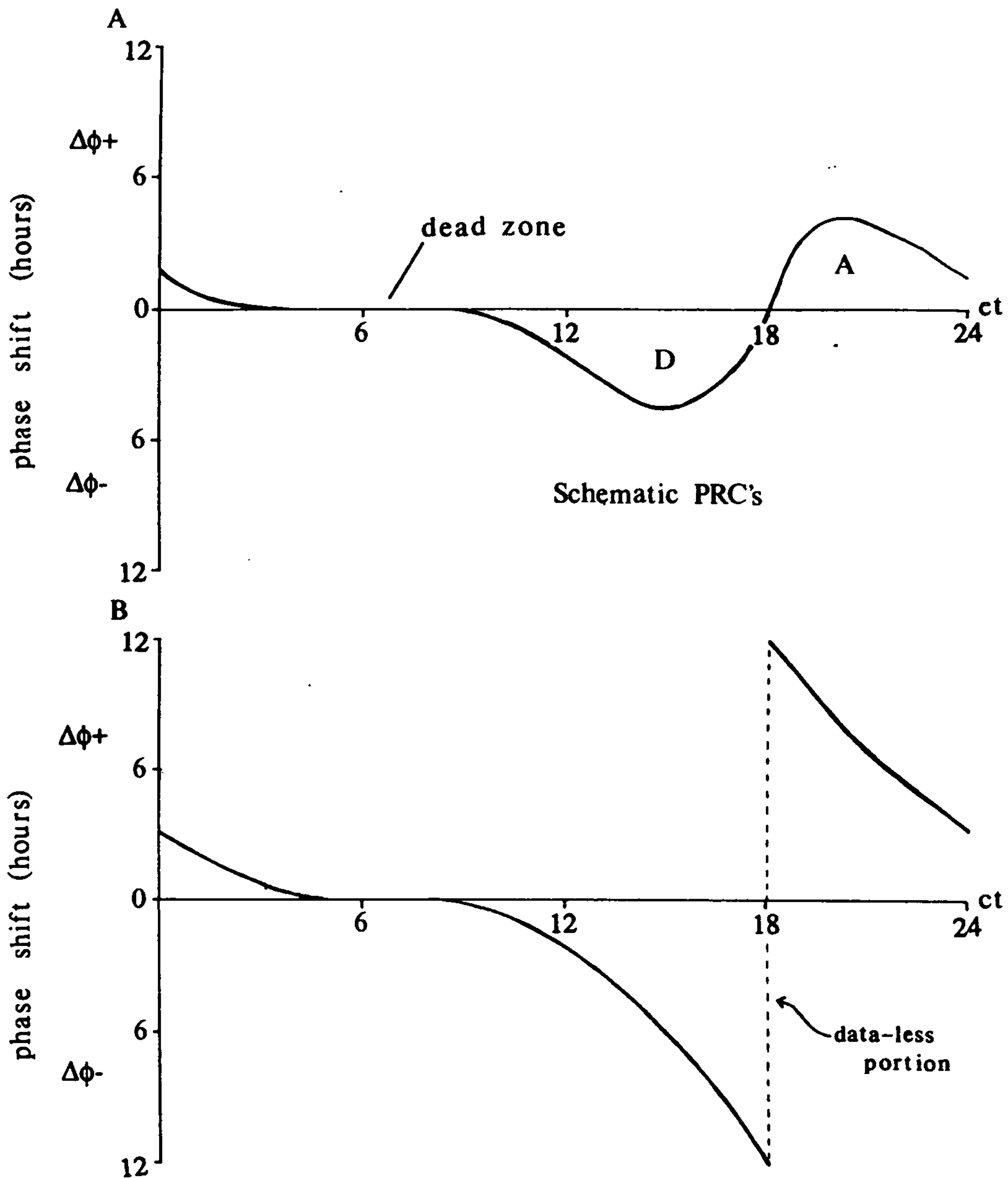


Fig 1.3 Two phase response curves (PRCs) showing the phase advance (Δϕ+) or phase delay (Δϕ-) induced in a free-running rhythm when a disturbing light pulse falls at a given circadian time (ct) of the rhythm for A) a Type 1 response and B) a Type 0 response. The Type 1 response has small maximum phase advances and delays whilst the Type 0 response has much larger phase shifts, with maximum advances and delays of 12 hours. The dotted line between the maximum phase delay and phase advance in B is a data-less portion of the curve drawn to assist in comparisons.

the Type 1 PRC. Because the maximum phase advance and delay sections are $\tau/2$ hours the PRC at the point of maximum phase delay and phase advance is actually continuous but drawn with a sharply defined data-less portion connecting the two for ease of comparison with Type 1 PRCs. The phases at which phase advances and delays occur, however, are similar: advances in the late night/early day and delays in the late day/early night. The general shapes of PRC are conserved across many taxa, but there are also consistent differences in the shapes of Type 1 PRCs between species. Fig 1.3A shows the PRC divided into a region of phase delays (D) and a region of phase advances (A). It would appear that the ratio of the region of delays to advances is dependent upon the period of the free-running rhythm. This trend is found both within and between species: the longer tau is,the lower is the ratio of D/A (Daan & Pittendrigh, 1976) or vice versa.

A PRC can be used to predict the shift in phase which a solitary light pulse of a given duration,administered at a set phase in the free-running rhythm,will produce. PRCs are also capable of explaining how a rhythm can entrain to a series of light pulses,and will be used to explain the phase relationship between entraining agent (light) and rhythm as well as phase jumps when individuals are exposed to skeleton photoperiods. For a rhythm to entrain to a daily Zeitgeber (the 24 hour light-dark cycle) with a photophase of, for example, 6 hours, then the successive light pulses have to ensure that the free-running rhythm is either shortened or lengthened so that it is equal to 24 hours. This change in tau is accomplished by a daily phase shift in the rhythm of 24 minus tau hours. In steady state, therefore, the light pulse needs to fall in each cycle at a point in the rhythm which will produce this required phase shift, and the PRC can be used to predict the phase the light pulse will need to illuminate. This also predicts the phase relationship between the light and the rhythm. If the initial light pulse falls at some phases or if the light pulse is sufficiently strong (Type 0 PRC) then entrainment may be almost immediate. However, for a Type 1 PRC steady state

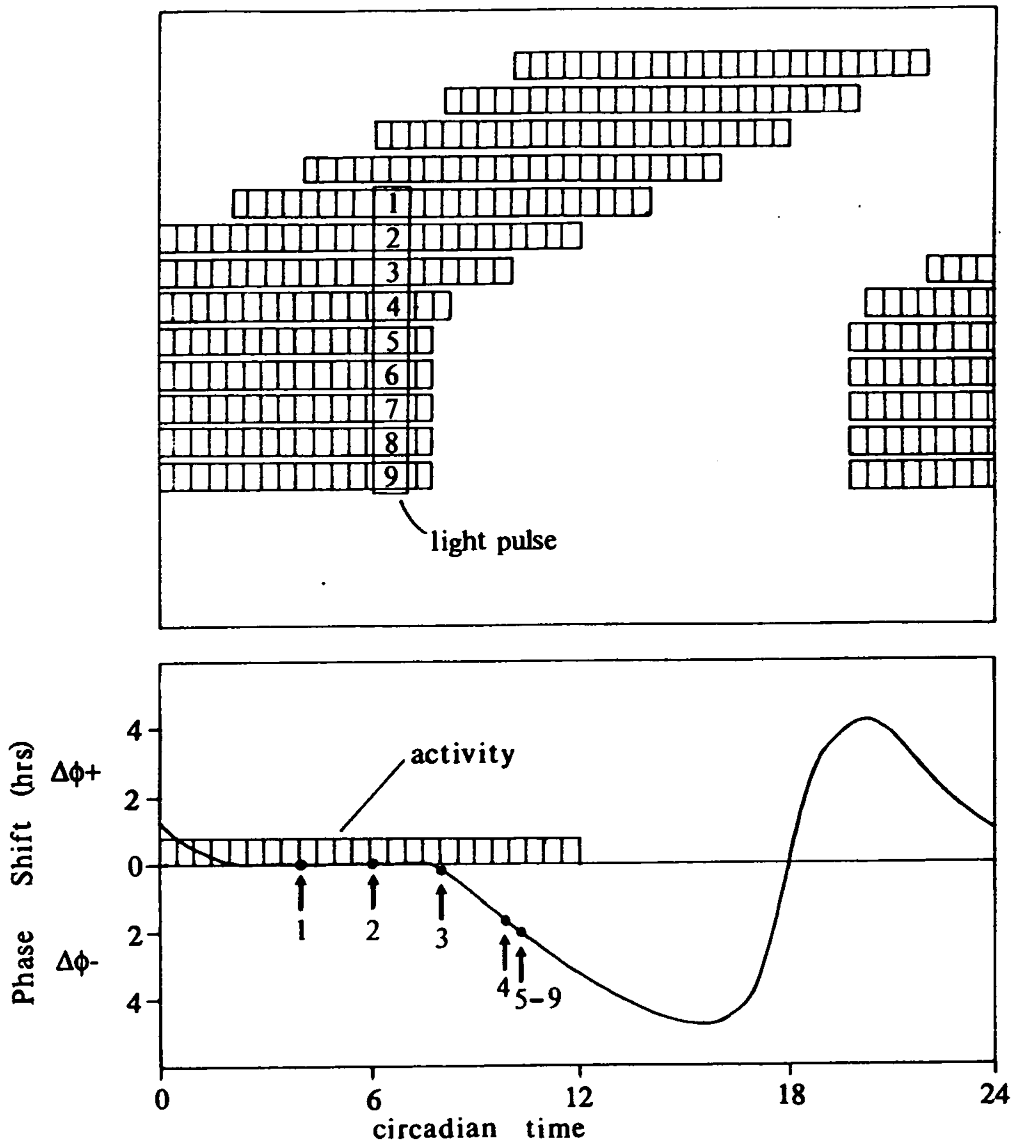


Fig 1.4 A diagram showing the entrainment of a diurnal animal to a daily one hour light pulse. The free-running period of the un-entrained rhythm is 22 hours, shorter than the 24 hour entraining cycle. The lower panel shows the schematic PRC associated with this rhythm and demonstrates the approach to steady state entrainment through daily phase shifts of the rhythm when the light pulse falls at the time shown by the arrows on days 1 to 9 until the 22 hour rhythm is delayed every cycle by two hours due to the light pulse illuminating this portion of the rhythms PRC. The upper panel also shows the phase relationship assumed by the activity (hatched horizontal box) to the light (open vertical box) under entrainment.

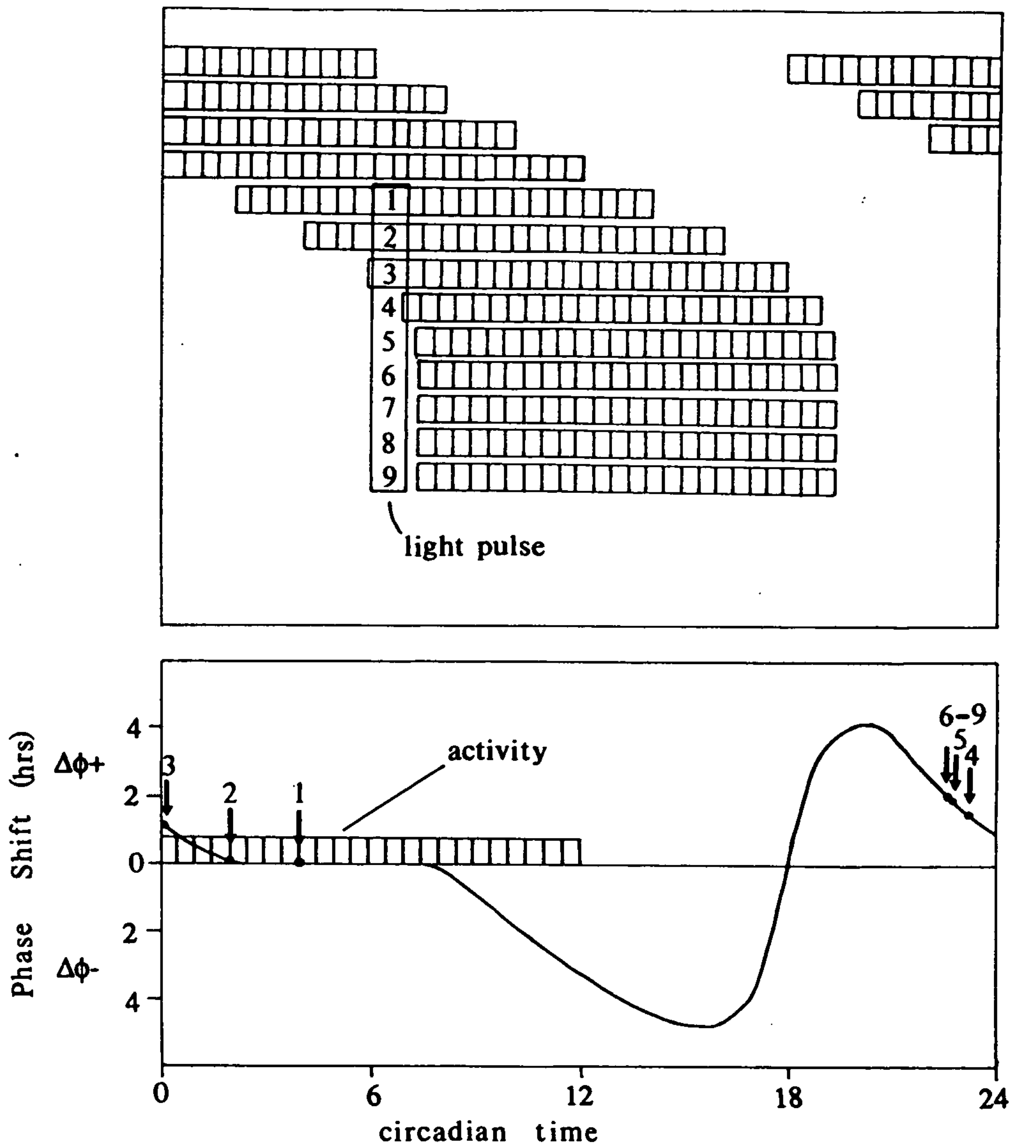


Fig 1.5 A diagram showing the entrainment of a diurnal animal to a daily one hour light pulse. The free-running period of the un-entrained rhythm is 26 hours, longer than the 24 hour entraining cycle. The lower panel shows the schematic PRC associated with this rhythm and demonstrates the approach to steady state entrainment through daily phase shifts of the rhythm when the light pulse falls at the time shown by the arrows on days 1 to 9 until the 26 hour rhythm is advanced every cycle by two hours due to the light pulse illuminating this portion of the rhythms PRC. The upper panel also shows the phase relationship assumed by the activity (hatched horizontal box) to the light (open vertical box) under entrainment.

entrainment can only be reached through a series of transient cycles, shown in Fig 1.4 for a hypothetical activity rhythm. The rhythm is phase-shifted each cycle by the light which causes the phase at which the light falls in the next cycle to change; the light appears to track back along the PRC until steady state entrainment is reached. Entrainment cannot occur through light pulses falling in the rapidly changing part of the PRC between the maximum phase delay and maximum phase advance because small changes in the phase illuminated by light produce large phase shifts, either advances or delays, in the rhythm. This will not result in stable entrainment at these phases because daily changes in tau, and hence in the PRC, will result in small daily changes in the phase of the rhythm illuminated by light which may produce large phase shifts in the rhythm. These phase shifts, furthermore, may as easily be phase advances as delays, and the rapidly changing nature of the PRC in this section does not result in stable entrainment. The PRC also explains phase jumps seen when diurnal animals are entrained to skeleton photoperiods with skeleton photophases greater than about 11 hours. When entrained to skeleton photoperiods the total phase shift required is produced by two short light pulses. The first, 'subjective dawn', results in a phase advance, the second, 'subjective dusk', results in a phase delay. The total phase shift (advance + delay) is equal to 24 minus tau hours. Steady state entrainment can be achieved for skeleton photophases up to 11 hours in duration, with the dawn signal producing a phase advance and the dusk a phase delay. As the skeleton photophase becomes greater than 11 hours a phase advance due to the subjective dawn signal has to be followed by another phase advance due to the subjective dusk signal falling in the advance section of the PRC. The next light pulse, which was the dawn signal, now lands in the delay part of the PRC and is taken to be a dusk signal. Steady state entrainment follows but the dawn light pulse is now taken to be the dusk pulse and vice versa; in other words a phase-jump has occurred

In diurnal animals skeleton photoperiods replicate complete photoperiods up to a point, showing that circadian rhythms are entrained in a discrete fashion. However, continuous entrainment is also involved in preventing phase jumps occurring in diurnal animals entrained to long photoperiods. This continuous entrainment is partly due to the tendency of long photoperiods to result in Type 0 PRCs in which light pulses reset the rhythm to a fixed phase irrespective of the phase at which the light pulse falls. The size and direction of a phase shift is still determined by the phase at which the light falls, but the final phase of the rhythm after a pulse is fixed at a set phase, about ct12 (See Fig 1.3B). This is not the case, however, in some nocturnal animals which are naturally entrained by skeleton photoperiods rising at dusk and returning to their shelters around dawn. The duration of the skeleton photophase experienced by a nocturnal animal living away from the equator changes through the year, long subjective nights in winter, short ones in summer. Although some animals have been shown to demonstrate a seasonal phase jump, at certain times of the year being diurnal and at others nocturnal, it has been shown in rodents that seasonal phase jumps are avoided, in part, because they have PRCs with depressed advance sections, large delay sections and circadian periods of less than 24 hours. Together, these properties ensure that entrainment can be reached through phase delays alone (Daan & Pittendrigh, 1976).

Photoperiodism

Away from the equator the seasonal changes in daylength mentioned above assume a mathematical accuracy. The duration of the day (or night) can be used to accurately predict the time of year at any given latitude (except the equator or poles). Many developmental processes and behaviours need to occur at certain times of the year when seasonal changes in environmental conditions are most favourable (for example, giving birth). But, because these processes may take many months to complete they often need to begin some time before the 'favourable conditions' required in the environment can be detected. Responses like these, geared to suitable environmental conditions but which take such a long time to develop, often use daylength as a cue to begin developing in a seasonally appropriate fashion. This is photoperiodism.

The relative advantages of a photoperiodic response can be seen by comparing quiescence or developmental arrest with diapause as overwintering strategies in insects. Quiescence is a direct response to low temperatures or desiccation in which physiological reactions cannot occur and development is stopped until favourable conditions return. This can produce fitful development in mild winters with a few warm days, and such a direct response to cold relies upon the insect being able to survive the first cold days whenever they arrive. Photoperiodic diapause, however, is a developmental process that begins in response to the shortening days of autumn and which is usually complete before the first cold days of winter arrive. The process often incorporates physiological adaptions which increase the insect's chance of surviving very cold winters, for example, the production of cryoproteins protecting against sub-zero temperatures. As one looks further north (in the northern hemisphere) and winters become harsher and more predictable, diapause tends to be a more prevalent method of overwintering.

Diapause induction is by far the best studied photoperiodic response in insects, recorded in over 200 species from about 12 orders (Saunders, 1982). However, photoperiod has also been shown to control diapause termination (Baker, 1935), the appearance of seasonal morphs (Marcovitch, 1923), growth rates (Saunders, 1972), migration (Dingle, 1974), colouration (MacLeod, 1967), sexual behaviour (Perez, Verdier & Pener, 1971), sex ratio (Hoelscher & Vinson, 1971), fecundity (Atwal, 1955), insecticide sensitivity (Fernandez & Randolph, 1966), and recovery from heat stress (Pittendrigh, 1961). In all these cases the changing daylength stimulates the physiological response ensuring the correct timing of seasonally appropriate responses. In 1936 Bunning first suggested that the circadian system provides the 'clockwork' behind daylength measurement. Since then, the circadian basis of photoperiodism has been suggested by evidence from a large number of organisms (Saunders, 1982).

Photoperiodic responses to changing day/nightlength are most often experimentally replicated using a succession of stationary photoperiods. Separate groups of the test organism are exposed to different light-dark cycles with a period of 24 hours for a part or all of their 'sensitive period' (the phase of the life cycle when photoperiodic induction can take place). The response to each light-dark cycle is measured, and when this is plotted against the light-dark cycle a photoperiodic response curve (PPRC) is produced (Fig 1.6). This figure shows that at some photoperiods a large proportion of individuals enter diapause, whilst at other photoperiods a small proportion do so. The point at which 50% of the individuals switch from one developmental pathway to the other is called the critical daylength (CDL) [or critical nightlength (CNL)]. The steepness of the curve around this critical point is a function of the similarity of the photoperiodic responses of individuals within the population and may reflect the genetic variability of the response. Fig 1.6

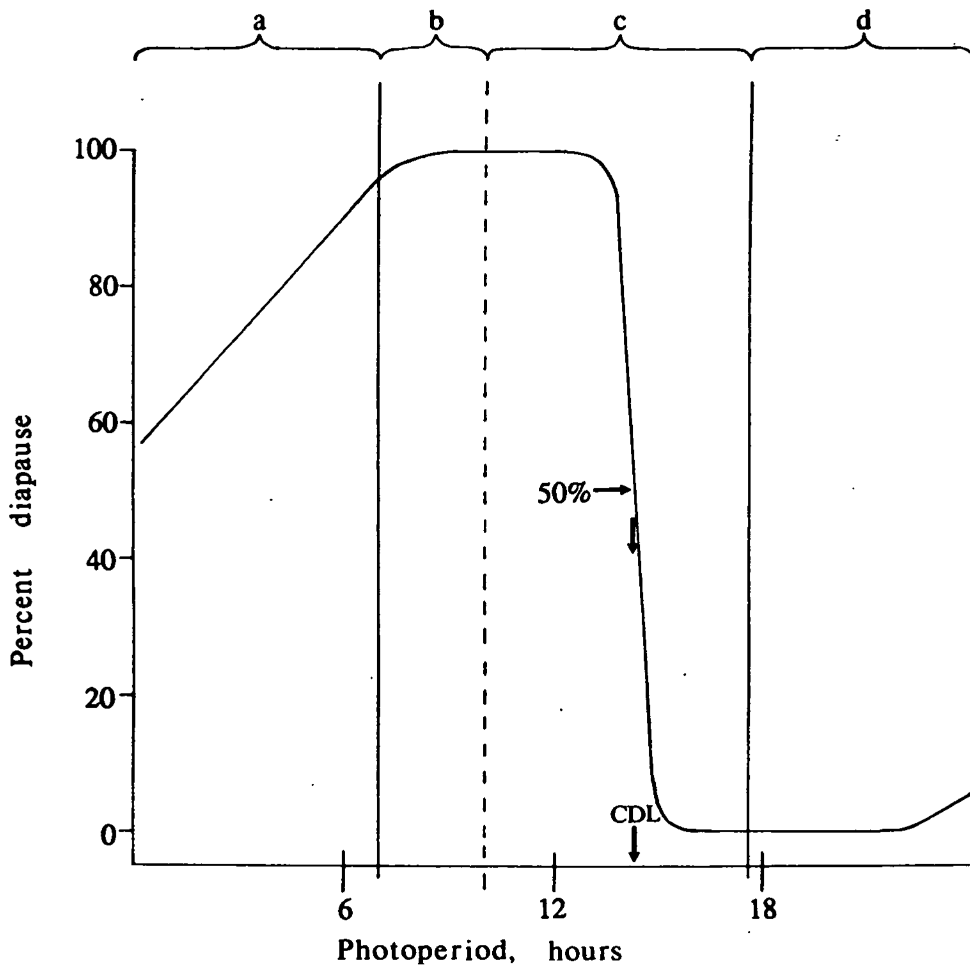


Fig 1.6 A schematic long-day photoperiodic response curve (PPRC) showing its properties. The solid vertical lines indicate the range of natural photoperiods at 55°N. Regions a and d are therefore never experienced in nature at this latitude. Region b only occurs during winter when the temperature is probably below the minimum for development (and the insect is in diapause). Only region c is of ecological importance and this region is dominated by the critical daylength (CDL) which operates the seasonal switch in metabolism (From Saunders, 1982).

shows the PPRC for a long-day insect, in which long days of spring and summer result in individuals which grow, develop and reproduce and it is not until the short days of autumn and winter arrive that diapause is induced. However, at ultra-short daylengths there is a drop in the response and at ultra-long days an increase in the response may occur. Both of these reflect processes within the photoperiodic system which occur outwith the normal photoperiods experienced by the organism, but which still need to be explained in any comprehensive description of photoperiodism.

PPRCs do not demonstrate a circadian basis to photoperiodic responses, and to support Bunning's hypothesis, experiments involving 'extended' or interupted nights need to be undertaken. The most widespread of these experiments is a 'resonance' or 'T-experiment', in which groups of individuals are exposed to photoperiods containing an inducing short daylength followed by nightlengths which range from 8 up to 74 hours or more. In this way the photoperiodic response of individuals exposed to the same 'daylength' (for example, 10 hours) but different 'nightlengths', and so different photoperiods, is measured. Evidence for the circadian measurement of nightlength is found when peaks of diapause induction are found in photoperiods of around 24 hours and modulo 24 hours (actually modulo τ of the photoperiodic system which is close to 24 hours). Troughs in the photoperiodic response are found in photoperiods of around 36, 60 and 84 hours (modulo τ + half τ). Some of the strongest evidence however comes from experiments in which the 'nights' of extended light-dark cycles are systematically probed by light pulses. When time measurement involves the circadian system the results of such an experiment show periodic maxima of long-day or short-day effect which occur with a circadian periodicity. Such experiments have been performed with a number of insect species and clear evidence for a circadian control mechanism in photoperiodic diapause induction has been found in *Nasonia vitripennis* (Saunders, 1970), *Sarcophaga argyrostoma*, its flesh-fly host (Saunders, 1976), the mosquito *Aedes atropalpus* (Beach & Craig, 1977) and *Pieris brassicae* (Claret *et al*,

1981). However in other insects the evidence is controversial and in the green vetch aphid *Megoura viciae* photoperiodic time measurement appears to involve an hourglass/interval timer, not a circadian component (Lees, 1973).

Several 'different' models for photoperiodic time measurement have been proposed (see Saunders, 1982 for review of these) which can be used to explain experimental data. These different models may reflect a large amount of convergent evolution producing several different mechanisms for completing the same function (nightlength measurement) or may be less useful. The several different models can be simply classified providing a key to the photoperiodic modelling which follows;

Q1 Is the circadian system involved in the measurement?

NO -> Q2

YES -> Q3

Q2 Is the photoperiodic system based upon one, or more than one component?

ONE -> HOURGLASS MODEL

MORE THAN ONE -> RESONANCE MODEL

Q3 Is the photoperiodic system based upon one, or more than one oscillator?

ONE -> EXTERNAL COINCIDENCE

MORE THAN ONE -> INTERNAL COINCIDENCE

Such a key will be helpful if the questions asked are valid. Any key can only classify things in an interesting and meaningful manner if the questions asked are themselves meaningful. It would seem pertinent, then, to spend some time discussing these questions and the merits of the models and how they relate to each other.

Evidence for a circadian involvement in diapause has been found in some species, not in others, and in some insects the evidence is controversial. When comparing *Megoura viciae* with *Sarcophaga argyrostoma* the division between hourglass and oscillator control appears straight forward. However, if resonance experiments are undertaken using 12 hour light pulses at 16°C, then the photoperiodic response in *S.argyrostoma* becomes apparently controlled by an hourglass (Saunders, 1973). Furthermore, it is not only temperature which can cause a switch from one method of nightlength measurement to another. In *Pieris brassicae*, the large cabbage

white butterfly, photoperiodic time measurement appears to switch from an oscillatory control system to an hourglass when larvae are reared on an artificial diet (Dumortier & Brunnarius, 1989). Is it feasible that insects have evolved two systems for time measurement, and they use each one in different environmental conditions, or, as in *P.brassicae*, one is redundant until larvae are reared on artificial medium in the laboratory? A more parsimonious solution to this hourglass/oscillator dichotomy might be that both systems are different expressions of the same underlying time measuring system which the environmental conditions impinge upon. The clearly classifiable results from *M.viciae* and *S.argyrostoma* or *Nasonia vitripennis* may be extremes of a spectrum, where damping oscillators measure nightlength as suggested by Saunders & Lewis (1987a). Using computer modelling they argue that the division between hourglass and oscillator is unreal and unnecessary when one can think of an hourglass as an oscillator which damps out after one cycle. Individuals use oscillators to measure nightlength, but the amount of damping upon the oscillator is a function of the physiology of the species and its environment.

The resonance model of photoperiodic time measurement was devised by Pittendrigh (1972, 1974) basing his argument upon the multioscillator nature of the circadian system. In this model the circadian system is involved in photoperiodism, but not necessarily in time measurement itself. Whatever system measures nightlength, the success with which this is achieved is a function of the proximity of the circadian system to resonance with the environmental light-dark cycle. This model draws support from observations that organisms 'do better' or 'differently' when driven by light-dark cycles which have periods close to the natural circadian period, tau, or modulo tau. One possible prediction from this model would be that within a population expressing a photoperiodic response in natural light-dark cycles, those individuals with endogenous periods closer to 24 hours ought to have a more successful photoperiodic response. Therefore, as photoperiodism is a major tool used

to avoid unfavourable conditions there ought to be selection upon a population forcing the mean tau to approach 24 hours, as those individuals with taus close to 24 hours are selected in favour of individuals with circadian periods further from the period of the environmental light-dark cycle. The period mutants, discussed later, of *D.melanogaster* would appear to provide a means of testing the resonance model. Short, or long, period mutants with circadian periods of about 19, or 29, hours ought to exhibit less successful photoperiodic responses than wild-type flies with taus of about 24 hours. As will be seen later, this is not the case.

A second means of dividing photoperiodic response mechanisms is by deciding whether they are based upon one, or more than one component. This specifically can be reduced to the debate between external coincidence (Pittendrigh & Minis, 1964) or internal coincidence (Pittendrigh, 1972). External coincidence relies upon a minimum of one circadian oscillator which is entrained by the light-dark cycle and also contains some photoinducible phase which, depending upon whether it is illuminated or not, determines whether the insect enters diapause or develops without arrest. Internal coincidence relies upon the multioscillator nature of the circadian system. The success of photoperiodic induction is due to whether two or more oscillations have suitable phases overlapping. In short days, for example, two oscillations entrained to the light-dark cycle may have phase points out of phase so that an inhibitory reaction occurs and diapause supervenes, However, in long days entrainment of the oscillators results in the phase points being close enough in time for the opposite reaction to occur and non-diapause will follow. This difference in models need not exist. Multicellular organisms will almost certainly have more than one circadian oscillation entrained to the external light-dark cycle, either directly or through some hierarchical arrangement of oscillators, and the internal coincidence model seems intuitively more appropriate for multicellular organisms. However, internal and external coincidence models are very similar, and external coincidence is

certainly the simpler to use for predicting results or modelling. Both are, as models, intended to explain the experimental data in as simple a way as possible, and both are very successful. However, the external coincidence model is simpler and the internal coincidence model does not offer any more accurate predictions. The external coincidence model whilst not ideal does appear to have an advantage, in terms of simplicity and ease of use which is lost to some extent in the internal coincidence model.

One further model which has not been mentioned is the Dual System Theory (DST) originally described by Beck (1974a,b; 1980) to explain diapause in *Ostrinia nubilalis*. Vaz Nunes & Veerman (1979a,b; 1982) developed a DST model which they ultimately found inadequate for *Tetranychus urticae*. This model is also very complicated and offers no greater depth of understanding of photoperiodism than the external coincidence model. It would appear then that Bunning's original hypothesis (1936) that the circadian system provides the clockwork behind the photoperiodic mechanism is correct, but, bearing in mind that the nature of the circadian oscillation/s is/are unknown, does this help? All we can say is that an oscillatory system which is as yet unexplained can somehow measure nightlength, amongst its other many functions, and this is used to control the development of seasonally appropriate behaviours and physiologies. Clearly there is a lot of work left to be done.

Circadian rhythms in Drosophilidae

The formal properties of circadian systems have been elucidated mostly by work on *Drosophila pseudoobscura*. The rhythm most investigated has been the eclosion rhythm, where individuals emerge from their puparia in daily 'gates'. Where comparisons have been made between this species and other Drosophilidae the results are contiguous and this comparative similarity within the family is believed to occur across the whole circadian system. However, although a lot of work on the eclosion rhythm of *D.pseudoobscura* has been documented, comparatively little work has been done on the locomotor activity rhythm. This is most probably because locomotor activity rhythms have traditionally been recorded in large insects, like cockroaches or crickets.

Work on drosophilid locomotor activity rhythms suggests that in general these flies are crepuscular in habit (Dyson-Hudson, 1956; Lewis & Taylor, 1965; Hardeland & Stange, 1973), and in artificial light-dark cycles in laboratory conditions Drosophilidae appear to have bimodal locomotor activity rhythms (Hardeland & Stange, 1971; Engelmann & Mack, 1978). In some cases the activity rhythm appears unimodal (Roberts, 1956) but as Engelmann & Mack (1978) found, when 'bimodal' activity in *D.pseudoobscura* is allowed to enter continuous darkness and 'free-run' the 'dusk' peak continues with a period of between 21.3 and 23.9 hours. The 'dawn' peak however, disappears apparently being an exogenous effect of the lights going on.

By far the most work on the circadian control of locomotor activity has involved *D.melanogaster*, specifically in connection with the *period* locus. Originally isolated in 1971 by Konopka & Benzer, mutant strains with a short period, long period and an essentially arrhythmic strain have since been utilized extensively. A second long period mutant and another arrhythmic strain have been isolated (see Konopka,

1987). These mutants were isolated by massive 'brute force' eclosion monitoring where a large population of flies were exposed to a chemical mutagen and the eclosion rhythm of their offspring recorded. Any adults which emerged at 'unusual' times outwith the normal eclosion gate of the population were isolated and bred from, tested and the mutant strains characterized. Whilst the details of the *period* locus and its effects upon the circadian expression of locomotor activity rhythms are given later, experimental evidence indicates that the *period* gene is involved, somehow, in the production and/or maintenance of the flies central pacemaker.

Populations of *Drosophila* adults express an eclosion rhythm which is probably the best known circadian phenomenon. Whilst the emergence of adults occurs around dawn,the exact phase relationship between the light/dark cycle and the peak in eclosion depends upon the photoperiod (Pittendrigh, 1965). In continuous darkness this rhythm free-runs with a period very close to 24 hours and is temperature compensated with a Q_{10} of 1.02 (Zimmerman *et al*, 1968). The properties of this eclosion rhythm are obviously selectively advantageous for *D.pseudoobscura* and any other flies which have a subterranean pupal stage.

Although the eclosion rhythm free-runs in constant temperature and darkness, if flies are raised from eggs in constant darkness an arrhythmic eclosion pattern can be seen. Transfer to light-dark cycles (LD) at any stage of larval or intrapuparial development rescues the eclosion rhythm (Bunning, 1935) as does a short single pulse of light, as little as 1/2000 seconds, or a temperature pulse. Since a 12 hour pulse of high temperature can rescue the arrhythmic eclosion rhythm, irrespective of when it is applied, and because when these pulses are applied to a rhythmic population only weak phase shifts are produced, Zimmerman (1969) concluded that the arrhythmia is 'primary'. This means that flies inherit their circadian system controlling eclosion 'at rest' and require a light or temperature disturbance to start them oscillating. Pupal

eclosion rhythms in *D.pseudoobscura* and *D.melanogaster* can be initiated by light pulses, temperature pulses or transfer from continuous light to continuous darkness at any stage of larval or intrapuparial development (Bunning, 1935; Pittendrigh, 1954; Brett, 1955; Zimmerman & Ives, 1971). Furthermore, the oscillation governing the eclosion of adults can be seen to operate throughout the developmental process from the embryo onwards and is therefore unaffected by the extensive morphological reorganization that metamorphosis entails.

Phase response curves (PRCs) for *Drosophila pseudoobscura* eclosion rhythms to light pulses of 15 minutes (500 lux) (Pittendrigh, 1965), 12 hours, 4 hours and 1/2000 second (electronic flash) were the strong, Type 0, kind (Pittendrigh, 1960). The magnitude of phase shifts, however, has been shown to be a function of signal energy (Engelmann, 1969). PRCs for the eclosion rhythm of *Drosophila melanogaster* to 60 second light pulses have also been completed (Hall & Rosbash, 1987) and appear to be Type 1. The PRC for *D.pseudoobscura* was used by Chandrashekeran (1967) to support the hypothesis that the eclosion rhythm was a gated response produced by a pacemaker entraining to the light-dark cycle and in turn entraining a slave oscillator controlling eclosion. The response to light pulses was shown to be instantaneous, transient cycles being the product of the slave oscillator entraining to the instantaneously phase-shifted pacemaker.

Like other developmental rhythms, the eclosion rhythm in *D.pseudoobscura* (and therefore presumably in other Drosophilidae) damps out to arrhythmicity quite rapidly in continuous light, however when released into darkness again the rhythm always appears to start close to the start of the subjective night, circadian time 12 (Pittendrigh, 1960, 1966).

A second population rhythm observed in *D.melanogaster* is an oviposition rhythm in light-dark cycles. With a light intensity of less than about 60 lux the main oviposition peak occurs shortly after lights off with a bimodal rhythm expressed when light intensity is greater than this (Rensing & Hardeland, 1967; Gruwez *et al*, 1972; David & Fouillet, 1973; Allemand, 1976a, 1976b, 1977). Similar rhythmicity has been seen in other drosophilids (Allemand, 1974). The rhythm in oviposition is parallelled by a similar daily rhythm in light-dark cycles in the development of oocytes and retention of developed eggs within ovarioles (Allemand, 1976b). When flies reared in continuous dark had their ovaries dissected over a 24 hour period no rhythm in vitellogenesis was observed. This can be contrasted with results from similar flies raised in LD 12:12 and then released into continuous darkness. Although the oviposition rhythm was not observed in these conditions, Allemand (1976c) observed a persistent rhythm in vitellogenesis. This resembles observations made on the eclosion rhythm in *D.pseudoobscura* (specifically: its primary arrhythmicity as shown in Zimmerman & Ives, 1971) and suggests that there may be a two oscillator control of vitellogenesis, which then possibly forces, in conjunction with light-dark cycles, a rhythm in oviposition. Furthermore, single female *D.melanogaster* had a clearly rhythmic oviposition rhythm in LD 12:12 which upon transfer to continuous light was observed for one cycle before oviposition became apparently random (Fleugel, 1978).

Clearly there has been considerable work on the eclosion rhythm of *Drosophila pseudoobscura* and where comparisons can be made with other drosophilids the responses seem similar. However, the circadian control of locomotor activity has not been investigated quite so rigorously. Most of the work on locomotor activity rhythms has been done in *D.melanogaster* and the *period* mutants (see later).

Whilst the eclosion rhythm was used to isolate these period mutants, the free-running period of the locomotor activity rhythm is also altered by the mutations. The direct alteration of this rhythm suggests that the *period* gene is somehow involved in the production and maintenance of a biological oscillator controlling both eclosion and locomotor activity. The period of the free-running activity has been documented (Konopka, 1987; Rosbash & Hall, 1989) and a phase response curve for the eclosion rhythm of the wild-type and the short period mutant to 60 second light pulses has been published (Hall & Rosbash, 1987); the response was Type 1 for both the wild-type and short period mutant strains. Furthermore, the PRCs suggested that the alteration in the free-running period in the short period mutant was due wholly to a decrease in the subjective day of these flies.

The free-running period of the locomotor activity rhythm has also been recorded at various temperatures ($15^{o}C$ to $26^{o}C$) in per^{s}, per^{L}, and per^{+} (Konopka *et al.*, 1989). Whilst in the wild-type flies the period of the locomotor activity rhythm (τ) was temperature-compensated with a Q_{10} of approximately 1, in per^{s} the Q_{10} value was less than 1 and in per^{L} it is greater than 1. Aside from this reciprocal temperature dependence in τ, observations of the free-running rhythm also show that the two mutants have other reciprocal behaviours. The average period in constant darkness changes as a function of time: in per^{s} τ lengthens in the second ten days in darkness when compared to the first ten days, whilst in per^{L} τ shortens. Finally, in constant light (intensities less than 0.1 lux) the periods of per^{s} flies have a tendency to shorten, whilst in per^{L} flies there is a tendency for τ to lengthen.

The *period* gene appears to effect a circadian pacemaker in *D.melanogaster*. It was originally hoped that genetic and molecular analysis of the *period* locus and its expression would describe the functioning of this molecular circadian clock. However, this has proved to be no mean task. The *period* locus has been mapped and

cloned, its areas of expression have been localised down to the subcellular level and circadian expression of the *per* mRNA and protein gene product have been shown to oscillate with a circadian period. Clearly, the *period* gene has something to do with the production and maintenance of a circadian clock, but how it does this is still a mystery (for reviews see Rosbash & Hall, 1989; Hall, 1990; Hall & Kyriacou, 1990).

The *period* gene is a non-vital locus, with flies surviving after deletion of the relevant section/s of the X-chromosome (Smith & Konopka, 1982; Bargiello & Young, 1984; Reddy *et al*, 1984). It maps to the 3B1-2 zeste-white region of the distal X-chromosome (Smith & Konopka, 1982). The gene has been sequenced and its structure (in terms of exons and introns) resolved (Jackson *et al*, 1986; Reddy *et al*, 1986; Citri, 1987; Yu *et al*, 1987). When comparisons between *per*'s structure and other genes were made with homology searches, it was not found to produce a protein with a known biochemical function. However, comparison of the amino-acid sequence encoded by the *period* gene has provided one similar *Drosophila* gene. Single-minded (*sim*) (Crews *et al*, 1988) encodes for a vital locus which controls the development of ventral midline neural cells from the ectoderm. However, this similarity is of very little help when discerning the molecular function of *per* as almost nothing is known about the function of *sim* either.

The *period* locus is a 6kb piece of X-chromosomal DNA (Hamblen *et al*, 1986). P-element transformations using DNA fragments of the *period* gene show several subsegments of the locus that can restore rhythmicity to per^{0} and per^{-} flies. These fragments overlap in a region which complements a 4.5kb RNA transcript, which is probably the *per* message (Hamblen *et al*, 1986). Analysis of cDNA indicates that *per* codes for a 1218 amino-acid protein and at least two minor variants (Citri, *et al* 1987). Most of the protein sequence is common among these three proteins, and biologically significant differences between these polypeptides are unknown. The

mutant alleles at the period locus are point mutations: per^{0}, per^{02} and per^{03} turn out to be identical 'null' genetic variants (Yu *et al*, 1987; Baylies,*et al*, 1987; Hamblen-Coyle *et al*, 1989) due to an 'amber' stop codon at amino-acid 464; per^{S} is due to a serine to asparagine missense mutation at amino-acid 589; and per^{L} (and per^{L2}) due to a valine to aspartic acid missense mutation at amino-acid 243 (Baylies *et al*, 1987). What these changes mean in terms of the biochemistry of the *period* gene product are far from clear. The nonsense codon in per^{0} presumably results in a short gene product with no or very little function and is phenotypically comparable with per^{-}, in which the *per* gene has been deleted (Smith & Konopka, 1981; Bargiello and Young, 1984; Reddy *et al*, 1984). It has been postulated that the change in per^{S} means that this gene product cannot be meaningfully phosphorylated (Yu *et al*, 1987). Although this in itself means very little it might lead to hyperactivity in the per^{S} gene product (Hall & Kyriacou, 1990).

The *period* gene, like other X-linked genes in *Drosophila*, appears to be dosage compensated. Males with only one dose of any allele have periods similar to females with two doses. An extra dose of per^{+} in a female shortens the period by about 30 minutes, but an extra dose in males, equivalent to two doses in females, shortens the rhythm by about one hour (Konopka, 1987). The removal of one dose of per^{+} has the opposite effect lengthening tau in a similar fashion. This supports the suggestion that per^{S} represents a hyper-activity in *per* gene product, and per^{L} hypo-activity. However, the shortening effect upon tau of increasing the dosage of per^{+} reaches a maximum when tau has been shortened by about 1.5 hours.

The genetic analysis of the *period* locus points to a possible hyper and hypoactivity associated with per^{S} and per^{L}/per^{L2} resulting in the short and long periods expressed in both the locomotor activity and eclosion rhythms. Mosaic analysis of gynandromorphs (with part male and part female bodies) has located the

head as the most probable site for *period* gene control of a circadian pacemaker. Rescue of circadian rhythmicity in *per*^{0} flies has been achieved by surgical implantation of the brains of *per*^{s} flies into abdomens of the recipients. Some of the resulting flies expressed 19 hour locomotor activity periods suggesting, not only that the *period* gene is expressed sufficiently in the brain to produce a rhythm, but that this rhythm can be transmitted humorally from the brain (Handler & Konopka, 1979). Rhythms have also been rescued in *per*^{0} flies by means of a heat-shock protein-70 promotor-*per* fusion gene (Ewer *et al*, 1988) which causes *per* to be transcribed at certain temperatures and not at others. It was found that the *per* product is only required in the adult during expression of rhythmicity and that *period* is necessary and sufficient for pacemaker function (Ewer *et al*, 1990). The speed with which periodicity can be turned on or off suggests that *per* is involved in the on-going production of circadian rhythmicity as opposed to being involved in the construction of a circadian clock through development.

The localisation of *period* expression resulting in circadian oscillations has also been investigated within the brain. Immunofluorescent assay of *Drosophila* heads reveals a circadian rhythm in wild-type flies in *period* gene messenger RNA (Hardin *et al*, 1990) in both light-dark conditions and constant darkness. Similar cycling in *per*^{s} has been observed but with a shorter endogenous period in constant darkness. Further analysis of the CNS has revealed a circadian oscillation in the *period* gene product which again persists in constant darkness and which has an altered time course in *per*^{s} (Zerr *et al*, 1990). The localisation of the *period* gene product in nervous cells appears to be nuclear (Liu *et al*, 1992) where this localisation used immunoelectron microscopy. In an earlier report the *period* gene product appeared to be located, at least in embryonic salivary glands, at cell membranes around gap junctions (Bargiello *et al*, 1987). This has now been retracted (Saez *et al*, 1992).

Since 1971 the *period* gene has been extensively investigated, sequenced, cloned and its areas of expression located. This has not, however, produced a clear picture of how the *period* gene may be involved in the production of circadian rhythms. There are several hypotheses. Dosage experiments suggest that per^S is somehow hyperactive and per^L (and per^{L2}) hypoactive and this has been used to explain altered circadian rhythms in two ways. Either the *period* mutants somehow alter the circadian clock during its development, which then runs either quickly (per^S) or slowly (per^L/per^{L2}). Or the altered gene changes the way in which a circadian oscillation is maintained. Early work suggested that the former developmental process may be involved with mutant-like rhythms being produced in wild-type flies raised in constant darkness (Dowse & Ringo, 1989). The location of the *period* gene product at cell membranes in embryonic salivary glands and changes in diffusion rates between mutant cells (Bargiello *et al*, 1987), however, suggested an on-going maintenance of the rhythm through cell-cell interactions. *Period* was thought to couple together oscillators, and the strength of this coupling produced different periods in the composite pacemaker (Dowse, Hall & Ringo, 1987). This model was originally formalised with *period* gene product coupling together a group of ultradian pacemakers.

When heat shock promoter proteins were fused with the *period* gene and used to rescue per^0 flies by switching on and off *period* gene expression at different stages of the lifecycle it became clear that *period* is not involved in the developmental construction of a circadian pacemaker (Ewer *et al*, 1990). It appears that the *period* gene product is involved in the continuous maintenance of a circadian oscillator. This does not, however, exclude the possibility that the *period* gene is involved in cell-cell interactions and acts as a coupling agent between pacemakers. A model proposed by Konopka & Orr (1980) was based upon the analysis of the free-running rhythm in per^+ and per^S in which period encodes for a transmembrane protein which gates ion

flow across the membrane (in theory either cellular or nuclear). The subsequent change in ion concentrations is circadian and the *period* gene specifically controls the subjective day part of the circadian cycle, with a second unknown membrane protein controlling the subjective night.

In general it is thought that the expression of the *period* gene has an immediate effect upon the expression of circadian rhythmicity and the hyperactivity of *per*^{S} and hypoactivity of *per*^{L} or *per*^{L2} is one way in which this effect can be produced. The *per*^{S} allele is a gain-of-function mutation resulting in a gene product that is somehow more active than wild-type, *per*^{L} or *per*^{L2} is a loss-of-function mutation resulting in a less active gene product. However, as Rutila *et al*, discuss (1992) there are several problems associated with this. They have produced a number of new short period circadian rhythm mutants by altering amino-acid 589 suggesting that the original *per*^{S} mutant is not rare and therefore probably a more traditional loss-of-function mutation like *per*^{L} or *per*^{L2} is defective in some aspect of its biochemical interactions. They cite the recent observations of circadian fluctuations in cellular levels of *per* mRNA and PER protein (Hardin *et al*, 1990; Zerr *et al*, 1990) and evidence of possible post-transcriptional regulation of *per* protein levels (Zwiebel *et al*, 1991) to support the suggestion that the *period* gene is somehow involved in an autoregulatory loop producing these circadian fluctuations, and *per*^{S} and *per*^{L}/*per*^{L2} are defective in some aspect of this, resulting in their expressed periodicities.

Thus this gene is somehow involved in the production and maintenance of a circadian oscillator and evidence suggests that the *period* mutants are defective in some step in a biochemical system resulting in altered periods of the locomotor activity and eclosion rhythm. Circumstantial evidence that the *period* gene plays a fundamental role in the production of a circadian oscillator in *Drosophila*, and that this system may have common elements across diverse groups comes from the comparison

of the *period* sequence with the *Neurospora* clock gene *frq* which share a common sequence element (McClung *et al*, 1989). Also antibodies to *per* protein have been shown to recognize known circadian pacemaker neurons in the marine molluscs *Aplysia* and *Bulla* (Siwicki *et al*, 1989) and also label antigens in the suprachiasmatic nucleus of the rat, the known location of the circadian pacemaker in mammals (Siwicki *et al*, 1992).

The *period* gene is not the only component of the circadian clock, other genes have been found which effect the free-running and entrained rhythms of locomotor activity and eclosion. An X-chromosomal 'clock' (*clk*) mutation which results in an expressed rhythm about 1.5 hours shorter than the wild-type has been isolated. *Clk* maps quite close to the *period* gene on the X-chromosome (Dushay *et al*, 1990). Another X-chromosomal rhythm mutant, 'Andante' (*And*), causes rhythms of either eclosion or locomotor activity to free-run with a period about 1 to 1.5 hours longer than in the wild-type (Konopka *et al*, 1990). *And* however maps to a classical pigmentation locus called dusky (*dy*). In a classical *dy* mutant normal circadian rhythms have been recorded, but in newly induced *dy*s 25-26 hour rhythms and one new *dy* with a shortened rhythm were seen (Hall, 1990). This suggests that *And-dy* could be either a biochemical defect indirectly affecting rhythms or could perhaps be one of a number of genes directly involved in pacemaker functioning, which also has a pleiotropic effect upon pigmentation.

Systematic artificial selection for 'early' and 'late' eclosing strains of *Drosophila pseudoobscura* has produced two strains which differ in their peak eclosion time by about four hours (Pittendrigh, 1967). This difference was maintained in all photoperiods, but the phase response curves for the two strains were identical implying that selection had altered the phase angle between the primary A-oscillator (pacemaker) and the secondary B-oscillator (slave) controlling eclosion. This

experiment has been repeated once for *D.melanogaster* (Clayton & Pietta, 1972) where it was demonstrated that laboratory maintained strains responded to selection more favourably than recently captured wild-type parental stocks. This phase angle difference is similar to results obtained by genetic mutation by Jackson (1983) who isolated three autosomal mutants.

Photoperiodism in Drosophilidae

Drosophilidae have proved to be very 'popular' experimental animals and a great deal is known about them. However, because they have very little economic importance some areas of their life histories, including their ecology, have not been extensively studied. In temperate and northern zones one of the most fundamental factors of the ecology of any organism is its breeding season, and overwintering strategy. The duration of the breeding season dictates how many generations can be produced in one year, when breeding can start and when it needs to end. The low number of drosophilid species found in northern zones attests to the qualitative problems incurred in adapting to the generally lower temperatures throughout the year, harsher winters and shorter breeding season.

Overwintering appears to be the fundamental way that populations synchronise their breeding season (Lakovaara *et al*, 1972), and in a large number of temperate zone and northern drosophilids the onset of overwintering diapause appears to be regulated by photoperiod. To date, of 18 species of Drosophilidae which have been found to have a photoperiodically controlled diapause, 17 overwinter as reproductively inactive pre-vitellogenic adults, and one, *D.deflexa*, as a larva (Basden, 1954). Of this 17, most attention has been paid to *D.phalerata* (Geyspits & Simonenko, 1970; Tyschenko *et al*, 1972); *D.littoralis* (Lumme *et al*, 1974; Lankinen, 1986); and *D.auraria* (Iwao *et al*, 1980; Pittendrigh & Takamura, 1987). In most of the diapausing drosophilids the length of the breeding season results in a multivoltine life history. However, further north in the subarctic region, univoltine races of *D.bifasciata* and *D.littoralis* have been found and one univoltine species, *D.subarctica* (Hackman, 1969; Lakovaara & Saura, 1970).

In *D.phalerata* the photoperiodic control of diapause has been found to respond strongly to changes in temperature with an increase resulting in a decrease in the critical daylength (CDL) (Geyspits & Simonenko, 1970). The interaction of photoperiod and temperature has been found to be very important in Drosophilidae and clearly in this case the two adaptively alter the time at which breeding stops each year. The CDL determines, for a given geographical location, when a population will stop breeding and overwinter in a developmentally more appropriate form; *i.e.* not wasting valuable resources on vitellogenesis and laying eggs which will not survive the harsh winter. But if the temperature is higher than average, in for example a mild autumn, the resultant shortening of CDL will allow breeding to continue for longer until the photoperiodic system demands diapause.

D.auraria has also been investigated in the laboratory. Various strains representing a geographical cline in CDL have been collected. Temperature effects upon CDL and the photoperiodic response at all photoperiods have been looked at in all the strains (Pittendrigh & Takamura, 1987). The surprising conclusion from this study was that temperature appears to affect CDL by raising and lowering the response to all photoperiods. The assumption that systematic changes in CDL are derived from systematic changes in the circadian system's entrainment behaviour was also unfounded. "Major changes in CNL can be realized by selection without any detectable impact upon the circadian rhythm studied" (the eclosion rhythm) (Pittendrigh & Takamura, 1987).

Lakovaara *et al* (1972) found that *D.littoralis* had both multivoltine and univoltine races, univoltinism being exhibited by more northerly races, presumably as a result of the shorter breeding season in these conditions. At 16°C 8 inbred strains of *D.littoralis* display CDLs which spread from 13.5 hours to 18 hours, whereas at 22°C this response was abolished in all these strains. When a series of crosses between a

univoltine and a multivoltine strain was analysed it was found that multivoltinism appeared to be dominant over univoltinism and the genetic determination of diapause polygenic. Furthermore, a recent extension to this work on *D.littoralis* by Lankinen (1992) has found that despite an initial study finding a partial co-inheritance of diapause and eclosion rhythm characteristics (Lankinen & Lumme, 1984), a more recent analysis of the same crosses after 40-70 generations has found a complete separation of eclosion rhythm characters from photoperiodism.

The best known species in the Drosophilidae is *Drosophila melanogaster*, an animal extensively used with a great deal known about it (Ashburner, 1989). Until recently *D.melanogaster* was thought to be a day-neutral species with no photoperiodic control of diapause. As a human commensal a specific overwintering strategy was believed to be either not necessary or involve simple quiescence in cold weather. However, Saunders *et al* (1989) found that at a temperature of 12°C the Canton-S strain of *D.melanogaster* demonstrated a photoperiodic diapause with a CDL of 14 hours and in which short days (<14 hours) result in pre-vitellogenic ovaries. In vitro studies have shown that the production of juvenile hormone III and B3 by the corpora allata (c.a.) in short day flies is about a quarter of that produced by glands from long day flies (Saunders *et al*, 1990). The reduction in the output of the c.a. results in the inability of the ovaries to extract yolk polypeptides (yps) from the surrounding haemolymph (which contains similar amounts of yps in long day and short day flies) and so remain pre-vitellogenic (Saunders *et al*, 1990).

Obviously, the period mutants in *D.melanogaster* with their altered circadian rhythms, seemed to provide a way to test Bunnings hypothesis that the circadian system provides the clockwork used in daylength or nightlength measurement. Strong *a priori* predictions based upon entrainment theory can be made about the CDLs of the short and long period mutants when compared to the wild-type. The short period

mutant will entrain to light-dark cycles with the pacemaker oscillation 'phase leading' the light (Fig 1.4). Assuming an external coincidence model, Φ_i will occur in the late subjective night shortly before the start of alpha. Entrainment of the rhythm is brought about in longer daylengths by the end of each light pulse being set to a particular circadian phase of the rhythm (usually about Ct12) and so as the duration of the light pulse increases the start of the light pulse tracks back into the late subjective night. Because in the short period mutant the pacemaker phase leads the light, entrainment theory predicts that for Φ_i to be illuminated (and non-diapause development to proceed) then a longer light duration is required than in the wild-type. In the long period mutant, entrainment of the rhythm by the light results in a phase lag (Fig 1.5) and so, by a similar argument, Φ_i is illuminated by light of a shorter duration than the wild-type. The short period mutant ought to have a CDL longer than the wild-type (14 hours) and the long period mutant ought to have a CDL shorter than the wild-type. The arrhythmic mutant, *per*^{0}, and *per*$^-$ (with deleted period locus) ought to have severely disrupted CDLs. These predictions were not found to be the case, *per*$^+$, *per*^{s} and *per*^{L2} had similar CDLs and *per*^{0} and *per*$^-$, whilst having CDLs shorter than *per*$^+$ could still tell the difference between long and short days (Saunders *et al*, 1989; Saunders, 1990). Furthermore, resonance experiments revealed a weak 24 hour periodicity to the photoperiodic response in all mutants (Saunders, 1990) leading to the conclusion that whilst *period* has some involvement in the photoperiodic response, shortening the CDL in *per*^{0} and *per*$^-$, the period gene is not causally involved in photoperiodic time measurement.

This conclusion is supported by work on *D.auraria* (Pittendrigh & Takamura, 1987) where prediction from two major and widely held assumptions were not experimentally supported. The assumptions made were that changes in CDL will be the result of changes in the entrainment behaviour of the circadian system,and that the eclosion rhythm can be used as a marker of all other components in the multioscillator

circadian system. The first assumption here is also made in the experiments with *D.melanogaster* in *a priori* prediction of changes in CDL due to alterations in the circadian system. However the second assumption that the eclosion rhythm is a suitable marker is replaced by the assumption that the locomotor activity rhythm (and implicitly the eclosion rhythm) is a suitable marker. The results from the resonance experiments (Saunders, 1990), which indicate a 24 hour periodicity in the photoperiodic response, imply that this second assumption may not necessarily be correct. Pittendrigh and Takamura (1987) consider this to be of minor importance. The recent results of Lankinen (1992) which clearly show the eclosion rhythm and photoperiodism in *D.littoralis* to derive from separate genetic systems suggests that the assumption that the eclosion rhythm or the locomotor activity rhythm can be used as accurate markers of the phase of the photoperiodic oscillation is erroneous. Modelling of the circadian system and its involvement in the photoperiodic response in *D.melanogaster* and its mutants (see chapter 5) has replicated experimental results by assuming two oscillators which interact to a small degree through a one way coupling from a pacemaker which involves the period gene to a second pacemaker or slave which does not. This second pacemaker can entrain to the external light-dark cycle but is also influenced by the entrainment behaviour of the period oscillator and is responsible for the measurement of nightlength for the photoperiodic response (Gillanders & Saunders, 1992).

Despite the possibilities that the *period* mutants and the discovery of an ovarian diapause in *D.melanogaster* provide for genetic dissection of the photoperiodic response, there are several problems. The exact role that *per* plays in the maintenance of a circadian pacemaker is not yet known and the photoperiodic response in *D.melanogaster* is very shallow. It appears that the photoperiodic response in this species is 'evolutionarily' quite novel and the alleles controlling the

diapause response are not yet present at all possible loci involved in all members of the population.

Chapter 2

General Materials and Methods

Stock populations, consisting of 30 or 40 flies, of strains of *Drosophila melanogaster*, Canton-S (wild-type), per^{s}, per^{L2}, per^{0} and per^{-} were kept at 25°C (+/-1°C) in a light-dark cycle of 12 hours light to 12 hours dark (LD 12:12). All stock populations were kept in labelled glass shell vials (75mm depth, 25mm diameter) stoppered with cotton wool and containing 7ml of standard Lewis agar-cornmeal medium. The flies were transferred to fresh vials twice a week and the old vials were kept. The eggs laid in these vials developed under the same environmental conditions and the adults which emerged were used either to replace the stock populations every five weeks or used as expanded egg laying adult populations providing experimental flies.

The wild-type population has been maintained in laboratories since its collection in Canton, Ohio over 70 years ago. Three of the mutant strains, per^{s}, per^{0}, and per^{L2}, were isolated by Konopka and Benzer (1971) and Konopka (1978) and have been maintained in laboratories since then. The mutant strains were isolated from the Canton-S population maintained here as a wild-type stock (Saunders, 1990). The per^{-} is a balanced stock carrying two overlapping deletions of *per* (provided by Dr M Young). The breeding stock of this strain consists of *y Df(1)TEM202(w⁻) / y Df(1)64j4 w*^{a} females with *y Df(1)TEM202(w⁻) / w*$^{+}$*Y* and *y Df(1)64j4w*^{a} / *w*$^{+}$*Y* males. Females are yellow bodied with apricot eyes, and carry one of each deletion (both of which are recessive lethals) and have therefore had the *period* locus completely removed (Saunders, 1990). The males are red-eyed, behaviourally rhythmic and per^{+}. About one to two percent of the females of this

stock are red-eyed and behaviourally rhythmic, and most probably are 'exceptional' females of the genotype *y Df(1)TEM202(w) / y Df(1)64j4w^a / w^+ Y* produced from the *per^-* stock by non-disjunction. Red-eyed females were not included in the stock populations or in the experiments. The period deletion in these *per^-* stock has been confirmed by Southern blotting (Maniatis *et al.*, 1982) using a 7.1kb *Hind* III fragment containing the *period* gene (Bargiello & Young, 1984) as a probe. The *per^-* females lacked the DNA corresponding to the *period* locus, while their male siblings and 'exceptional' females showed the same hybridization pattern as *per^S* and Canton-S wild-type (Saunders, 1990).

Whilst the stock populations could provide enough adult flies for locomotor activity experiments they could not provide enough material for experiments where the photoperiodic response was measured, so the stock populations had to be expanded to provide experimental populations of laying adults producing an almost daily supply of freshly eclosed adults. To expand a stock population, between 50 and 100 flies collected from a stock population were placed into each of 3 or 4 1/3 pint bottles containing approximately 250ml of Sugar-Yeast medium. The bottles were stoppered with cotton wool bungs and kept at 25°C (+/-1°C), LD 12:12. The laying adults in these bottles were transferred to fresh S-Y bottles twice a week. The old bottles containing the developing flies were kept at 25°C and the emerging experimental adults collected daily. Every few weeks the adult laying populations in the S-Y bottles were replenished with the appropriate adults from the stock populations.

Since only small numbers of adults were required for locomotor activity experiments it was possible to collect these females directly from the stock populations. Fat, healthy looking females, which were under about 4 days old (age being in terms of days since eclosion) were collected under CO_2 anaesthesia and

placed in the locomotor activity recorders. However, much larger numbers of females were required on a daily basis for the photoperiodism experiments and, furthermore, these females needed to be less than about 10 hours old when they entered the experimental conditions (a decrease in temperature to around 12°C, Saunders *et al*, 1989). These large numbers were collected from S-Y bottles using a double collection procedure. Pupae on the inside of the bottles were checked daily to see if any had turned black. This change in colour occurred when the developing adults became pharate, and indicated that emergence of the adults would begin the following day. On the following day those bottles containing adults which were ready to emerge were cleared of any adults, of an unknown age, at 'dawn'; these adults were discarded. The bottles were then left for 6 to 7 hours. After this time the bottles were again emptied by carefully removing the cotton wool bung and quickly turning the bottle upside-down placing the mouth of the bottle into a plastic funnel which led into a glass shell vial containing 7ml of standard Lewis medium. The flies in the bottle were then dislodged by gently but firmly banging the mouth of the bottle against the funnel. The adults collected in this way had all emerged since the bottles were cleared, 6 or 7 hours previously, and were therefore less than 10 hours old.

Recording the locomotor activity rhythm

In order to record the locomotor activity rhythm of female *D.melanogaster*, flies were collected from the stock populations. Because the females needed to be as large as possible so that the recording device could detect them more easily, they were left for two or three days in labelled glass shell vials containing standard Lewis medium. In this time the females built up large fat stores which combined with the start of vitellogenesis meant their abdomens were bloated. However, even though egg production had started, the restriction of suitable egg laying sites within the recording device meant that throughout this investigation there were no larvae, which could interfere with the recording of the adult locomotor activity rhythm, found in the recording devices. The recorders consisted of a black perspex 'holder' housing an infra-red light emitting diode (Radio Spares [RS] 306-077) and a matched detector (RS 306-083) arranged so that the beam passed vertically through a cylindrical hole which held a glass tube (50-70mm length, 5mm outer diameter) horizontally, in which was placed the experimental female (see Fig. 2.1). Food was provided for the flies in one of two ways. In the first, sugar crystals were placed at one end of the tube as a food source and a water source, a wick leading from a water reservoir in a bijou bottle, was at the other end. The second method involved supplying a sugar solution by means of the wick which therefore provided water and sugar simultaneously.

Clean,empty tubes were prepared several days before an experiment was due to begin. Of the two means of feeding the flies, the first was more difficult to set up. In addition it did not greatly improve locomotor activity records by inducing the flies to walk from one end of the tube (the food source) to the other end (the water source), when compared to flies which had both the sugar and water sources combined at one end. The first arrangement used a pipette to place a quantity of hot, saturated sugar solution at one end of each of the tubes. This sugar solution cooled and

EX BIBL. UNIV. EDINBURGH

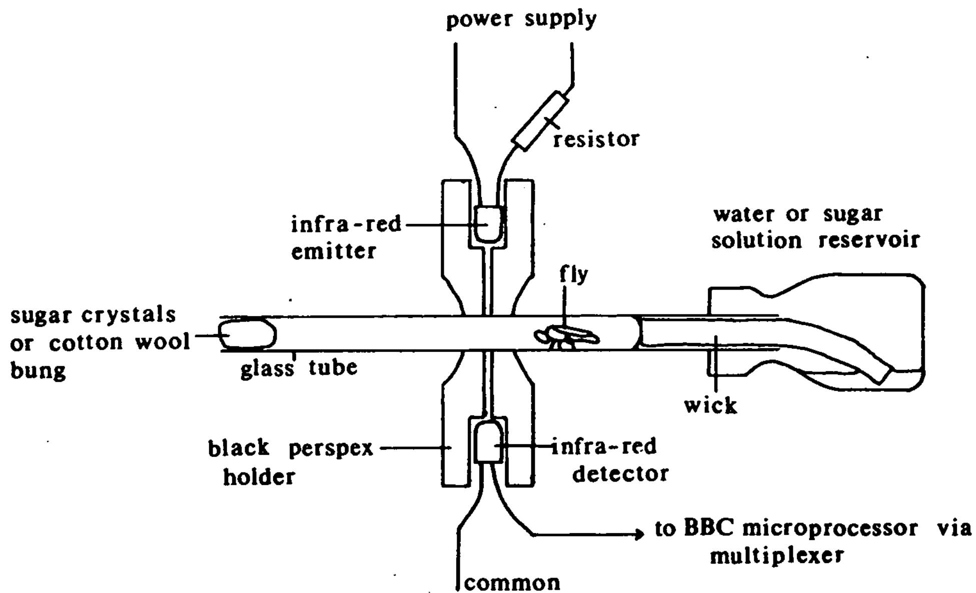


Fig 2.1 Cross sectional diagram of the apparatus used to record the locomotor activity of *Drosophila melanogaster*. Each of 32 devices are connected together, the emmitters in parallel to a 12V supply, the detectors share a 'common' earth, with individual signal leads connected to the computer via a 32 channel multiplexer. This allows the 8 input channels of the BBC to read all of the 32 recording channels.

crystallized onto the inside edge of the tubes and the sugar crystal which formed then had a small air hole drilled through it using a pin; this hole was sealed with cotton wool, so air could get through to the fly. Selected females were carefully placed into the open end of the tubes under CO_2 anaesthesia using a fine paint brush. A damp wick leading from a water reservoir in a bijou bottle was then used to seal the open end of the glass tube and the female inside the tube was allowed to recover from anaesthesia. When the experiment was due to start the glass tubes with their water reservoirs were placed in the holders.

The second method was exactly the same except that instead of blocking one end of the tube with a sugar crystal it was closed with a cotton wool bung, which allowed the passage of air. Once the fly was in the tube it was sealed using a damp wick which, instead of leading from a water source lead from a weak sugar solution. The holders were set up in light-tight boxes in a walk-in constant temperature room. 4 Watt fluorescent lights inside the boxes, regulated by commercially available timers, were used to produce experimental light-dark cycles and light pulses, the mean light intensity in the area of the flies in these boxes was 1.13 Wm^{-2} (+/-0.05Wm^{-2}).

Two similar recording systems were used, one collected and recorded data from 32 flies simultaneously whilst the other took data from 30 flies. Each system consisted of the emitter-receiver pairs housed in the holders connected to a BBC B+ Master computer by a 32 channel multiplexer. This multiplexer enabled the computer's eight input channels to monitor the 32 (or 30) recording devices. When a fly in the recorder was active it would walk along the length of the glass tube and break the infra-red beam. This event was detected by the computer which then recorded the total number of times each beam had been broken by the fly in ten minute bins for seven days onto a 5.25" floppy disc. If the locomotor activity rhythm had to be recorded for longer than 7 days then the program was restarted. The

programs used to record and analyse the data were written in BBC BASIC and are listed in Appendix i.

The original recorded data file needed to be initially split up to produce the individual activity records of the 30 or 32 flies recorded together. Once this had been done the records of the flies could be viewed in an actogram format. This plots subsequent days below each other with every day having the data from the following data plotted beside it as well. This means that for a seven day record an actogram would plot the locomotor activity recorded on day one to seven below each other and then to the right of day one plot day two again and below this day three to seven (see Fig 2.2).

The free-running nature of the activity rhythm in constant conditions can then be analysed in a number of different ways. If the rhythm is quite clear a least squares regression line can be plotted through the middle of the activity band and the slope of this line represents the period of the free-running rhythm. Alternatively two programs can be used which can calculate this period if the activity bands are not clear, periodogram analysis and mass entropy spectral analysis (MESA). The periodogram is a generally accepted and widely used method of time series analysis producing clear measures of the period of the rhythm; MESA however is believed to be more sensitive to subtler oscillations in the activity record. However, MESA does not produce confidence limits when calculating the presence and period of rhythms, and Edinburgh University does not hold a license for part of the Fortran control routine necessary to implement MESA (the data were consequently analysed using MESA at Birmingham University with the kind assistance of Dr Elfed Morgan).

Comparison of the two methods of analysis can provide evidence that MESA is not necessary however. Fig 2.2 shows an actogram for a wild-type fly recorded

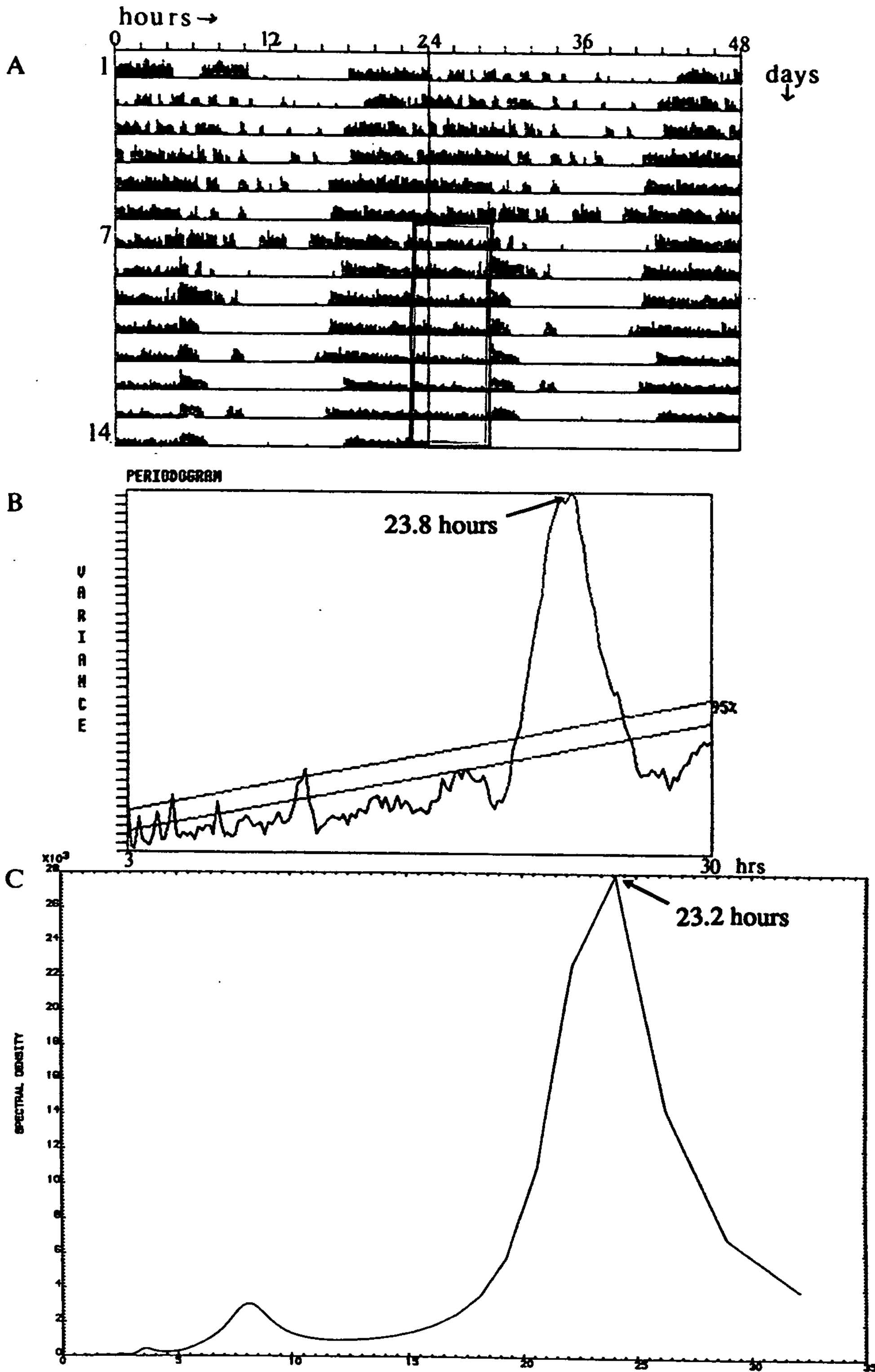


Fig 2.2 A) an actogram showing the double plotted locomotor activity rhythm of a wild-type *D.melanogaster* free-running for six days at 20°C and subsequently exposed to a light dark cycle (the light pulses are shown by the red boxes). Comparisons of two methods of period analysis B) periodogram picks out a significant rhythmic element within the first six days of 23.8 hours and C) Mass Entropy Spectral Analysis (MESA) picks out a rhythm of 23.2 hours over the same six days.

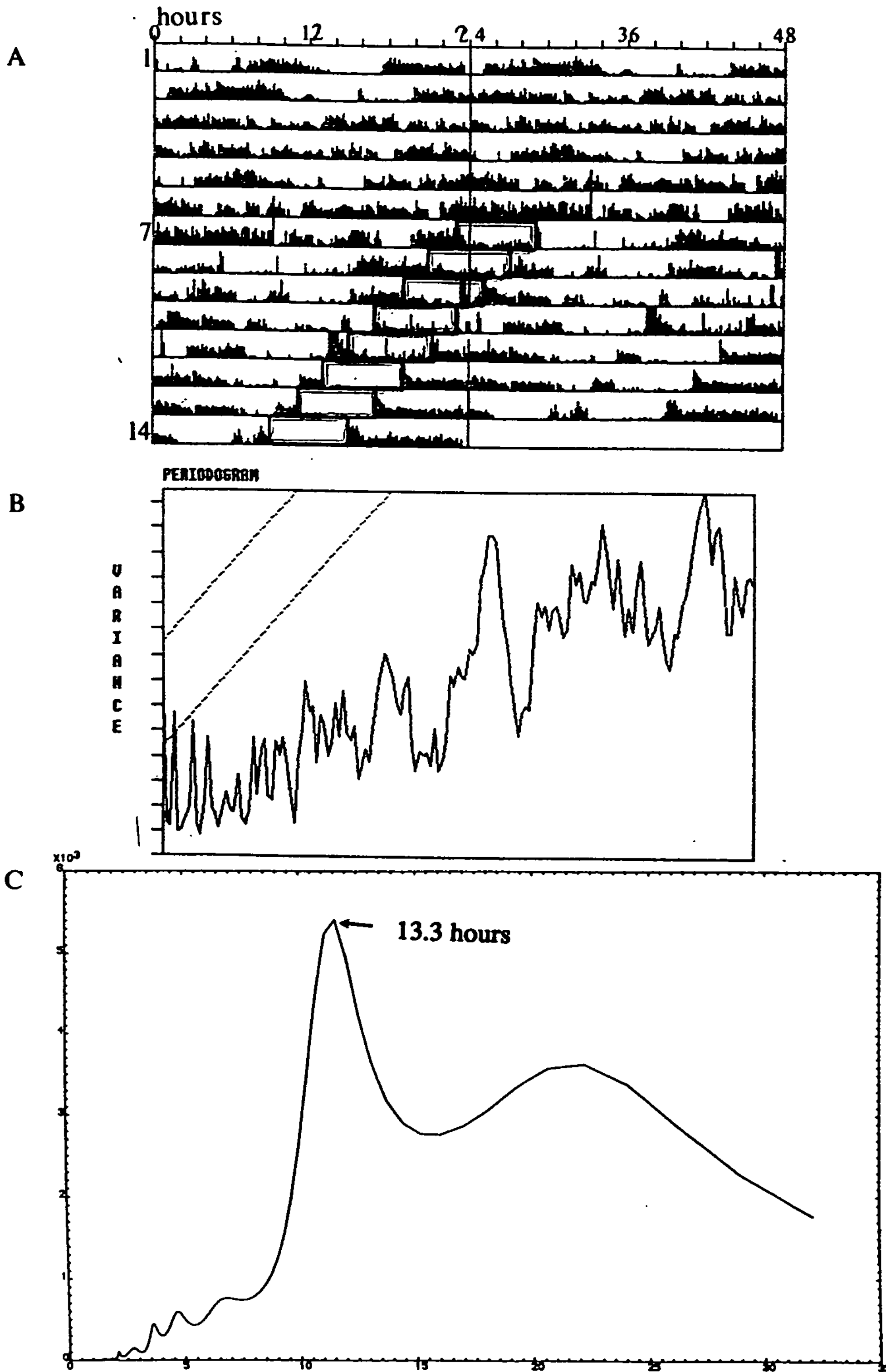


Fig 2.3 An actogram showing the double plotted locomotor activity rhythm of a mutant per^{02} strain of *D.melanogaster* free-running in constant darkness for 6 days and then exposed to a 22 hour light-dark cycle (LD 6:16) (the light pulses are shown by the red boxes). Comparison of the two methods of analysis highlight a difference between them. B) periodogram analysis determines no significantly rhythmic elements in the record whilst C) MESA picks out an ultradian rhythm of 13.3 hours.

over 14 days. Below this, periodogram analysis of the first six days of activity pin-points a locomotor activity rhythm with a period of 23.8 hours whilst MESA shows the rhythm to be 23.2 hours. In Fig 2.3 the same two methods have been used to analyse the activity of a *per*^{02} fly. During the first six days of this record, the periodogram picks out no clear periodicity, whilst MESA finds evidence of an ultradian rhythm with a period of 13.3 hours. This sort of analysis has been used to argue that the locomotor activity rhythm is the product of a group of ultradian oscillators, which when coupled together are expressed as a circadian rhythm, the MESA test being more 'sensitive' to subtle underlying rhythms than periodogram analysis. However, there is no indication in the MESA test whether the periods identified are significant (which periodogram analysis does give). There is also an argument that the "more sensitive" MESA may be confusing the issue by highlighting rhythms which are not significant. Furthermore even if the periods identified by MESA are significant they are not necessarily the product of underlying ultradian oscillators; they could be the product of an interaction between a number of rhythmic elements with much longer periods.

For these reasons it was decided not to use MESA for routine analysis of the locomotor activity rhythm. Periodogram analysis and least-squares regression through midpoints were taken to be accurate tests for detecting periodicities and did not have the theoretical problems associated with MESA.

Analysis of the photoperiodic response

For a fly to enter diapause in short day conditions it has to experience a low temperature (about 12°C) within the first ten hours after emergence from its puparium. At this temperature, short days can maintain the flies in a state where vitellogenesis does not begin. In long days, however, a slow cycle of vitellogenesis occurs. Because of this temperature requirement it is essential that only flies which are less than 10 hours old are used. To collect the large number of flies necessary, the procedure described earlier was used. Each daily sample was divided into groups of approximately equal numbers of flies and these were placed into labelled glass shell vials containing 7ml of standard Lewis medium. These vials were placed in experimental photoperiods at 12°C in light-tight boxes inside a walk-in constant temperature room, or placed in two plant growth cabinets which could provide light cycles (LEEC model PL2). In the light-tight boxes the photoperiods were regulated by commercially available timers controlling 4W fluorescent strip lights. The flies were left in these conditions for two weeks. The females were then dissected and the ovaries visually inspected to determine whether each fly had entered diapause or not. A female was said to have entered diapause if none of the egg follicles had developed passed stage 7 (King, 1956), that is none of the oocytes had begun to collect yolk proteins from the surrounding haemolymph (see Fig. 2.4). The number of females dissected from each vial and the number of these which had entered diapause were recorded. For each strain three separate groups of flies were placed in each experimental photoperiod and the percentage of adult females which entered diapause was calculated. To produce mean diapause responses for each strain the three separate percentages were combined by inverse sine transformation. This geometrical method of combining percentages is excellent for binomial data points which cover a large range. The average number of females dissected, in each condition, was 99·24.

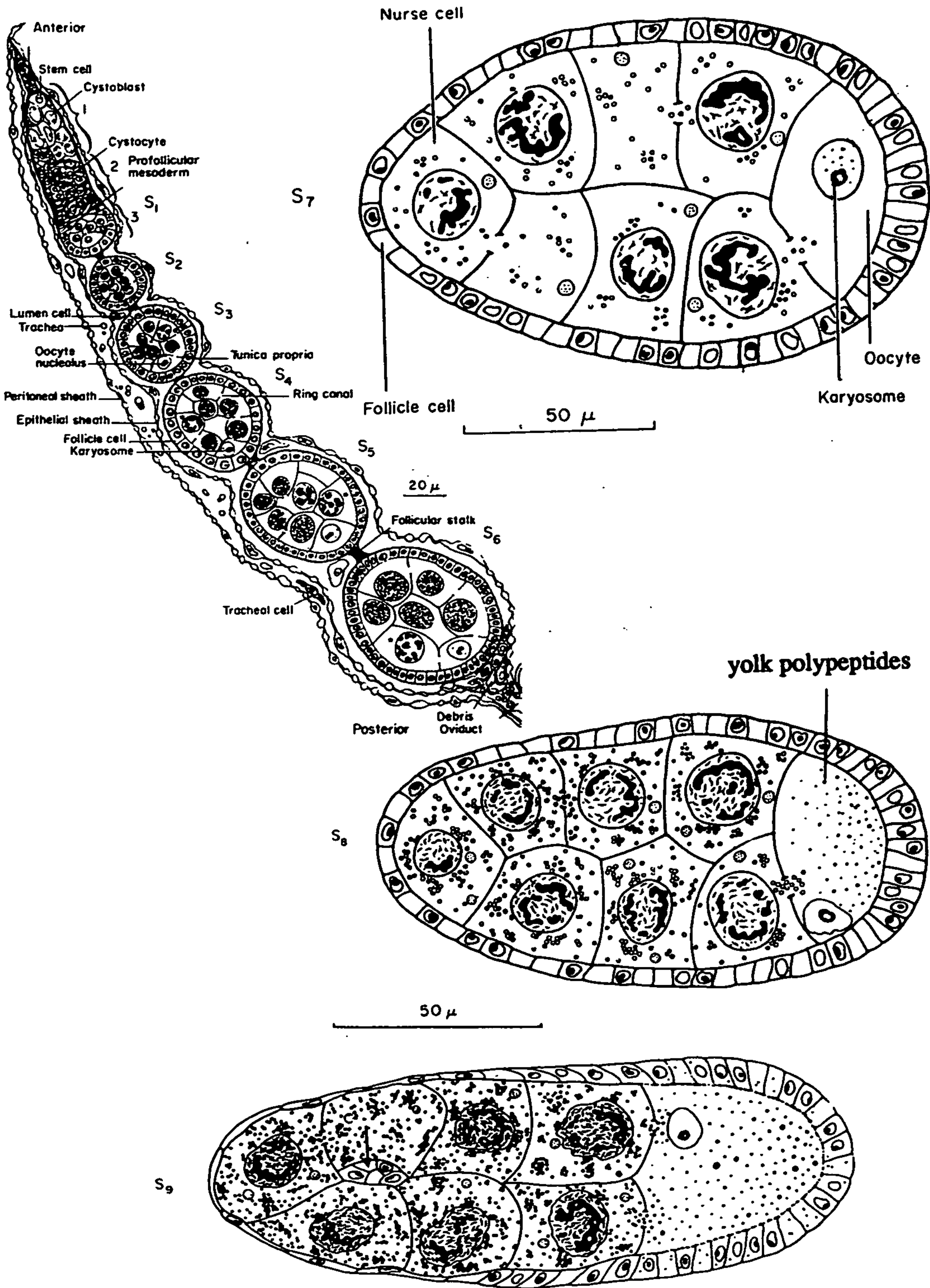


Fig 2.4a Cross sections taken from King (1970) of ovarian maturation from stages 1 to 9. Stages 1 to 6 are shown within the vitellium. Stage 7 is still considered to be pre-vitellogenic whilst any flies found to contain oocytes developed beyond this stage after two weeks of post eclosion development are considered to be undergoing full ovarian maturation, *i.e.* not in diapause.

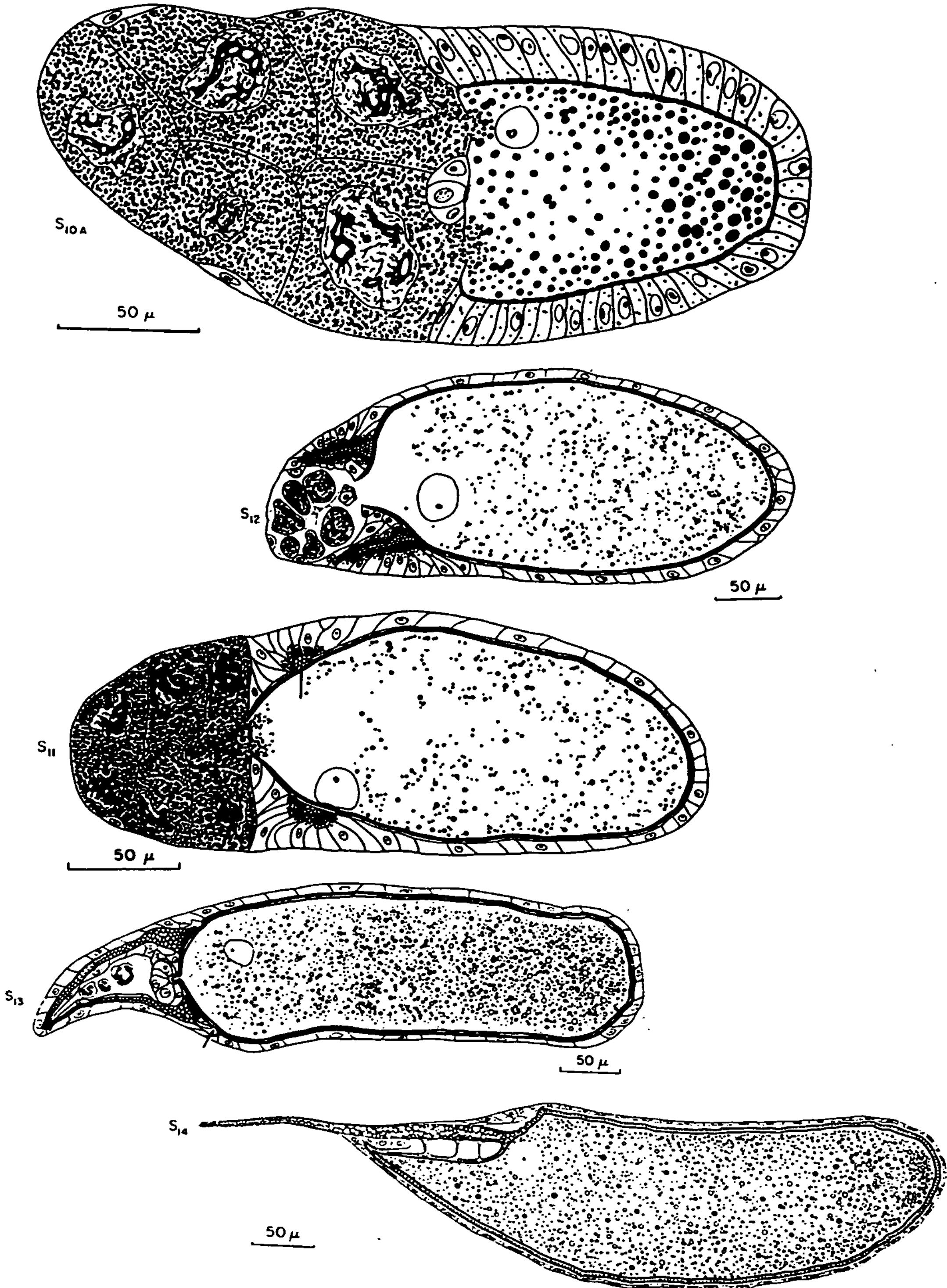


Fig 2.4b Further cross sections taken from King (1970) of ovarian maturation from stages 10 to 14 demonstrating complete, non-diapause development.

CHAPTER 3

The Locomotor Activity Rhythm in *D.melanogaster*

Introduction

Analysis of the locomotor activity rhythm in wild-type and *period* mutants of *Drosophila melanogaster* has involved the automatic recording of the rhythm by computer in constant darkness. The exact nature of the arrangement varies from laboratory to laboratory but most recordings are made using an infra-red beam which is broken by the moving fly in a manner similar to the one used here (see chapter 2). Analysis of the free-running rhythm has involved measuring the period of the rhythm, and in two cases the production of light phase response curves (PRCs)[i]. For a gene believed to be directly involved in the production of the locomotor activity rhythm very little behavioural analysis of this phenomenon has been undertaken. Most of the work done on the *period* locus has involved the investigation of the molecular biology of the gene.

Konopka (1987) recorded the locomotor activity rhythm in per^{+}, per^{S} and per^{L2} at a constant temperature of 22°C in constant infra-red light. Per^{S} had a mean endogenous rhythm of 19.0 hours (+/-0.3 hours) whilst per^{+} had a rhythm of 23.9 (+/-0.3) hours and per^{L2}, 29.2 (+/-1.6) hours. Per^{L} is reported to have a rhythm of 29.6 (+/-0.7) hours. It is now known that per^{L} and per^{L2} are identical mutants (Hall, *pers comm*). Further analysis of the free-running locomotor activity rhythm in per^{+} and per^{S} by Konopka & Orr (1980) has demonstrated that the reduction in tau in per^{S} is due solely to a shortening of alpha, the duration of the active portion of the rhythm. per^{+} had an active phase lasting 12 hours and an inactive phase which also lasted 12 hours. In per^{S}, however, whilst the inactive phase was 12 hours, the active phase of

------

i-Hall & Rosbash (1987)

the locomotor activity cycle was only 7 hours. This observation led Konopka & Orr (1980) to propose a model for the generation of locomotor activity rhythms in which the *period* gene controls the duration of the active portion of the cycle. A second, unidentified, gene controls the duration of the inactive portion. They predicted that the lengthening of tau seen in per^L mutants would be a direct result of an *increase* in alpha.

Two further *period* mutant strains, per^0 (which is the same as per^{02} and per^{03}) and per^-, appear to be arrhythmic in constant darkness. However, it has been suggested that this arrhythmia, which can be clearly seen in traditional actogram plots, is the result of noisy data masking an underlying rhythmicity. More 'precise' analysis, relying upon filtering of 'unwanted noise' from the records has revealed that over 50% of both per^0 and per^- flies contain periodic elements within their records (Dowse, Hall & Ringo, 1987). Six out of eleven male per^0 flies tested were found to have periodic activity rhythms with a mean value of 13.1 (+/-5.3) hours. Eight of sixteen per^- females also expressed locomotor activity rhythms with a mean period of 14.6 (+/-4.2) hours. These rhythms were discovered using correlograms and Mass Entropy Spectral Analysis (MESA). The same tests calculated periods for per^s of 20.2 (+/-1.3) hours, per^+ of 24.0 (+/-1.0) hours and per^L of 30.6 (+/-3.7) hours. These tests found ultradian rhythms in the records of apparently arrhythmic flies and also found periods for the three strains per^s, per^+, and per^L which agree with data collected by other workers and analysed in different ways. Whether these analyses find evidence of rhythmicity in the records of per^0 and per^- flies which is intrinsic to the oscillator controlling locomotor activity and which periodogram analysis is too 'insensitive' to locate, or whether the periods pin-pointed by MESA are functions of some other component is still unknown.

Rearing wild-type flies in continuous darkness for several generations results in flies with locomotor activity records which resemble those of the mutant strains (Dowse & Ringo, 1989). In dark reared flies 33% of the recorded flies expressed wild-type rhythms, 16% expressed phenocopies of *per*^{L} flies, with long rhythms, and 52% were phenocopies of *per*^{0} flies. An interesting comparison can be drawn here between these dark reared flies with '*per*^{0}' locomotor activity rhythms, and the eclosion rhythm expressed by dark reared *D.pseudoobscura* (Zimmerman, 1969). In the later case, arrhythmic eclosion rhythms were recorded. The pacemakers in these individuals are inherited at rest and require a stimulation to 'kick start' them (Zimmerman, 1969). The alternative hypothesis used to explain the arrhythmia was that the individuals within the population were out of synchrony with each other and, although each expressed a circadian rhythm, the population rhythm was masked.

Obviously the locomotor activity rhythm in wild-type flies is not expressed for long periods of time in constant darkness outwith the artificial laboratory environment. Light-dark cycles entrain the rhythm so that it expresses a 24 hour period. As was discussed earlier, one clear way to understand how this entrainment can take place is through phase response curves (PRCs). PRCs also provide clues to the underlying dynamics of the oscillator producing the rhythm. Two PRCs are shown in Hall & Rosbash (1987), one for *per*$^{+}$ and the other for *per*^{S}, both to 60 second light pulses. Both are of Type 1. The shortening of tau in *per*^{S} is shown to be due to a decrease in the length of the 'dead zone', the animals subjective day, to 7 hours. This agrees with Konopka & Orr's (1980) evidence that tau is shortened in *per*^{S} because alpha, the subjective day, is shortened from 12 to 7 hours. There are, however, no published PRCs for *per*^{L} or *per*^{L2}, or *per*$^{+}$ and *per*^{S} to light pulses of a longer duration.

PRCs provide a way of describing free-running rhythms as well as predicting entrainment behaviour in natural and non-natural light-dark cycles. A second way of describing the response of a free-running rhythm to a light pulse is by plotting a phase transition curve (PTC) (Johnsson & Karlsson, 1972; Vaz Nunes, 1981). PTCs also provide a very convenient way of visualising the strength of the response to the light pulse and can be used to categorise the response to light into Type 1 or Type 0 (Winfree, 1970a, 1980; Vaz Nunes, 1981).

In 1987 Helfrich & Engelmann published results from a series of experiments in which wild-type and *per*^{0} flies were entrained to diel and non-diel light-dark cycles. Entrainment theory was used to predict the behaviour of *per*^{0} flies. If *per*^{0} flies truly did not contain circadian oscillators then they ought to respond equally well to light-dark cycles of any duration. In contrast, *per*$^+$ flies would demonstrate 'limits to entrainment', light-dark cycles of such a duration (either longer than or shorter than 24 hours) that the flies could not entrain to. Instead they would express a free-running rhythm despite the presence of light-dark cycles. The results, it was suggested, demonstrated that *per*^{0} flies contain circadian oscillators which respond to light-dark cycles in a way that would be predicted from oscillator theory (*i.e.* in T22 they would phase-lag the Zeitgeber and in T26 they would phase-lead the Zeitgeber as shown in Fig 1.4 and 1.5 on pages 17 and 18).

In a second, more recent paper (Hamblen-Coyle et al, 1992), locomotor activity has been recorded in males exposed to 24 hour light-dark cycles. In LD 12:12, the rhythm was seen to consist of two peaks, one around dawn the other around dusk. The entrained rhythms of the wild-type and a series of mutants were compared. The morning peak was found in a similar position in all the mutants tested, whilst the evening peaks of activity were found to occur earlier in short-period mutants and later in long-period mutants. Entrainment theory can predict changes in the phase

relationship of a rhythm to its entraining light dark cycle when the period of the rhythm changes. Fig 1.4 and 1.5 clearly demonstrate that when τ is shorter than the period of the entraining light dark cycle then the rhythm phase leads the light, and when it is longer than the period of this Zeitgeber cycle the rhythm phase lags the light. This difference in the phase relationship of the rhythm to the light is seen in the changing position (relative to the light) of the evening peaks in activity which are therefore taken to be the product of an endogenous oscillator, influenced by *period*. The morning peaks, however, do not exhibit this predictable changing phase relationship and are presumably due to an exogenous effect of the light coming on, possibly a 'startle' reaction to the sudden increase in light intensity.

There is still a large amount of work that can be done on the behavioural analysis of the locomotor activity rhythm in the wild-type and mutant flies. To this end, work here has concentrated upon three aspects of the locomotor activity rhythm. Firstly, the free-running rhythm has been analysed; secondly, a series of PRCs and PTCs has been produced for pulses of different duration; and thirdly, the behaviour of per^{+}, per^{0} and per^{-} flies entrained to light-dark cycles has been investigated.

Analysis of the free-running rhythm

Materials and Methods

The free-running locomotor activity rhythm of females of the wild-type Canton-S strain, the short period mutant, *per*^{s}, and the long period mutant, *per*^{L2}, were analysed at 20°C (+/-1°C). Flies were placed in the activity recording devices described in chapter 2 and entered constant darkness for six days. During this time the computer system recorded the amount of activity of each fly in ten minute 'bins'. These data were stored on disc and analysed at a later date. Analysis consisted of measuring the period of the free-running rhythm by periodogram analysis and by plotting a least squares regression line through the mid-point of the active portion of the rhythm. These two measures of tau were compared for thirty flies by means of a t-test. The mid-point of the active portion of the rhythm, or alpha, was found using a computer program in which a mouse was used to specify the start and end of alpha. The middle of this activity band was taken to be the middle of the subjective day, or circadian hour 6 (ct6). This computer program is listed in Appendix i. The same program simultaneously calculated the mean duration of alpha.

The mean period of the free-running locomotor activity rhythm and the mean duration of alpha were found by a regression line through ct6 for each of 50 females. From these two measures the mean value of the duration of the subjective night, or rho, was found by subtracting mean alpha from mean tau. These three parameters of the free-running locomotor activity rhythm were then statistically compared by means of a sequence of t-tests. The amount of locomotor activity recorded within alpha was also calculated and a mean value for each of fifty females compared between the three strains of *Drosophila melanogaster* using t-tests.

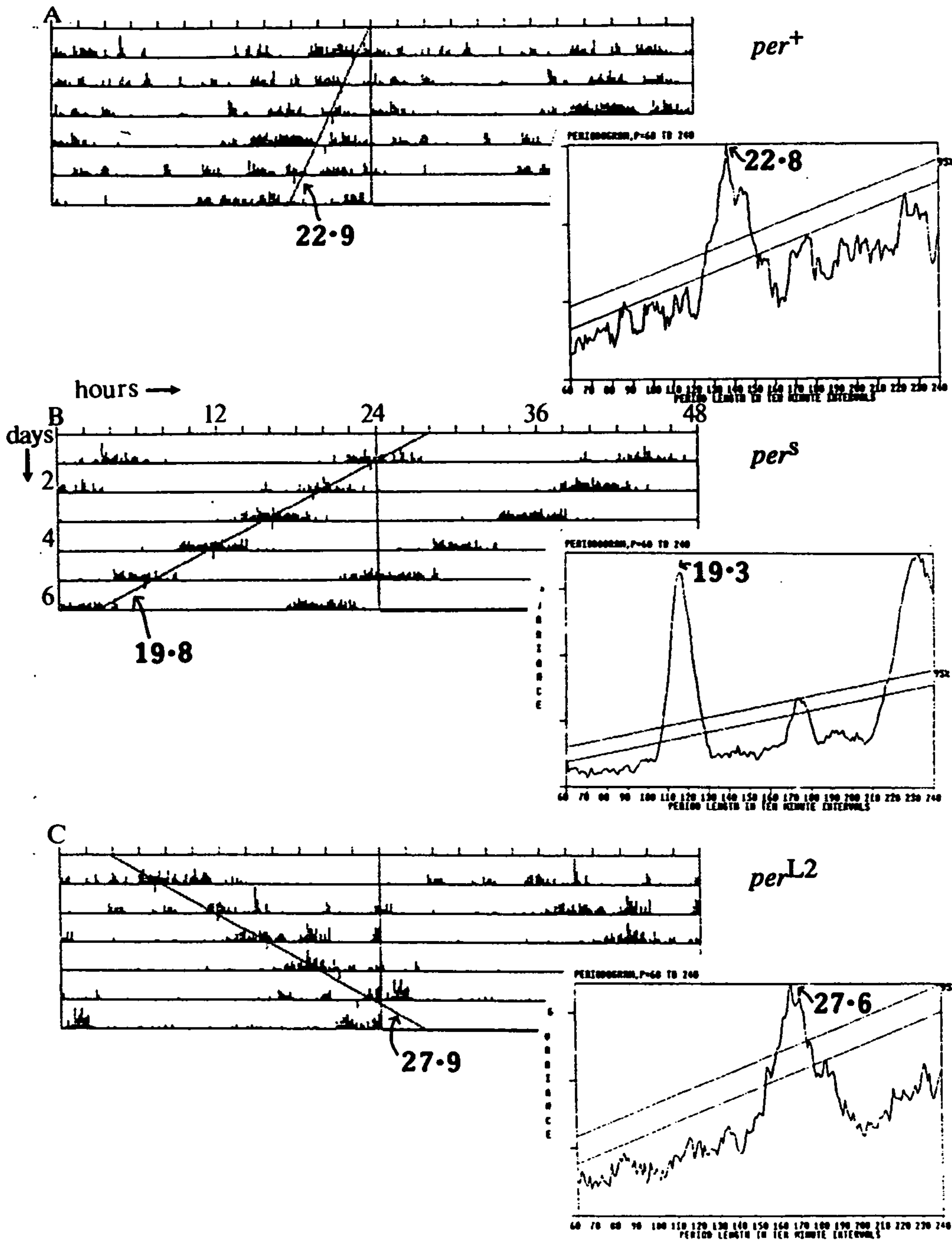


Fig 3.1 Actograms and periodograms of the locomotor activity rhythms of A) wild-type *D.melanogaster* and two mutants strains B) *per*^{S} and C) *per*^{L2} in constant darkness. The periods of the free-running rhythms have been calculated by regression curve through the midpoint of the activity band (circadian time 6) and by periodogram analysis.

Results

Examples of the free-running locomotor activity rhythm for all three types of fly are shown in Fig 3.1 with accompanying periodogram analysis and regression lines through ct6 predicting tau.

The two different ways of measuring tau, periodogram analysis and regression line, when compared by t-tests for each of the three strains did not prove to be significantly different (T for *per*$^{+}$ 1.880; T for *per*^{S} -0.310; T for *per*^{L2} 2.487). Mean tau values and standard deviations using periodogram analysis are given in Table 3.1. The parity between the two methods of calculating tau for individual flies means that the alpha and rho values calculated are representative of the duration of the subjective day and night respectively.

Using the regression line program to measure tau, alpha, rho and the mean locomotor activity within alpha, means and standard deviations for these parameters for the three strains are given in Table 3.2. The free-running rhythms for the three strains are significantly different as shown by Anova ($F = 848.3$ $P<0.001$) as are the values of alpha and rho. T-tests show that the differences revealed are due to differences between the periods of the free-running rhythms of all three strains (T *per*$^{+}$ vs *per*^{S} = 26.451 $P<0.001$; T *per*$^{+}$ vs *per*^{L2} = -19.434 $P<0.001$; T *per*^{S} vs *per*^{L2} = -36.569 $P<0.001$). However the difference in alpha (Anova F = 32.5) is due to alpha in the *per*^{S} strain being significantly shorter than alpha in both *per*$^{+}$ and *per*^{L2}, which are not themselves significantly different (T *per*$^{+}$ vs *per*^{S} = 6.613 $P<0.001$; T *per*$^{+}$ vs *per*^{L2} = -1.311; T *per*^{S} vs *per*^{L2} = -4.5244 $P<0.001$). The reverse is true in the case of rho, where *per*$^{+}$ and *per*^{S} have similar values which are both significantly shorter than rho in the *per*^{L2} strain (T *per*$^{+}$ vs *per*^{S} = 0.574; T *per*$^{+}$ vs *per*^{L2} = -6.510 $P<0.001$; T *per*^{S} vs *per*^{L2} = -7.139 $P<0.001$). No significant difference was found between the amount of locomotor activity within alpha in the three strains.

Table Showing Significant Relationship of T−tests Between period Mutant Flies

Period of Activity Rhythm

	per^{+}	per^{S}	per^{L2}
per^{+}	–	P<0.001	P<0.001
per^{S}		–	P<0.001
per^{L2}			–

Length of alpha

	per^{+}	per^{S}	per^{L2}
per^{+}	–	P<0.001	NS
per^{S}		–	P<0.001
per^{L2}			–

NS – T−test did not show a significant result

Table 3.1 Sample size and mean tau values for the three strains of *D.melanogaster* calculated by periodogram analysis

	Per^{S}	Per^{+}	Per^{L2}
N	30	30	30
Mean Tau (hrs)	19.73	24.35	30.19
STD	0.50	1.13	2.15

Table 3.2 Analysis of the free-running rhythm of the three strains of *D.melanogaster* calculated using least squares regression and measurement of alpha and rho using a computer program designed for this purpose

	Per^{S}	Per^{+}	Per^{L2}
N	50	50	50
Mean Tau (hrs)	19.70	23.72	29.10
STD	0.57	0.90	3.64
Mean Alpha (hrs)	8.47	12.18	12.99
STD	2.35	2.84	3.64
Mean Rho (hrs)	11.23	11.54	16.11
STD	2.37	2.87	3.99
Mean Activity within Alpha (no)	319.0	476.5	282.1
STD	393.6	455.4	289.5

The distribution of individual free-running periods and of alpha are shown in the histograms in Fig 3.2. The distribution of tau values (Fig 3.2a) about the mean for each strain is larger in the long period mutant, *per*^{L2} than in either the wild-type or *per*^{S}. The three strains clearly have separate mean tau values, each normally distributed about their means. This is not the case, however, with the mean duration of the subjective day (Fig 3.2b). The distribution of alpha in the wild-type and *per*^{L2} strains overlap, and both have the same mean value. The mean duration of α is shorter in *per*^{S} and its distribution does not overlap either of the other two strains. When correlations within each of the strains were sought between the period of the free-running rhythm and the length of alpha and rho no significant relationships were found.

Discussion

The values of tau for the three strains compare well with those recorded elsewhere (Konopka, 1987; Dowse, Hall & Ringo, 1987). A novel result is found when comparing how the free-running rhythm is divided into active and inactive portions. As Konopka & Orr (1980) found, tau in *per*^{S} is shortened by a shortening in alpha, the active phase, from 12 hours in the wild-type to 8 in this mutant. Surprisingly, though, and in contrast to the predictions made by Konopka & Orr (1980), the increase in tau in *per*^{L2} is not caused by a lengthening of the active portion but by a lengthening of the *inactive* portion. This categorically rules out their model for the generation of the rhythm. It appears that the *period* gene plays some role in the overall control of the rhythm and the two mutations causing different amino-acids substitutions in the PER product result in different sections of the rhythm being affected. This may be another example of the reciprocal behaviour of the *period* mutants which will need to be explained in any model of *period*-mediated rhythm production. A diagrammatic representation of the effects of the two mutations upon the free-running locomotor activity rhythm is given in Fig 3.3.

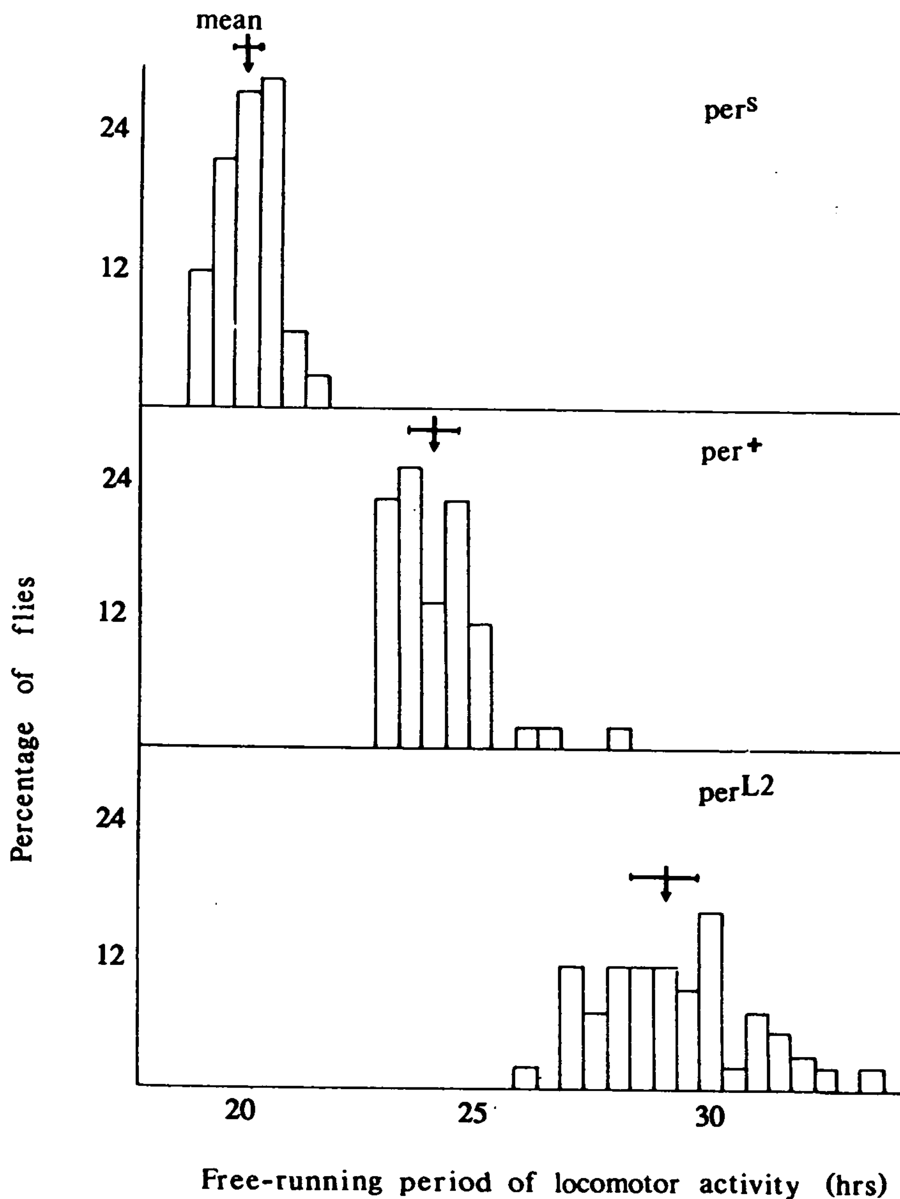


Fig 3.2a Histograms showing the distribution of τ values of wild-type, *per*^{S} and *per*^{L2} female *D.melanogaster* flies. The mean period of the free-running locomotor activity rhythm has been calculated by least squares regression through the midpoint of the activity for 50 individuals of each strain. Each strain has a normal distribution with *per*^{S} strain showing a smaller standard deviation. The two other strains have overlapping distributions, *per*^{L2} having the largest standard deviation.

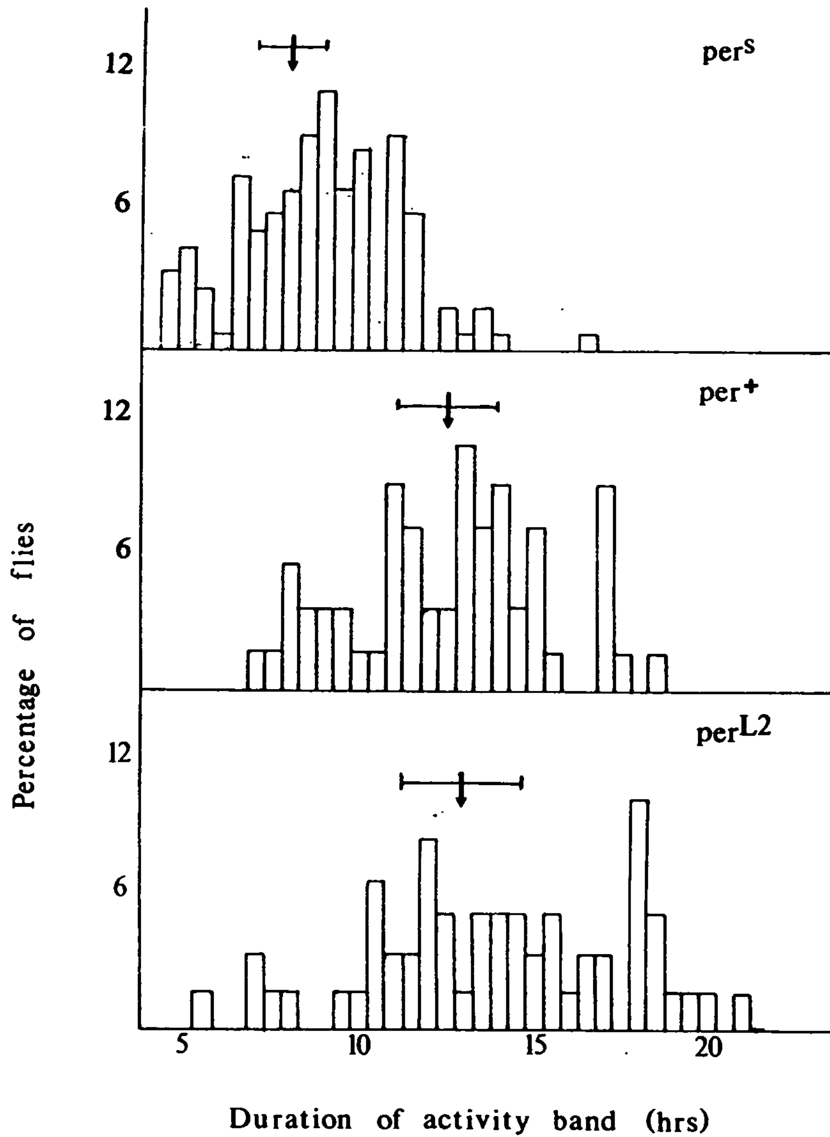


Fig 3.2b Histograms showing the distribution of α values of wild-type, *per*^{S} and *per*^{L2} female *D.melanogaster* flies. The duration of α was calculated by computer whilst the mean period of the free-running locomotor activity was calculated. The three strains appear to have a normal distribution about means. The short period mutant shows a distribution of values different from the other two mutant strains which appear to have very similar means and distributions about the mean.

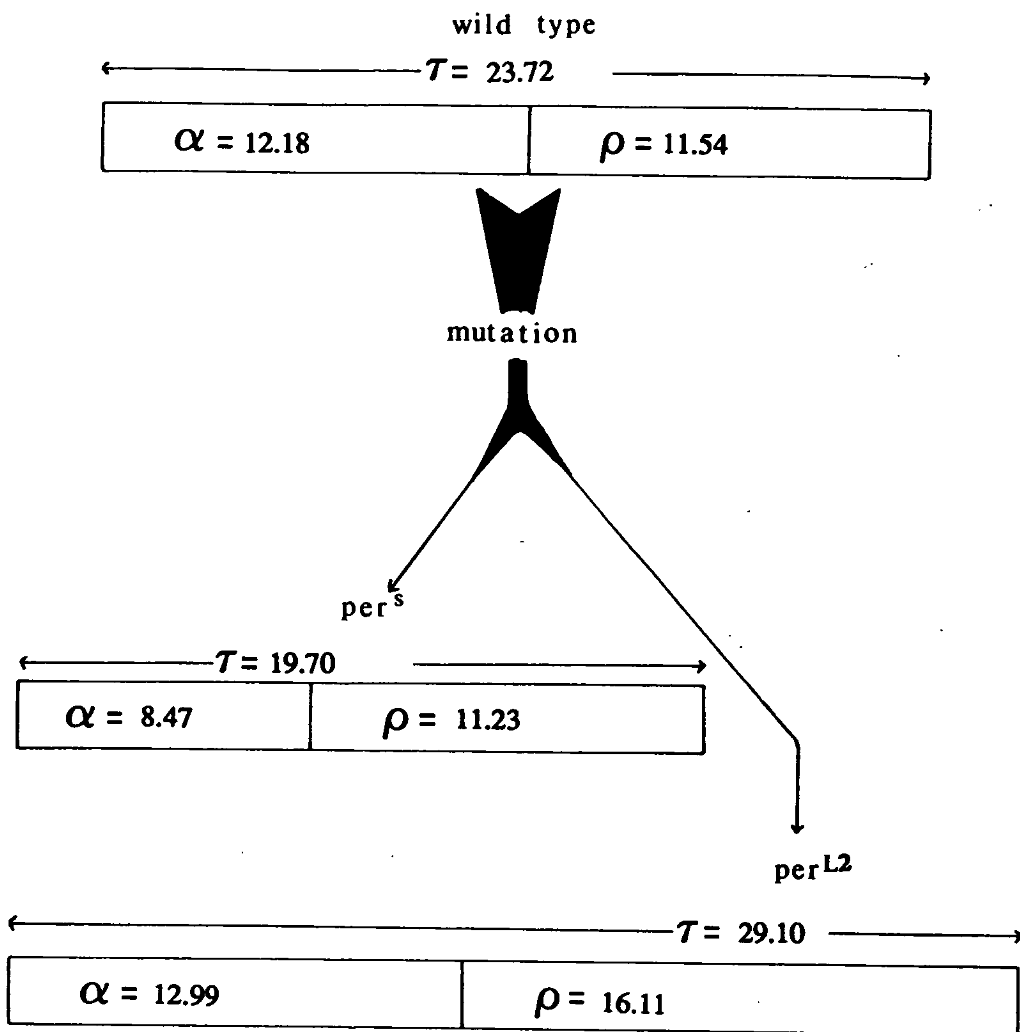


Fig 3.3 Diagrammatic representation of the locomotor activity rhythm of female *D.melanogaster*. The short period mutation reduces τ by shortening α, the active portion of the locomotor activity rhythm, and apparently does not change the inactive portion, ρ. The opposite is seen in the long period mutant, the mutation causes an increase in the duration of τ by increasing the duration of ρ, apparently not altering α.

The activity levels within alpha in the three strains investigated show that in *per*$^{+}$ and *per*^{L2}, where the mutation does not affect the duration of the active portion, the amount of locomotor activity is similarly not affected. However, in the *per*^{s} mutant, with its reduced duration of alpha, there appears to be compensation for this with flies as active in their 8 hours of activity as *per*$^{+}$ and *per*^{L2} flies are in their 12 hour subjective days. This could be either due to a pleiotropic effect of *per* or because activity levels are controlled by a separate genetic locus or loci. Given that most homeostasis is lost in the *period* mutants it would seem more appropriate to conclude that this constancy in mean activity level in the three strains is due to the second hypothesis. However, a *caveat* stems from the standard deviation involved in the calculation of the mean, which is so large (and therefore so is the standard error) that it would be difficult to find any significantly different activity levels. Whilst the locomotor activity recorders were similar they did have individual differences in their responsiveness, which was most probably due to deterioration in function through time. In order to control this source of variation the infra-red emitters and receivers were adjusted at the start of every run to maintain a maximum sensitivity. The large numbers of flies used to calculate the mean activity levels (50) would also probably result in any error variation being so small as to be negligible. Ultimately it is felt that the non-significant difference in levels of activity due to large standard deviations (and therefore standard errors) cannot be entirely the result of individual variation in the recording devices. There is probably a large amount of individual variation in the level of activity present in all three strains of fly and it would be unwise to argue for any one cause for the apparently similar locomotor activity levels in these flies. Perhaps in future, the activity levels could be investigated in greater detail.

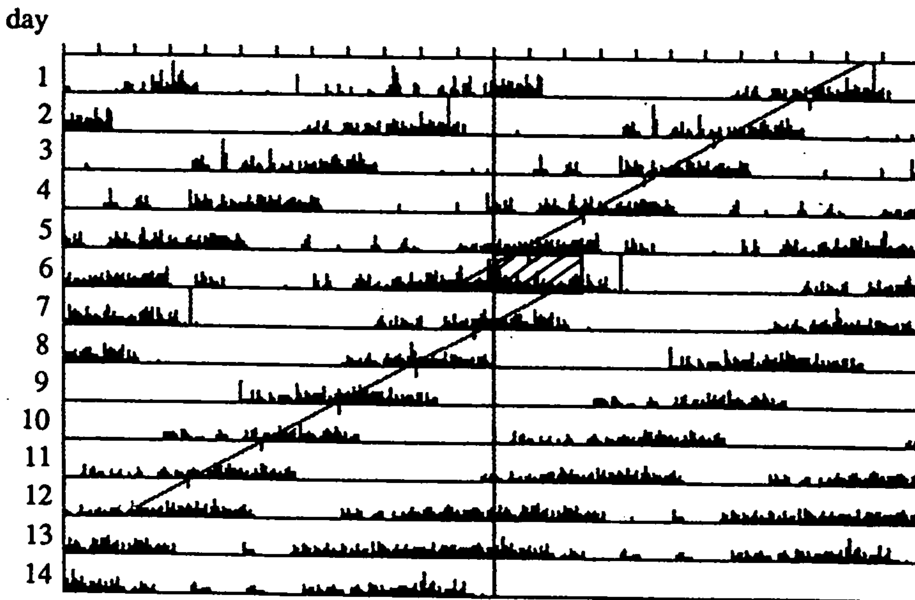
Within each of the strains the general relationship between a short period and a reduced alpha, and a long period and an increased rho, which is demonstrated in Fig 3.3 was not found. The normal distribution of tau in each of the three strains (Fig

3.2a), and the distribution of alpha in Fig 3.2b are not correlated, *i.e.* *per*$^+$ flies with taus shorter than the mean do not have significantly shorter active phases (alpha) and those with longer than average taus have no lengthened inactive phases (rho). It appears that the changes in these two parameters which lead to the periods measured in the two mutants are not exaggerated effects, due to mutation, of a phenomenon that can be seen in wild-type flies and which leads to the variation in tau seen in the population. The general (or mean) changes seen in alpha and rho in the two *period* mutants are separate from the changes causing individual variation in tau in wild-type populations of Canton-S, and also, indeed, different from the changes causing individual variations in tau in both *per*^{S} and *per*^{L2}

Production of Phase Response Curves

Materials and Methods

Female Canton-S, *per*^{S} and *per*^{L2} flies had phase response curves (PRCs) for their free-running locomotor activity rhythm produced for different durations of white light of intensity 1.13 Wm^{-2} (+/-0.05) at 20°C (+/-1°C). Flies were placed in the recording devices and left to free-run in constant darkness for 6 days. After this time a single pulse of light was administered to the flies of one, two, six or ten hours in duration at different phases of the free-running rhythm. The fly was then left in constant darkness for a further 7 days before the activity record was analysed. Analysis of the records was undertaken by means of a computer program given in Appendix i. For the first six days in constant darkness, alpha was demarcated on a computer drawn actogram using a mouse to highlight the start and end of the activity band. The middle of this band in each subjective day was taken to be circadian time 6 (ct6). A least squares regression line was drawn through these midpoints and extended on to include the day of the light pulse. This regression line marked when ct6 occurred during day 7 of the locomotor activity recording and the computer used this marker to calculate the phase at which the light pulse began. The same procedure was followed for the steady state rhythm after the light pulse (*i.e.* this did not include any obvious transient cycles) and this regression line was used to calculate when the light first illuminated the endogenous rhythm. The program therefore calculated two values for when the light illuminated the locomotor activity rhythm, the first with respect to the rhythm before the pulse was seen and the second with the respect to the rhythm after it had been disturbed by the light pulse. The difference between the two values is equal to the phase shift caused by the light pulse, measured in circadian time. Fig 3.4 shows this phase shift calculated for a short period fly with a light pulse of six hours starting in the late subjective day.



τ before the pulse = 20.06 hours

τ after the pulse = 19.96 hours

phase shift = -7.19 circadian hours

Fig 3.4 An actogram plot of the locomotor activity rhythm of a female per^S mutant fly exposed to constant darkness for fourteen days except for a short pulse of light on day 7 (arrow). The two lines are least square regression lines calculated through the midpoint of the activity band on days 1 to 6, before the light pulse, and on days 8 to 12, after the light pulse. The difference in the position of these two lines on day 7 is due to a phase delay in the rhythm caused by the light pulse illuminating the flies late subjective day.

The raw data are plotted so that the phase shift induced by a light pulse (in real time) is plotted against the number of hours since the midpoint of the previous activity band (Fig 3.5-3.8). The phase shifts produced, in circadian time, were then plotted against the phase at which the light pulses were administered in circadian time (with respect to the undisturbed rhythm) for the three strains, per^{+}, per^{s} and per^{L2} (Fig 3.9, 3.12 & 3.13). Such PRCs were produced for per^{s} flies exposed to one, two, six and ten hours of light, whereas the other two strains were exposed to only one and six hour pulse durations. The unusual practice of plotting the phase shift in terms of circadian time (where one circadian hour equals tau/24 real hours) enabled the PRCs of different strains to be directly compared, and also acted to normalize the phase shifts obtained from different individuals. Within a population with a mean free-running rhythm, different individuals have different free-running rhythms and presumably slightly different underlying oscillators. Given that one of the purposes of a PRC is to measure the time course of the underlying oscillator, it seems sensible to standardise the data from different individuals.

When the original data are plotted so that the phase of the cycle illuminated by the pulse with respect to the undisturbed rhythm (old phase) is plotted against this phase with respect to the disturbed pulse (new phase) then a phase transition curve (PTC) results (Fig 3.11). PTCs are useful in determining which type of phase response (Type 1 or Type 0) the light pulse produces, as Type 1 responses produce PTCs with an average slope of 1 and Type 0 reponses an average slope of 0. A further intricacy, discussed by Vaz Nunes (1981) following Johnsson & Karlsson (1972), involves the inclusion of the duration of the light pulse itself to produce phase transformation curves. Summation of successive light pulses can then be used to produce theoretical PTCs for long durations of light (equivalent to constant light, LL) because, for example, a 12 hour light pulse can be thought of as two six hour light pulses falling immediately after one another with no dark gap in between. This

information can be used to predict the phase of the rhythm at a transfer from extended periods of light into darkness (LL/DD transfer).

About 30 flies were used to construct each PRC. A PRC (and PTC) was produced by averaging the phase shifts in overlapping 2 hour bins. The bins overlapped by an hour on each side, and this produced sufficient data to calculate mean phase shifts and standard deviations, and also incorporated into the calculations a certain amount of 'smoothing'.

Results

The general conclusions that can be drawn from the PRCs and PTCs are that for *per*^{S}, all the responses are of Type 0, one, two, six, and ten hour light PRCs, whereas in *per*$^{+}$ and *per*^{L2} one hour PRCs are of Type 1 and six hour PRCs are of Type 0. The difference between the response of the *per*^{S} and *per*$^{+}$ and *per*^{L2} strains to one hour light pulses reflects some form of increased sensitivity to light in the short *period* mutant, which can also be seen to a lesser extent in the other two strains. The raw data are plotted in Fig 3.5 to 3.8 showing the phase shift in free-running rhythm in real time plotted as a function of the time (in real hours) since the previous midpoint of the activity band (ct6). These figures show areas where the phase shifts tend to be phase advances and areas where they tend to be phase delays. Because the *per*^{S} mutants have a free-running rhythm of about 20 hours there can only be a maximum phase advance and delay of 10 hours, in *per*$^{+}$ the maximum can be 12 hours and in *per*^{L2} the maximum can be about 14.5 hours. The raw data are used to plot PRCs and PTCs and these are given in Fig 3.9 - 3.16.

Fig 3.9 compares PRCs for *per*^{S} to increasing light pulses, plotting mean circadian phase shifts in two hour bins and Fig 3.10 compares the 'smoothed' curves taken from these. The one hour PRC has a large phase delay section and a much

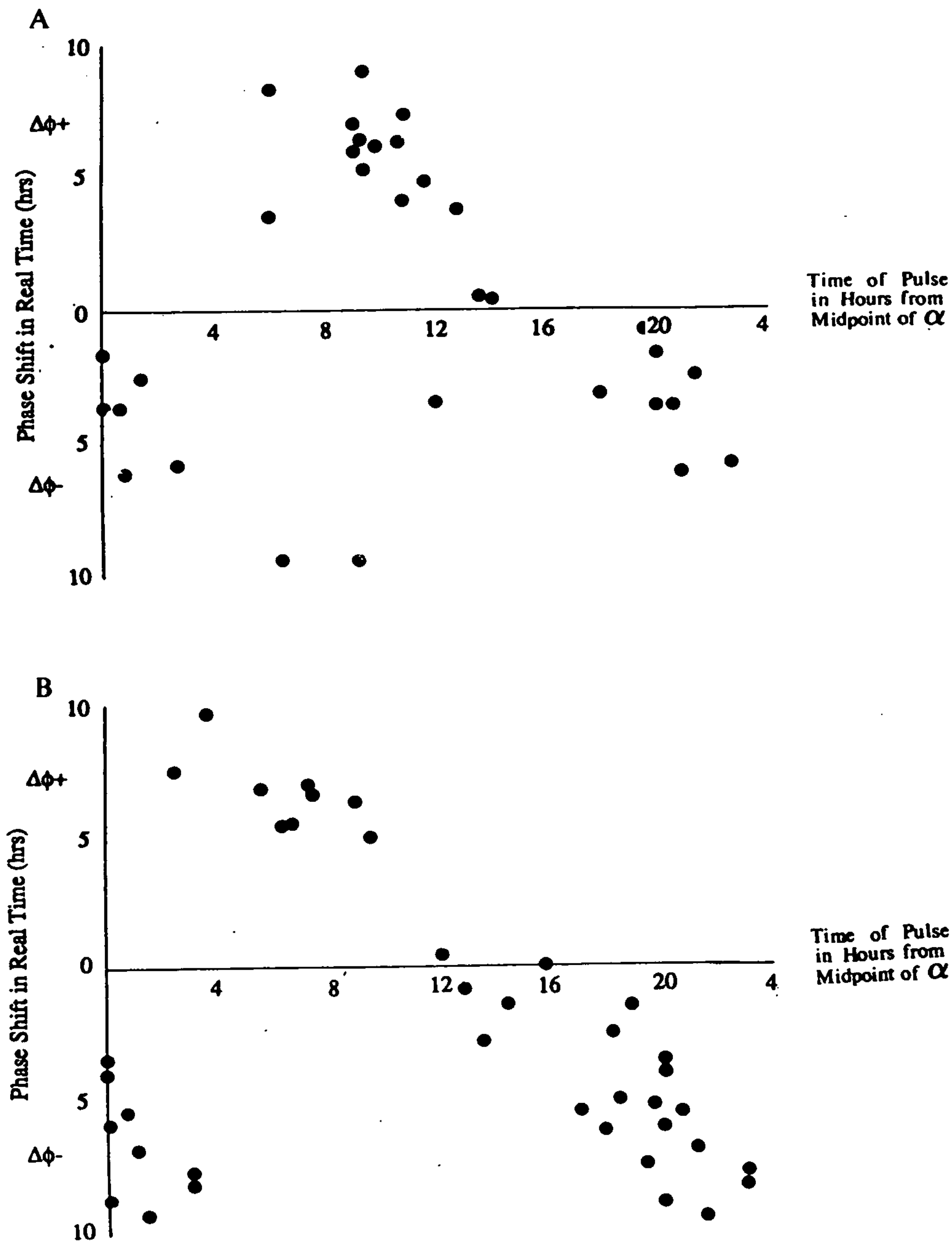


Fig 3.6 Raw data taken from per^S flies at 20°C showing phase shifts in the free-running locomotor activity rhythm in real time following disturbance of the rhythm by a light pulse of A) six hours and B) ten hours in duration falling at different times after the midpoint of the activity band.

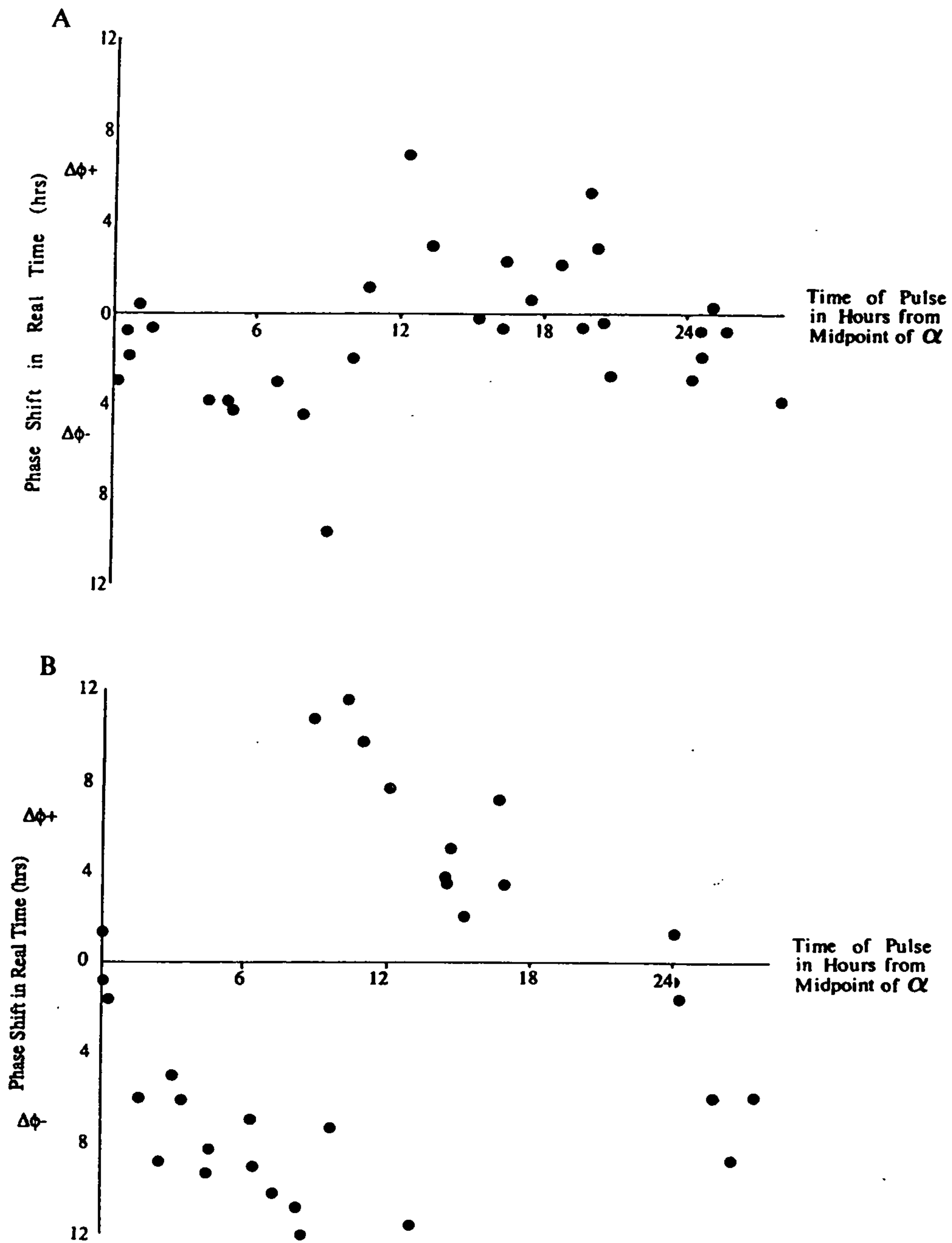


Fig 3.7 Raw data points for wild-type *D.melanogaster* at 20°C showing the distribution of phase shifts (real time) due to a disturbance of the free-running locomotor activity rhythm by A) one hour light pulses and B) six hour light pulses illuminating different phases of the rhythm, measured in hours since the midpoint of the activity band, α.

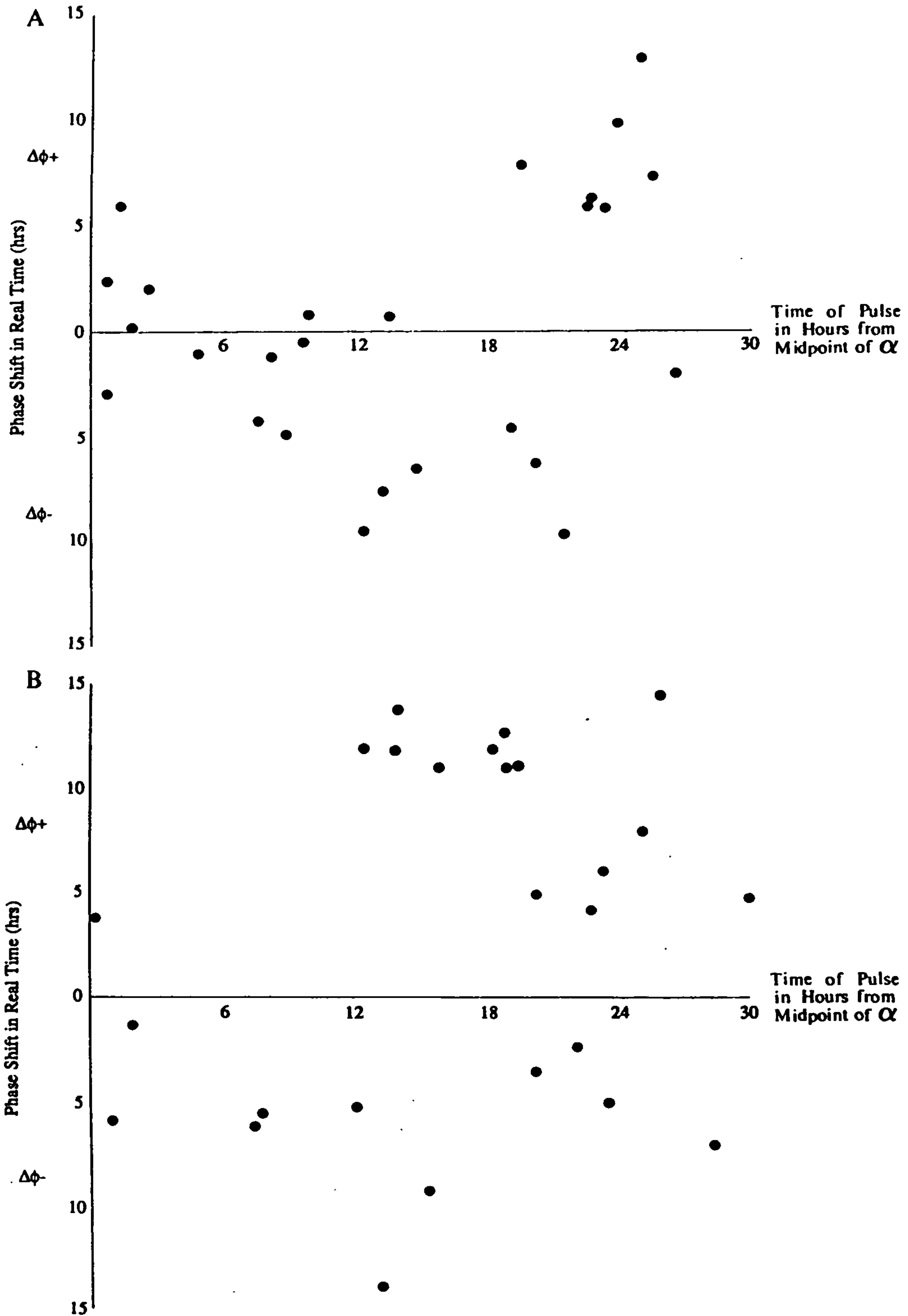


Fig 3.8 Raw data for *per*^{L2} mutants at 20°C showing phase shifts (real time) of the locomotor activity rhythm due to A) one hour light pulses and B) six hour light pulses illuminating various phases of the rhythm measured in hours since the midpoint of the activity band, α.

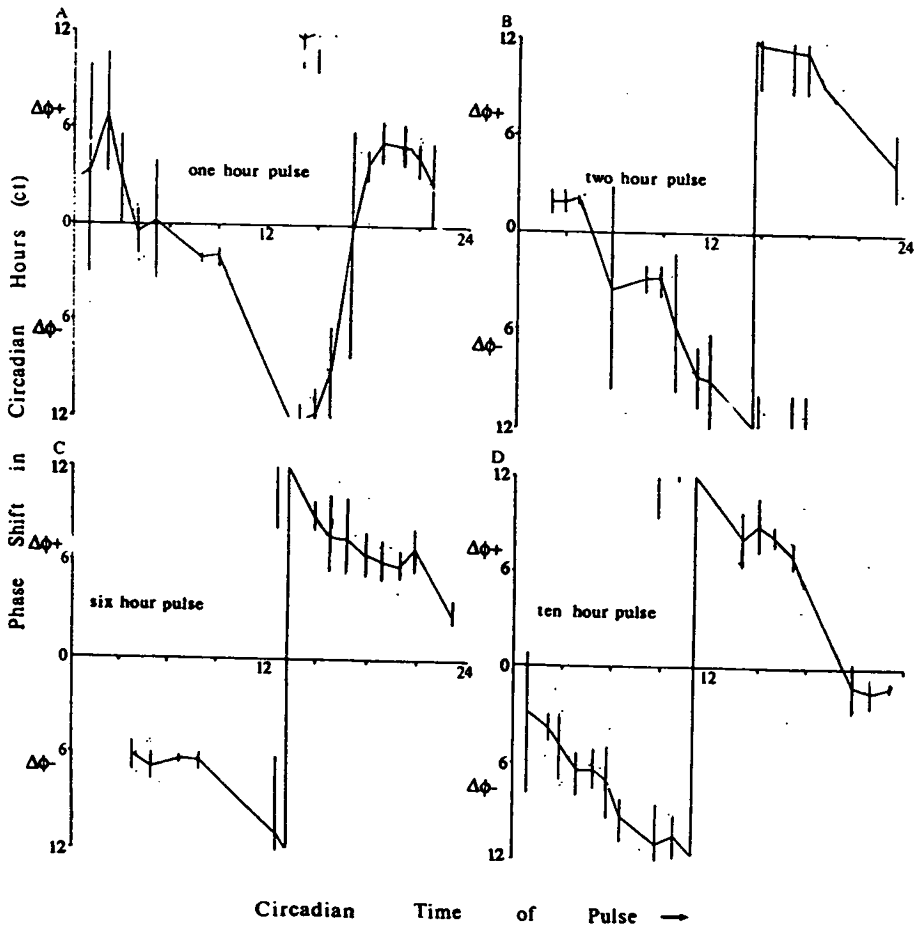


Fig 3.9 Four phase response curves at 20°C for the *per*^{S} mutants strain plotting the mean phase shift in the locomotor activity rhythm (in circadian time) and the standard deviation for consecutive overlapping two hour bins for light pulses of A) one hour in duration, B) two hours in duration, C) six hours in duration and D) ten hours in duration.

smaller phase advance section. The phase shifts do not stretch across a full 24 circadian hours, the phase advances reaching a maximum of about 7 circadian hours. However, following a graphical analysis of the PTC for a one hour light pulse (Fig 3.11) used by Vaz Nunes (1981) it may be shown that the response is Type 0 but borderline Type 1.

The PRCs for *per*$^{+}$ to one and six hour light pulses in Fig 3.12 demonstrate the change between a Type 1 and Type 0 response, the same occurring in Fig 3.13 for *per*^{L2}. The comparison of the form of PRCs to one and six hour light pulses for the three strains of fly are shown in Fig 3.14. An outstanding difference between these PRCs is in the position of the phase delay and advance sections. In *per*^{S} and *per*$^{+}$ these two portions of the PRCs occupy similar positions but differ in size. The delay section of the PRC of *per*^{S} is clearly larger than the advance section, whereas in *per*$^{+}$ both sections are about the same size. However, the PRC for *per*^{L2} is shifted right along the x-axis to such an extent that the delay section in this PRC lies in a similar position to the advance section of the PRCs for *per*^{S} and *per*$^{+}$.

Fig 3.15 shows PTCs for 1 hour light pulses and the PTCs for 6 hour light pulses for the *per*$^{+}$, *per*^{S} and *per*^{L2} strains. The change from Type 1 to Type 0 response in per+ and *per*^{L2} with an increase in the duration of the light pulses from one to six hours can be seen when these two figures are compared. In Fig 3.15A the average slope of the *per*^{S} curve is 0, indicating a Type 0 response to light, whilst in *per*$^{+}$ and *per*^{L2} the average slope is 1 (Type 1 response). In Fig 3.15B all three strains show PTCs with average slopes of 0, demonstrating that in *per*$^{+}$ and *per*^{L2} the increase in the duration of the light pulse results in a change in the response from Type 1 to Type 0.

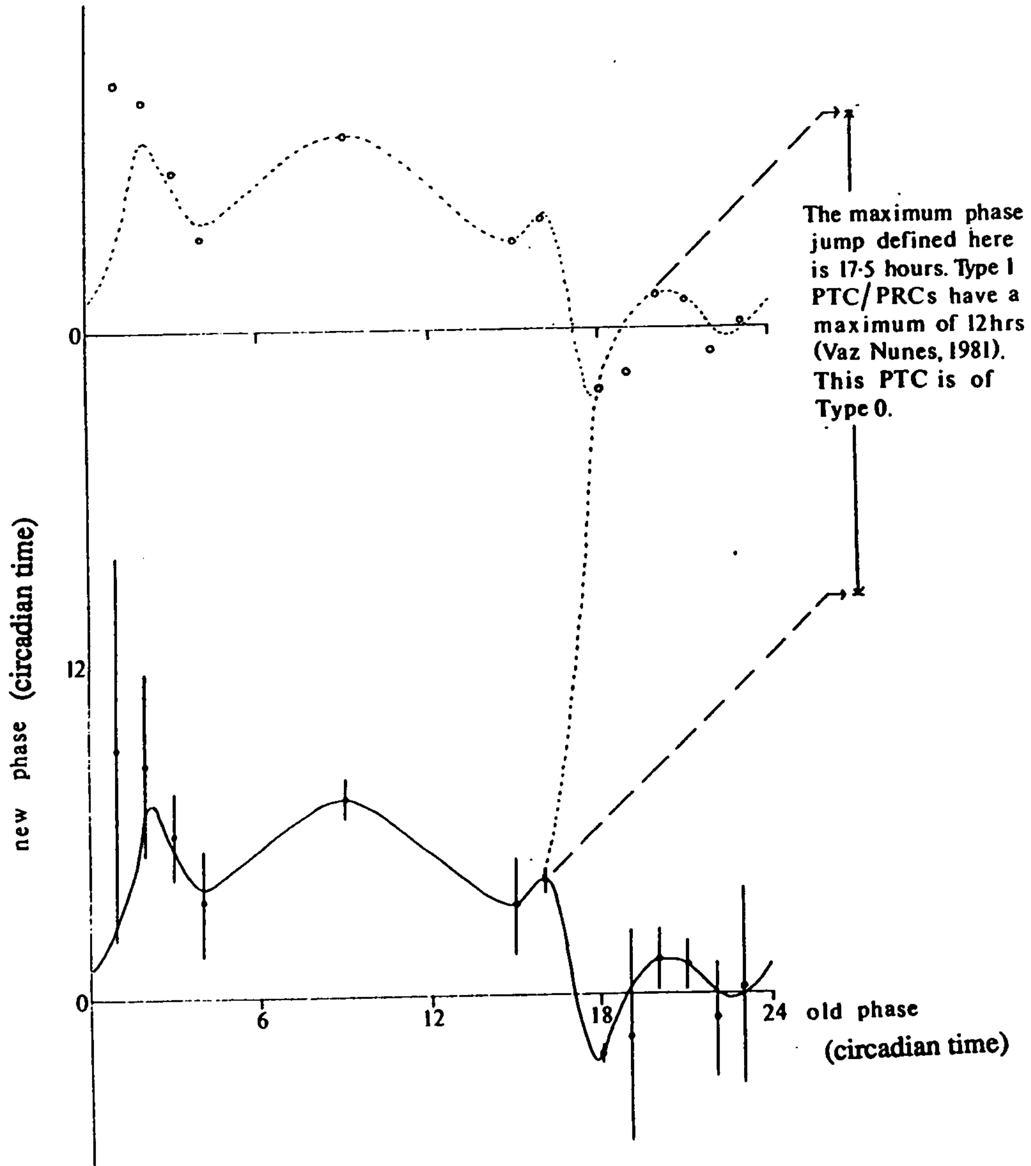


Fig 3.11 A phase transition curve for the *per*^{S} mutant strain at 20°C to one hour light pulses disturbing the free-running locomotor activity rhythm. The phase of the rhythm at which the light falls (old phase) is plotted in circadian time against the phase illuminated by the light with reference to the rhythm after the pulse (new pulse) in circadian time. This diagram demonstrates that the PRC of the *per*^{S} mutant strain to one hour light pulses is of Type 0

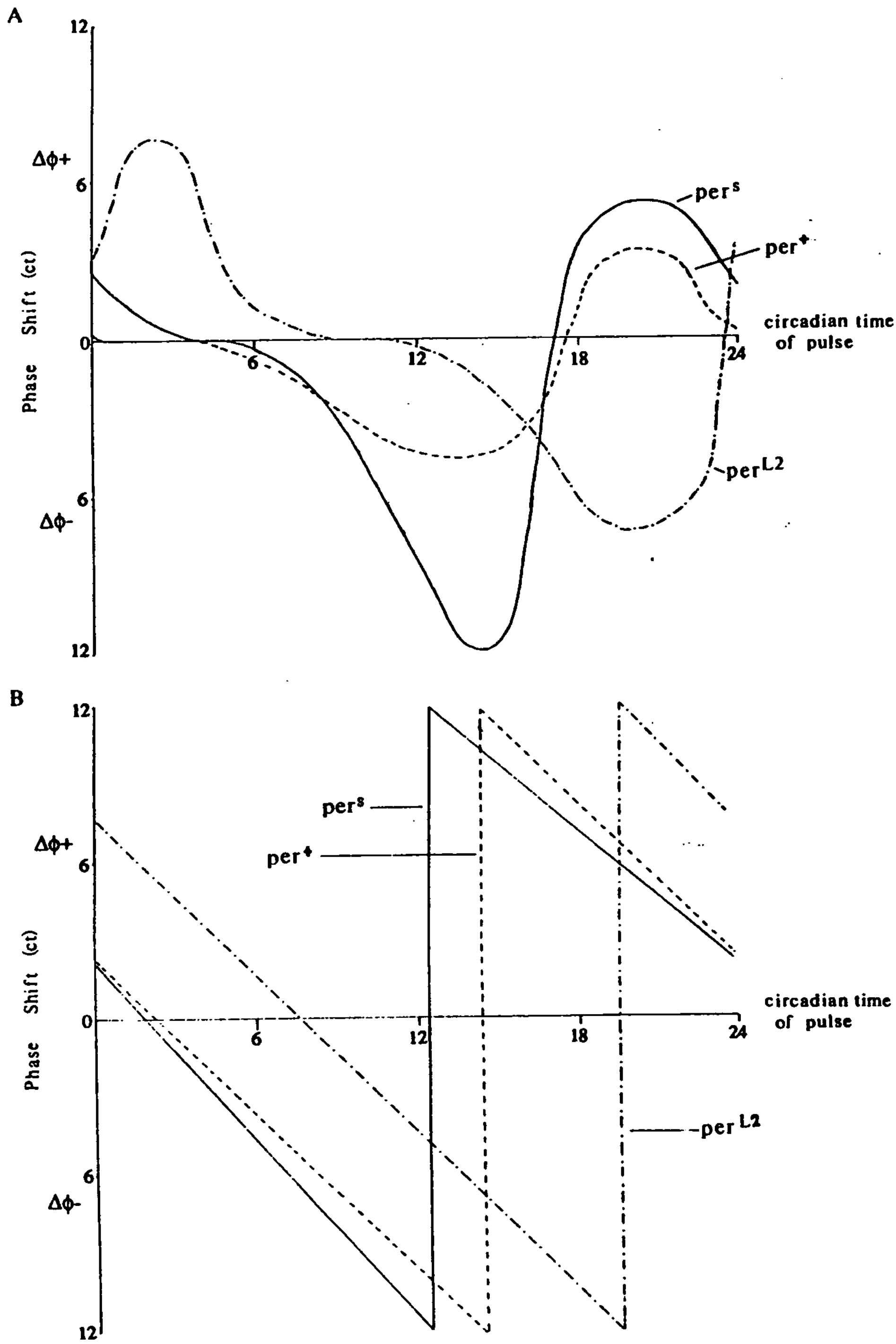


Fig 3.14 A comparison of the smoothed PRCs of the locomotor activity rhythm of wild-type, *per*^{S} and *per*^{L2} mutant flies at 20°C to A) one hour light pulses and B) six hour light pulses. The position of wild-type and *per*^{S} PRCs in both A) and B) are quite similar whereas the PRC of *per*^{L2} appears to be shifted to the right along the x-axis.

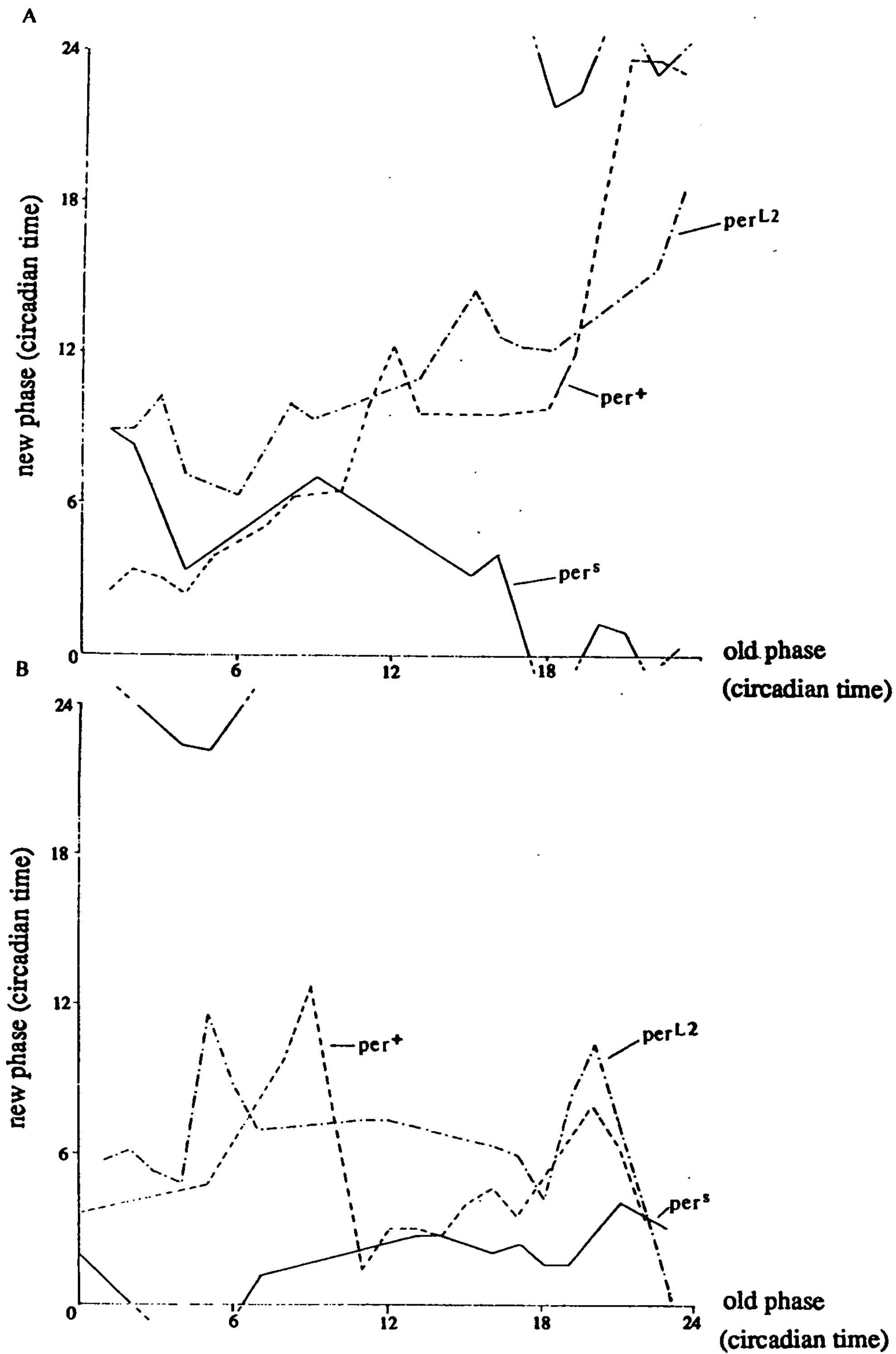


Fig 3.15 A comparison of phase transition curves of the locomotor activity rhythm of wild-type, *per*^{S} and *per*^{L2} mutant flies at 20°C to A) one hour light pulses and B) six hour light pulses. In B) all three strains demonstrate a Type 0 response whilst in A) *per*^{S} has a Type 0 response with the other two mutant strains showing a Type 1 response to light.

Discussion

Phase response curves can be used for two purposes: 1) they can describe the time course of the underlying oscillator, and 2) they can be used to predict phase relationships during steady state entrainment to diel and non-diel light-dark cycles.

Comparing the PRCs for six hour light pulses in *per*^{S}, *per*$^{+}$ and *per*^{L2} strains (Fig 3.14B) it appears that the underlying oscillator has a cycle, which as well as being shorter in *per*^{S} than *per*$^{+}$ which is shorter than *per*^{L2}, also has maximum phase delays and advances earlier in the cycle in *per*^{S} than *per*$^{+}$ than *per*^{L2}. In *per*^{S}, *per*$^{+}$ and *per*^{L2} 12 (circadian) hour phase delays occur at about ct 13.5, 14.5 and 17.5 respectively. This difference in the positioning of the PRCs can be more clearly seen when comparing the PRCs of the three strains to one hour disturbing light pulses (Fig 3.14A). The 'position' of a PRC along the x-axis is best defined by the circadian time when the curve crosses this axis with the phase shifts rapidly changing from maximum phase delay to maximum phase advances. There are several possible explanations for this shift in the PRC position. Light pulse duration can be thought of as being equivalent to light pulse intensity and Fig 3.10 shows the effect of increasing light pulse duration upon the position of the PRC, namely increasing duration shifts the position of the PRC to the left along the x-axis. Therefore we might expect to see a shift in the position of the PRC depending upon the intensity of the light pulse, or reciprocally depending upon the photosensitivity of the oscillator. This suggests that *per*^{L2} flies are less photosensitive than the other two strains, a finding which is supported by the PRCs shown in Fig 3.15A.

Comparing these PRCs demonstrates another difference in their shape. Daan & Pittendrigh (1976) have shown correlated differences in the duration of τ and the shape of PRCs, namely that individuals or species with mean τ's shorter than 24 hours

have larger phase delay sections than advance sections and those with τ's longer than 24 hours have advance sections larger than delay sections. This result has recently been elegantly demonstrated in the Djungarian hamster, where PRCs were plotted using data taken from a population of individuals but the data were plotted in one of two PRCs depending upon whether the individual tested had a mean free-running rhythm of less than or greater than 24 hours. The two PRCs produced were slightly different in shape (Puchalski & Lynch, 1992), the PRCs from those animals with τ's greater than 24 hours had advance sections larger than delay sections and the PRCs from animals with τ's shorter than 24 hours had delay sections larger than advance sections. In the present study the PRCs of the three strains of *D.melanogaster* to one hour light pulses also demonstrate this 'rule' (Fig 3.14A).

The PTCs show that *per*^{S} has a response to one hour light pulses of Type 0 whilst *per*$^{+}$ and *per*^{L2} have a Type 1 response (Fig 3.15A). This indicates that the *per*^{S} mutant is somehow more responsive to light than the other two strains, a conclusion also reached by Winfree & Gordon (1977) when they compared the eclosion rhythms of *per*^{S} and *per*$^{+}$ flies. Furthermore, when one compares the one hour PTCs of *per*$^{+}$ and *per*^{L2} in detail it appears that *per*$^{+}$ is more photosensitive than *per*^{L2} but not markedly so. The more photosensitive a strain is to the light pulse the greater the PTC departs from a 45 degree line and approaches the horizontal. In fact, the difference between the sensitivity of the *per*^{S} mutant and the other two strains is greater than the differences between the sensitivity of the *per*$^{+}$ and *per*^{L2} strains. This would lead one to expect the positions of the PRCs of *per*$^{+}$ and *per*^{L2} along the x-axis to be similar and the position of the PRC of *per*^{S} to be drastically altered. This, however, is not the case. In actual fact the positions of the PRCs of *per*$^{+}$ and *per*^{S} are similar and the position of the PRC of *per*^{L2} is drastically altered (Fig 3·14a,b).

How the *period* mutant affects photosensitivity is an important question providing clues to the nature of the underlying oscillator. The altered photosensitivity could be due to an alteration in the input pathway of light to the oscillator controlling the locomotor activity rhythm. However, this argument would require the *period* locus to have a pleiotropic effect upon both the oscillator, changing tau and alpha in constant darkness, and simultaneously changing the input pathway of the light. A more parsimonious argument would have the *period* mutant affecting photosensitivity within the oscillator itself and connected in some way with the changes in the free-running rhythm. One way that *period* could affect both tau and photosensitivity is by altering the amplitude of the oscillator (Pittendrigh *et al.*, 1991). An oscillator running with the same angular velocity but smaller amplitude than a second will have a shorter period. So assuming per^S has a smaller amplitude than per^+ which has a smaller amplitude than per^{L2}, and because a given intensity of light has a larger proportional effect upon an oscillator with a smaller amplitude, one could expect per^S to be more photosensitive than per^+ which will be more photosensitive than per^{L2}.

The second use PRCs have is in predicting steady state entrainment to diel and non-diel light-dark cycles and especially for predicting the phase relationship under such conditions between the light and activity. For the wild-type to entrain to a light-dark cycle of LD 6:18 it needs to alter its period from around 23.7 to 24 hours, that is to phase delay the oscillator by 0.3 hours every cycle. To do this the light pulse must, in steady state, illuminate a phase of the cycle which results in a phase delay of this duration, *i.e.* at ct 3.7. The active portion of the cycle is centred around ct 6 and so steady state entrainment will result with the light pulse starting near the start of the activity band. For per^S to entrain to LD 6:18 a phase delay of 4.3 hours is required every cycle. This phase delay is equal to a phase delay of 5.2 circadian hours which is produced when light falls at ct 10.6 near the end of the activity band. per^S entrains with the activity beginning several hours before the light comes on and is said to

'phase-lead' the light. When the same procedure is followed for *per*^{L2} flies which require a phase advance of 5.1 hours, or 4.2 circadian hours, then the light has to illuminate ct 3.7, the same point required to entrain the wild-type. This prediction is quite unexpected as within natural populations long taus are associated with 'phase-lags' during entrainment where the activity band lags behind the light pulse during steady state entrainment (refer to Fig 1.4 and 1.5). However, the *per*^{L2} mutant cannot be classified as natural, and the mutation has, as well as lengthening tau, somehow caused the PRC to shift to the right, which in turn results in this entrained phase relationship.

Another possible explanation for this change in the PRC position is that PRC shape is altered in order to conserve the phase relationship between the rhythm and the Zeitgeber during steady state entrainment. This would appear to be successful for the *per*^{L2} mutants but not for the *per*^{s} mutant strain. However, this conservation of phase relationship is not successful at all photoperiods as the *per*^{L2} strain was isolated after mutagenesis by collecting flies which emerged at 'unusual' phases of a population eclosion rhythm. This may explain why the two mutants have differently positioned PRCs in LD 1:23 and 6:18. If with increasing light intensity this phase conservation is lost then we could expect the *per*^{s} mutant to lose its phase conserving properties at light pulses of shorter duration than the *per*^{L2} mutants. If at lower light intensities or shorter pulse durations the *per*^{s} mutant begins to display phase conservation then this would require the PRC to be displaced to the right of the wild-type PRC.

However, another explanation for the difference in relative positions of the PRCs when compared to the wild-type may lie in our definition of the midpoint of the locomotor activity band as being ct6. If this were not the case then whilst the shape of the PRCs would not change, the relative positioning of the PRCs along the x-axis would be altered. How likely is it then that the position of ct6 is not the same in the

three strains of fly? Circadian time 6 is defined as the midpoint of the activity band, which is unaltered in *per*^{L2} when compared to the wild-type. It would appear to be a fair assumption that ct6 is correctly defined in the wild-type and *per*^{L2} mutant. Whereas, from the analysis of the free-running rhythm of the *per*^{S} mutant we know that the activity band, alpha, is altered by the mutation. If the duration of alpha is altered because a section of the activity band is 'lost' from its end then the predicted position of ct6 will be wrong. In fact ct6 will be nearer the end of the activity band, not in the middle, and this will have the effect of repositioning the PRC to the left of its current position. The two mutant PRCs would then be positioned on either side of that for the wild-type. However, it is equally likely that if a section of the activity band is missing in the *per*^{S} mutant then it is missing from the front of alpha which would result in repositioning the PRC to the right of the wild-type and in similar position to the *per*^{L2} mutant.

Ultimately, however, there is no reason why the two mutations in *per* ought to have reciprocal effects upon the position of their PRCs. The observation that the *per*^{S} and *per*^{L2} mutations have different effects upon PRC shape may reflect different effects of the mutations that are not the product of a hypothetical reciprocal loss-of-function (*per*^{L2}) and gain-of-function (*per*^{S}) of the *period* locus. This conclusion draws support from the recent findings of Rutila *et al.* (1992), where data from a large number of recently produced short period mutations indicate that a short endogenous oscillator is not the result of a gain-of-function (which proves to be very rare).

PRCs can also be used to predict the phase relationship between light and activity during steady state entrainment to non-diel light-dark cycles. For example, a six hour light PRC can be used to predict the entrainment pattern of *per*$^{+}$ flies to LD 6:16 (T=22), 6:18 (T=24) and 6:20 (T=26). These predictions are used as the basis for the next set of experiments in which evidence for circadian oscillators controlling the

locomotor activity rhythm in *per*^{0} and *per*$^-$ flies is sought. Entrainment theory demands that the phase relationship between the activity and light will change as the T-cycle changes. Just as Fig 1.4 and 1.5 show that the phase relationship between a rhythm and its entraining light-dark cycle changes as the period of the rhythm changes, so the phase relationship changes as the duration of the light-dark cycle (T-cycle) changes. If the duration of this is longer than the period of the free-running rhythm then this rhythm phase-leads the light-dark cycle (as in Fig 1.4) whereas if the T-cycle is shorter than the period of the rhythm then this rhythm phase-lags the light-dark cycle (as in Fig 1.5). However, if no circadian oscillators control activity and light-dark cycles simply force the activity into bands, then the phase relationship between light and activity ought not to change as T changes. This was tested in wild-type, *per*^{0} and *per*$^-$ flies in the next section in an attempt to find evidence for a circadian basis to the locomotor activity rhythm in these arrhythmic mutants.

Entrainment to light-dark cycles

Materials and Methods

Wild-type, *per*^{0} and *per*$^-$ females were entrained to light-dark cycles which consisted of six hour light pulses of intensity 1.13 Wm^{-2} (+/-0.05) and dark intervals of either 16, 18 or 20 hours thus producing T-cycles of 22, 24 or 26 hours in duration. The flies were placed in the locomotor activity recorders and allowed to free-run in constant darkness (DD). They were maintained in DD for six days and then exposed to the experimental light regimens. The flies were maintained in these T-cycles for seven further days before they were again released into DD.

The PRC for Canton-S flies for a six hour light pulse (Fig 3.12) was used to predict the phase relationship of the wild-type flies to each of the three T-cycles. These predictions were then compared with the mean form estimates of ten wild-type flies in each condition. The form estimates for the individual flies were produced by an adapted periodogram analysis program (listed in Appendix ii) which also merged standardized individual form estimates together to produce a mean strain form estimate from the ten flies entrained to each T-cycle. Similar mean strain form estimates were produced for the *per*^{0} and *per*$^-$ strains for each T-cycle and these were compared with those produced for the wild-type, and the theoretically produced ones.

Results

The mean form estimates for each of the three strains entrained to the three T-cycles are shown in Figs 3.16-3.18. Clearly for each T-cycle the three strains do not show the same entrained phase relationship between the locomotor activity rhythm and the light. There is, however, one similar peak in all nine form estimates, namely a sharp increase in locomotor activity when the lights go off. The similarity between

this point in all the records and because it does not change its phase relationship with the light pulse in different T-cycles, suggests that it is an exogenous effect of the sharp transition from illumination to darkness (a 'startle' reaction). It may be similar to the dawn peak reported in *D.pseudoobscura* which disappeared when flies entered free-run conditions after entrainment (Engelmann & Mack, 1978). Fig 3.19 shows a 21 day actogram for a wild-type fly entrained to LD 6:16 from day 7 to 14. The increase in activity at the end of the light pulse during entrainment corresponds to the peak seen in the form estimate. This increase in activity is not seen in the free-running record of the fly after its release into DD. As well as predictable changes in the phase relationship between entraining light pulses and an entrained rhythm indicating an endogenous control of the rhythm, release into DD after entrainment will be accompanied by a continuation of endogenously controlled characteristics of the rhythm or a few cycles of the free-running rhythm. This further suggests that the peaks seen in Figs 3.16 to 3.18 at the end of the light pulse are exogenous products of a startle reaction. The same thing is demonstrated in Fig 3.20 and Fig 3.21 for per^{02} and per^{-} flies respectively, exposed to T-cycles of 24 and 26 hours in duration.

The wild-type strain demonstrates a shift in the phase relationship of activity to light dependent upon the duration of the light-dark cycles to which it is entrained as would be predicted from entrainment theory (Fig 3.16). However, both the per^{-} and per^{02} strains do not appear to show this change. Comparison of the form estimates of these two strains of fly, however, shows them to be different. The per^{02} strain looks to be almost as active during the dark as the light with activity levels only slightly raised during the light, especially around the light to dark and dark to light transitions (Fig 3.17). There appears to be no consistent change in phase relationship between the light and activity, and no suggestion of any phase leads or phase lags. The per^{-} strain, on the other hand, demonstrates a clear drop in activity levels after 'lights off' with an increase in activity before 'lights on' (Fig 3.18). Measurements show the time before

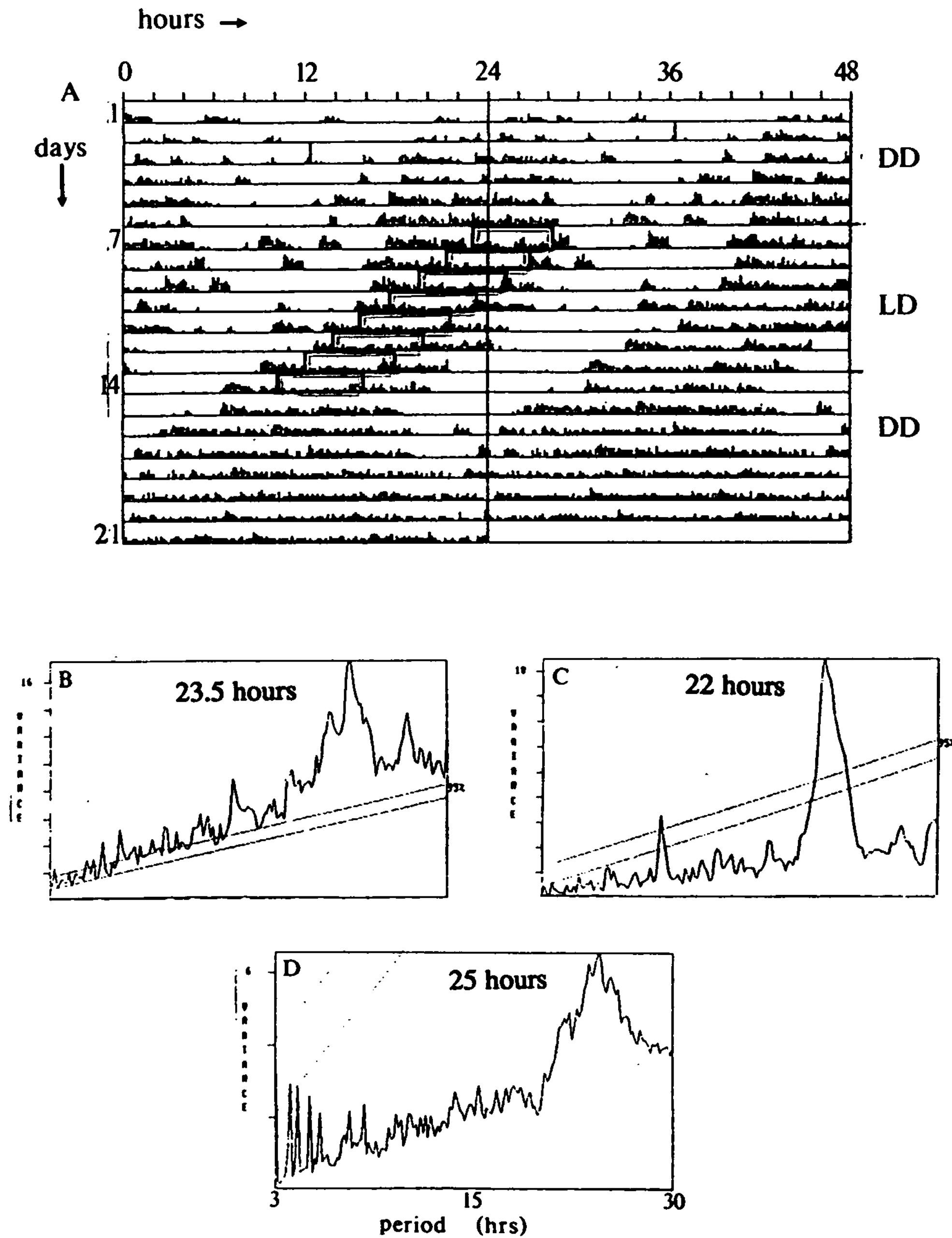


Fig 3.19 An actogram of a wild-type flies locomotor activity rhythm (A) free-running for 7 days before being exposed to a T-cycle of 22 hours and containing 6 hours of light for a further 7 days and then finally being released into DD. The light pulses are shown highlighted in red. B) is a periodogram analysis of the rhythm free-running for the first 7 days demonstrating a significant periodicity of 23.5 hours. Periodogram analysis in C) of the 7 days exposed to the T-cycle highlights a 22 hour period, whilst in D) a peak is seen in the analysis of around 25 hours which does not reach significance levels. The arrhythmicity of this wild-type fly is possibly due to its age.

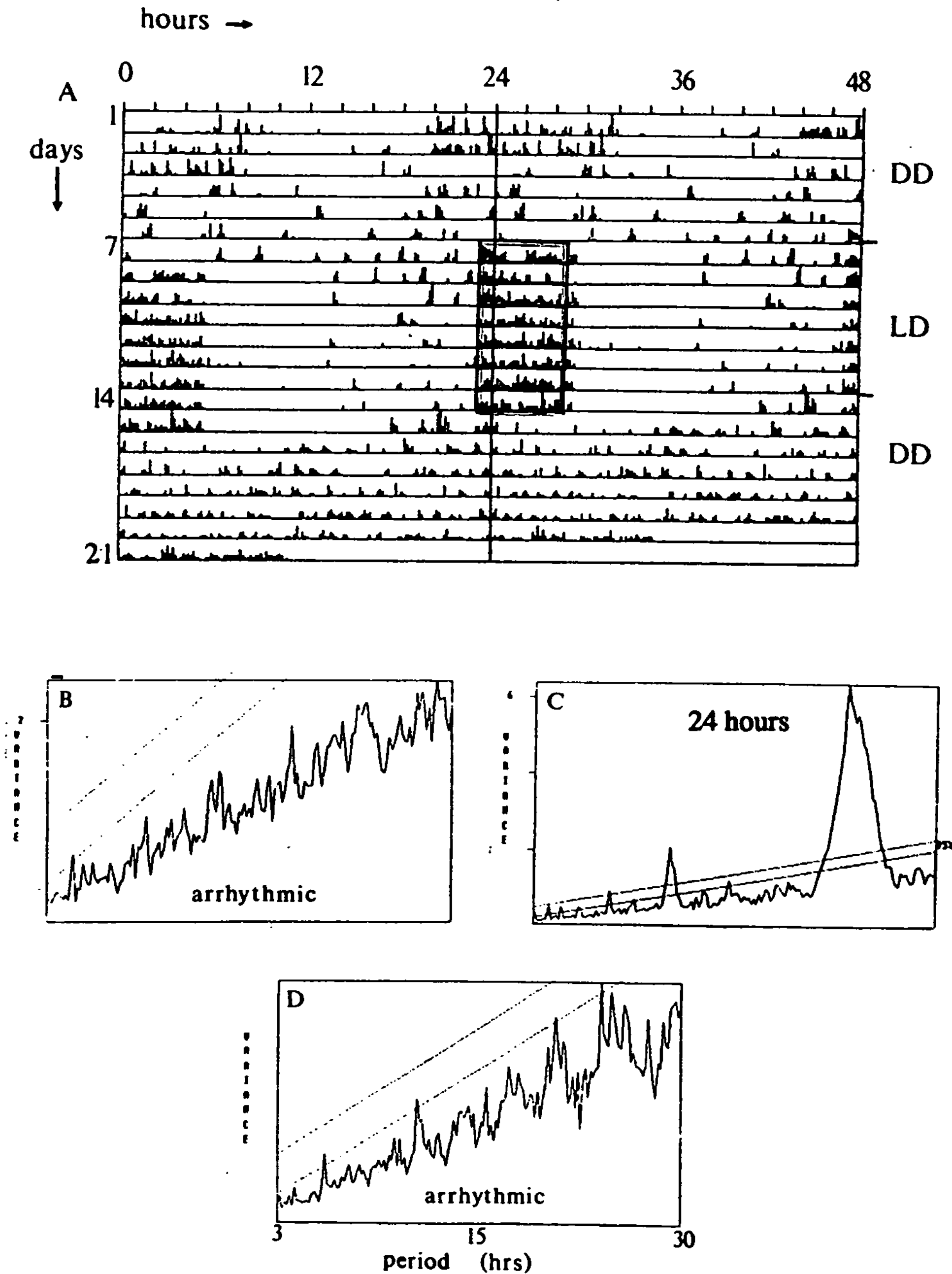
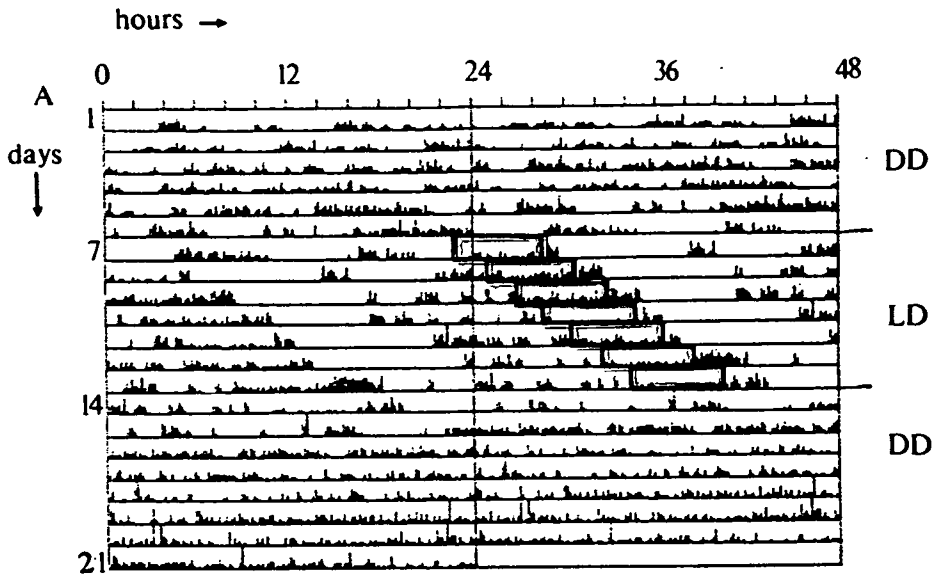
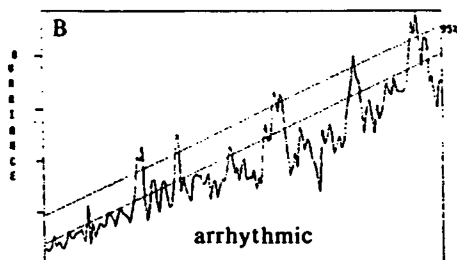
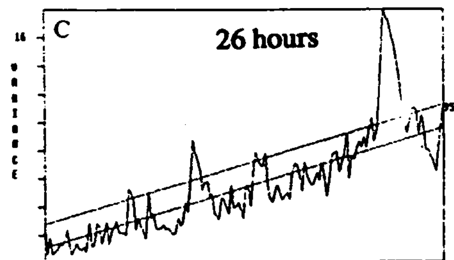


Fig 3.20 An actogram of a per^{02} flies locomotor activity rhythm (A) free-running for 7 days before being exposed to a T-cycle of 24 hours and containing 6 hours of light for a further 7 days and then finally being released into DD. The light pulses are shown highlighted in red. B) is a periodogram analysis of the rhythm free-running for the first 7 days demonstrating an arrhythmic pattern. Periodogram analysis in C) of the 7 days exposed to the T-cycle highlights a 24 hour period, whilst in D) another arrhythmic pattern is seen.







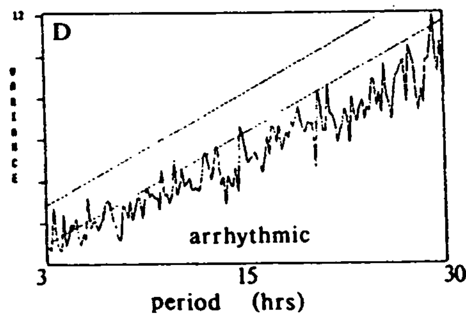


Fig 3.21 An actogram of a *per*⁻ flies locomotor activity rhythm (A) free-running for 7 days before being exposed to a T-cycle of 26 hours and containing 6 hours of light for a further 7 days and then finally being released into DD. The light pulses are shown highlighted in red. B) is a periodogram analysis of the rhythm free-running for the first 7 days, demonstrating an arrhythmic pattern. Periodogram analysis in C) of the 7 days exposed to the T-cycle highlights a 26 hour rhythm, whilst in D) another arrhythmic pattern is seen.

this rise in activity brings the level of activity above the mean level varies with the T-cycle to which they are entrained. In T = 22 hours (LD 6:16) this time is 4.8 hours, in T = 24 hours (LD 6:18) this is 7.5 hours and in T = 26 hours (LD 6:20) it is 8.4 hours.

The way that *per*^{02} and *per*$^{-}$ enter constant darkness and subsequently free-run is also important. In *per*$^{-}$ (Fig 3.21) after the final light pulse on day 14 there is a period of inactivity, in the absence of further light pulses the locomotor activity becomes arrhythmic. There is no gradual break-down of the rhythmic pattern into arrhythmicity over a number of cycles in DD.

The data taken from the wild-type PRC to six hour light pulses have been incorporated into a computer program which predicts entrainment patterns and can produce theoretical square-wave form estimates. These are shown in Fig 3.22 superimposed on the experimentally produced mean form estimates for the wild-type flies entrained to the three T-cycles. Predictions from the PRC and the experimental data are close: in T22 activity phase lags the light, whereas in T26 it phase leads it when compared to T24. When the same model form estimates are superimposed upon the mean form estimates from *per*^{02} (Fig 3.23) and *per*$^{-}$ (Fig 3.24) no such phase relationships may be seen.

Discussion

It would appear then that the wild-type flies are entrained by 22, 24 and 26 hour light dark cycles in a way predicted by entrainment theory and the PRCs produced for this strain. The *per*^{02} and *per*$^{-}$ strains, however, do not. *per*^{02} does not show any evidence of a changing phase relationship with T-cycles and *per*$^{-}$ has behaviour which is not predicted by the wild-type PRC and is probably not due to an underlying circadian oscillator controlling the locomotor activity rhythm.

What explanation is there for these results? Clearly the hypothesis that *per*^{02} and *per*$^{-}$ flies have the same underlying oscillator as wild-type flies is not substantiated. The most obvious conclusion that can be drawn from this is that the truncated *period* gene product produced in *per*^{02} mutants does not have a phenotypically identical effect upon the locomotor activity rhythm as not having the product at all. The records for *per*$^{-}$ and *per*^{02} flies are different showing clearly that the shortened *period* gene product in *per*^{02} flies performs some function. This result is not wholly unexpected as the photoperiodic response in *per*$^{-}$ and *per*^{02} mutants are qualitatively different. It appears therefore that the truncated *per*^{02} gene product plays some part in the production or maintenance of a much weakened circadian rhythm.

The change in the shape of the mean form estimates of the *per*$^{-}$ strain with different T-cycles is not equivalent to the changes in phase relationship seen in the wild-type. There is, however, a difference in the beginning of activity relative to the end of the previous light pulse in the different T-cycles. This need not be because the light is entraining an endogenous oscillator and, if it is, then this oscillator is not the same as the one being entrained in wild-type flies. A possible explanation is that locomotor activity in DD is determined in purely energetic terms, energy input balancing energy expenditure. However in the presence of a light cycle, the organising effect of the light and the 'startle' reaction it produces may disturb the balance of energy so that shortly after the lights go off the flies have a lower activity level until they rest and build up their energy stores. Although the duration of the light pulse is constant in the three T-cycles, the length of the dark interval is not, and it may be possible that the increased inactivity in the longer T-cycles is due to a larger energy deficit after lights-off in longer T-cycles due to a greater amount of activity since the last lights off.

One model for *period*-gene involvement in the production and maintenance of a biological oscillator maintains that the circadian oscillator actually consists of a number of ultradian oscillators coupled together (Ringo & Dowse, 1987). The *period* gene product is then believed to be the coupling agent, the tighter the coupling, the shorter the composite rhythm. This model argues that apparent arrhythmicity in per^- and per^0 mutants is observed because the ultradian components free-run independently. The periods of these ultradian components can be seen when sensitive Mass Entropy Spectral Analysis (MESA) techniques are used to analyse the data, and when exposed to LD the ultradian components will coalesce into a forced activity band, gradually breaking down into arrhythmicity again upon re-entry into constant darkness.

Another explanation for these results could revolve around *period* having an intrinsic function in the production of an oscillation, as opposed to acting as a coupling agent. The hypothesis that *period* couples together ultradian components to form a composite circadian oscillator relies upon the assumption that ultradian rhythms detected by the sensitive MESA technique are fundamental indicators of an underlying organisation from which circadian rhythms are built. However, this may not be true; the apparent ultradian components detected by MESA may not be produced by ultradian oscillators but by a number of out-of-phase *circadian* oscillators which interact to produce apparent ultradian components in their total activity. Alternatively, the apparent ultradian rhythms may be the product of ultradian oscillators which under normal conditions are not observed because they are masked by the dominant rhythm of a circadian oscillator.

In this case, one explanation for the behaviour of per^- and per^{02} flies in LD and free-running conditions could be that the oscillator controlling locomotor activity is a highly damped circadian oscillator, which behaves to all intents and purposes like

an hourglass. Light 'kick starts' the oscillator which then expresses an inactive phase after the light is switched off, but this oscillation damps out very quickly, before one cycle of activity and rest is complete. Under damped conditions the hyperactivity expresses ultradian oscillations when the coordinating circadian oscillator is not organising activity and masking ultradian components. In this model *period* gene product (PER) plays some role in maintaining the free-running oscillation. Lack of PER means that the oscillatory system controlling locomotor activity damps out. The *period* gene thus plays some role in maintaining the oscillation.

<u>Summary</u>

1. The duration of the free-running rhythm compares well with those recorded elsewhere. Wild-type 23.7 hours, per^{s} 19.7 hours, per^{L2} 29.1 hours.

2. The duration of τ in per^{s} is shortened because α is shortened from 12.1 hours to 8.5 hours, the duration of τ in per^{L2} is lengthened because ρ is lengthened from 11.5 hours to 16.1 hours.

3. Individual flies free-running in constant darkness respond to light pulses which can induce a phase shift in the rhythm. The direction and magnitude of these phase shifts depend upon the duration of the light pulse, the phase of the free-running rhythm illuminated and the mutation at the *period* locus.

4. The short period mutant appears to be more light sensitive than the wild-type which is more light sensitive than the long period mutant.

5. The PRCs for the long period mutant have been found to be positioned to the right of the PRCs of the two other strains.

6. The behaviour of the wild-type flies exposed to light dark cycles of different durations, but all containing six hours light, is not found to be the same as that of the per^{02} mutant, or of the per^{-} strain which lacks the *period* locus altogether.

7. Whilst light PRCs of wild-type flies can be used to predict entrainment to different T-cycles, the same predictions do not produce comparable results in per^{02} or per^{-} strains.

8. Arrhythmia appears almost immediately in the records of per^{-} flies after the locomotor activity rhythm has been forced by a light dark cycle. There is a non-locomotory phase after the last light pulse and then in the absence of a further light pulse, activity appears completely arrhythmic. This can be compared with the gradual breakdown in rhythmia observed in per^{02}.

9. The arrhythmia observed in the per^{-} and per^{02} strains could be the result of a damped oscillator controlling locomotor activity, which in these two strains is so damped as to resemble an hourglass control mechanism.

Chapter 4

The Photoperiodic Response in *D.melanogaster*

Introduction

Photoperiodic time measurement relies upon a clock which can measure the duration of either the day or night and use this information to control seasonally-appropriate developmental strategies such as diapause. It was Bunning (1936) who first suggested that the circadian system could provide the 'clockwork' behind photoperiodic time measurement (PPTM). Since then a large body of evidence has accumulated which supports this hypothesis (Saunders, 1982). Specific experiments demonstrating the circadian involvement in PPTM have been described in chapter 1, the most generally used being the 'resonance' or 'T-experiment' in which an organism's photoperiodic response is compared in different light dark conditions containing the same duration of light but varying lengths of darkness and therefore different light-dark cycles. Maximum short day responses are observed with a short light pulse when this pulse is embedded in a light dark cycle with a period equivalent to the circadian clock or multiples of it (*e.g.* in approximately 24, 48 or 72 hour cycles). Minimum responses are observed between these peaks when the light dark cycle has a duration equivalent to modulo tau + 1/2 tau (*e.g.* in 36, 60 or 84 hour cycles). Clear results of this kind, indicating a circadian involvement, have been seen in *Sarcophaga argyrostoma* (Saunders, 1973a) and *Calliphora vicina* (Vaz Nunes *et al*, 1990), whereas peaks and troughs of diapause induction are not observed with other species such as *Megoura viciae*.

Further evidence of a circadian involvement in PPTM comes from comparative responses of the circadian system to symmetrical skeleton photoperiods

formed by two short and equal pulses of light per cycle. The entrainment of circadian rhythms to such skeletons was discussed in chapter one (p 16). Symmetrical skeleton photoperiods effectively replicate complete photoperiods up to about 10 hours, but when the simulated photophase is longer than about 14 hours, entrainment of the overt rhythm switches so that the smaller gap between the two pulses is taken as the day. Between about 10 and 14 hours the circadian system can adopt either of two steady states depending upon a) the duration of the first dark gap seen and b) the circadian phase illuminated by the first pulse in the train. The region between 10 and 14 hours is called the zone of bistabilty (Pittendrigh, 1966).

Symmetrical skeletons are known to mimic many of the diapause inductive effects of complete photoperiods. For example, in *Sarcophaga argyrostoma* (Saunders, 1975), the system has a tendency to take the shortest gap between the light pulses in the skeleton photoperiod as the photophase, inducing a short day response, in the same way that overt rhythmicity entrains to the skeleton. Such experiments have been used as evidence of a circadian control of photoperiodic time measurement which relies upon the same or similar oscillators that control overt rhythmicity. The fact that the two systems (overt rhythmicity and photoperiodism) appear to share an underlying circadian system has meant that overt rhythmicity has been used as a marker for the phase of the clock underlying photoperiodism, *i.e.* the overt rhythms are considered to be accurate "hands" of the photoperiodic clock. This assumption has often been used to great effect; for example the use of the eclosion rhythm in the analysis of photoperiodic timing in *Sarcophaga argyrostoma* (Saunders, 1978; 1982).

With the discovery of a photoperiodically controlled diapause in *Drosophila melanogaster* (Saunders *et al*, 1989) a genetic dissection of photoperiodic control appeared possible. Newly eclosed female Canton-S flies exposed to less than 14 hours of light a day at a temperature of 12°C were shown to enter a fairly shallow

reproductive diapause. Similar females exposed to 16 hours of light a day at this temperature avoided diapause, exhibiting a slow rate of vitellogenesis. The diapause response was maintained at LD 10:14 for 6-7 weeks before the females emerged from diapause spontaneously. Transfer to a higher temperature or to long days 'broke' the diapause at any time during the 6 to 7 weeks.

A comparison between four wild-type strains of *D.melanogaster* showed similar photoperiodic responses for two recently collected strains, with a minimal response found in a laboratory maintained Oregon-R stock (Saunders & Gilbert, 1990). Exposure of the pupae to long or short days at high or low temperatures did not effect the diapause response in the emerging adults, indicating that the 'decision' to enter diapause is made after the adult emerges from its puparium (Saunders & Gilbert, 1990). The temperature to which the females are exposed after eclosion is crucial to the strength of the response with 12°C being the most effective. It appears that experience of this low temperature enables a photoperiodic control system to influence ovarian development by altering the rate of synthesis of juvenile hormone by the corpus allatum (Saunders et al, 1990). Release of juvenile hormone by the corpus allatum causes the egg follicles to take up yolk polypeptides from the haemolymph and mature ova are produced. A photoperiodic block to juvenile hormone production and release means that the ovarioles remain immature and pre-vitellogenic.

Comparisons of the photoperiodic response curves (PPRCs) of wild-type (Canton-S) females and females taken from *period* mutant stocks, per^{S}, per^{L2}, per^{0} and per^{-}, ought to provide further evidence for circadian involvement in photoperiodic time measurement. For example, entrainment behaviour of per^{S} and per^{L2} flies could be used to predict changed PPRCs if the oscillator controlling the locomotor activity rhythm also controls nightlength measurement (see p 55). The external coincidence model (Pittendrigh & Minis, 1964) assumes a photoinducible phase (Φ_i) in the late

subjective night/early subjective day. In light-dark cycles an oscillation is set by the light-dark transition to a phase which is roughly constant irrespective of the daylength and usually about ct12 (Pittendrigh, 1966). Because this phase is fairly constant the effect of increasing the light pulse duration is that the 'light-on' signal tracks back across the late subjective night. *Per^S^* females, with a shortened period, entrain to light-dark cycles in such a way that the oscillator phase-leads the light (Fig 1.4). Assuming that this oscillation contains the photoinducible phase and that this occurs at the same phase in the mutant as it does in the wild-type, then the photoinducible phase will occur *earlier* in the dark period than in the wild-type and will therefore require a *longer* light pulse to illuminate it. The critical daylength will theoretically, therefore, be *longer* in *per^S^* than in wild-type. The same but opposite argument (that the oscillator in *per^L2^* mutants phase-lags the light-dark cycle) can be used to predict a *shorter* critical daylength in the *per^L2^* mutant than in the wild-type (Fig 4.1).

This hypothesis was tested by comparing the critical daylengths of per^{+}, per^{S} and per^{L2} (Saunders, 1990) along with their response to resonance protocols. The PPRCs of the three strains were found to be almost identical whilst the resonance experiments showed peaks of high diapause at T24 and T48. Since the CDLs were not altered, despite the known periods of 19 or 29 hours, the data suggested that whilst photoperiodic time measurement in *Drosophila melanogaster* was based upon the circadian system, the locomotor activity rhythm (and the *period* gene) was not an accurate marker of the phase of the photoperiodic clock, *i.e.* the locomotor activity rhythm and the *period* gene do not play a *causal* role in photoperiodism.

The present work on the photoperiodic responses of *D.melanogaster* attempts to investigate how photoperiodic timing may be controlled by the circadian system. Initially, photoperiodic response curves (PPRCs) were produced at 12°C for per^{+}, per^{S} and per^{L2} flies to ascertain whether the CDLs were similar. Responses to

symmetrical skeleton photoperiods were also investigated in an attempt to show circadian involvement in PPTM, and, using two temperature controlled cabinets, the photoperiodic responses of the three strains were measured at a range of temperatures.

Photoperiodic response curves in *D.melanogaster* strains

Materials & Methods

Flies from *per*$^{+}$, *per*^{s} and *per*^{L2} laying adults were raised from eggs in S-Y bottles at 25°C, LD 12:12 as described in chapter 2. Similarly, the collection procedure described in that chapter was followed to provide a large daily supply of virgin females and males which were less than 10 hours old. These experimental adults were placed in glass shell vials containing 7ml of standard Lewis medium in light-tight boxes held in a walk-in constant temperature room at 12°C. Illumination of the flies was by 4W fluorescent light bulbs, held in water jackets, and controlled by commercially available timers. The light intensity in the boxes in the region of the flies was measured by light meter and found to have a mean intensity of 1.13 Wm^{-2} (+/-0.05Wm^{-2}).

Collected flies were divided into a number of sub-groups: *per*^{s} and *per*^{L2} strains were divided into two groups, each of which was placed in reciprocal light-dark cycles; if one vial of flies was placed in LD 8:16 then the second vial would be placed in LD 16:8. The wild-type flies, however, were divided into four sub-groups. Two of these groups were wrapped in tinfoil so that they would be confined to constant darkness throughout the experiment. Then one vial in tinfoil and one of the other vials were placed in an experimental light-dark cycle, the other two vials of flies being placed in the reciprocal light-dark cycle. This meant that groups of wild-type flies were exposed to constant darkness but also experienced the small unavoidable temperature cycle associated with the light going on and off. This was done because accurate measurement of temperature inside the light boxes indicated that the fluorescent tubes had a heating effect upon the ambient temperature. This effect was

quite large when compared to the low temperature at which the photoperiodic response was observed.

Over a period of time, three experimental sub-groups of each strain were exposed to each experimental condition ranging from constant darkness through to constant light, in two hour incremental steps. Obviously those wild-type flies wrapped in tinfoil were not exposed to any light but did experience the varying temperatures associated with the changes in light intensity within the boxes. The flies were left in the experimental conditions for two weeks before being dissected to assess the vitellogenic state of their ovaries (in the manner described in chapter 2). The number of females with pre-vittellogenic ovaries and the number of females dissected were recorded, and the percentage of females in diapause in each group calculated. A mean response for each photoperiod was computed by arc-sin transformation of the raw data.

Results

The photoperiodic responses of the three strains, wild-type (*per*$^{+}$), *per*^{S} and *per*^{L2}, are shown in Fig 4.2. The PPRCs are clearly not the same as those predicted, either from previous data (Saunders, 1990) where the critical daylength (CDL) of all three strains were identical, or from entrainment theory (Fig 4.1) where *per*^{S} would be expected to have a longer CDL than wild-type and *per*^{L2} would be expected to have a shorter CDL. In addition, the wild-type does not demonstrate 100% diapause in short days whereas the mutants do. Because 100% diapause is never achieved in *per*$^{+}$ the CDL is taken to be the duration of the light pulse which induces diapause in half of the maximum number of flies which can enter diapause, in this case 40%. This gives *per*$^{+}$ a CDL of 10 hours. The PPRCs of *per*^{S} and *per*^{L2} are similar to each other with a maximum level of diapause of 100% and CDLs of about 16 hours. The PPRCs also

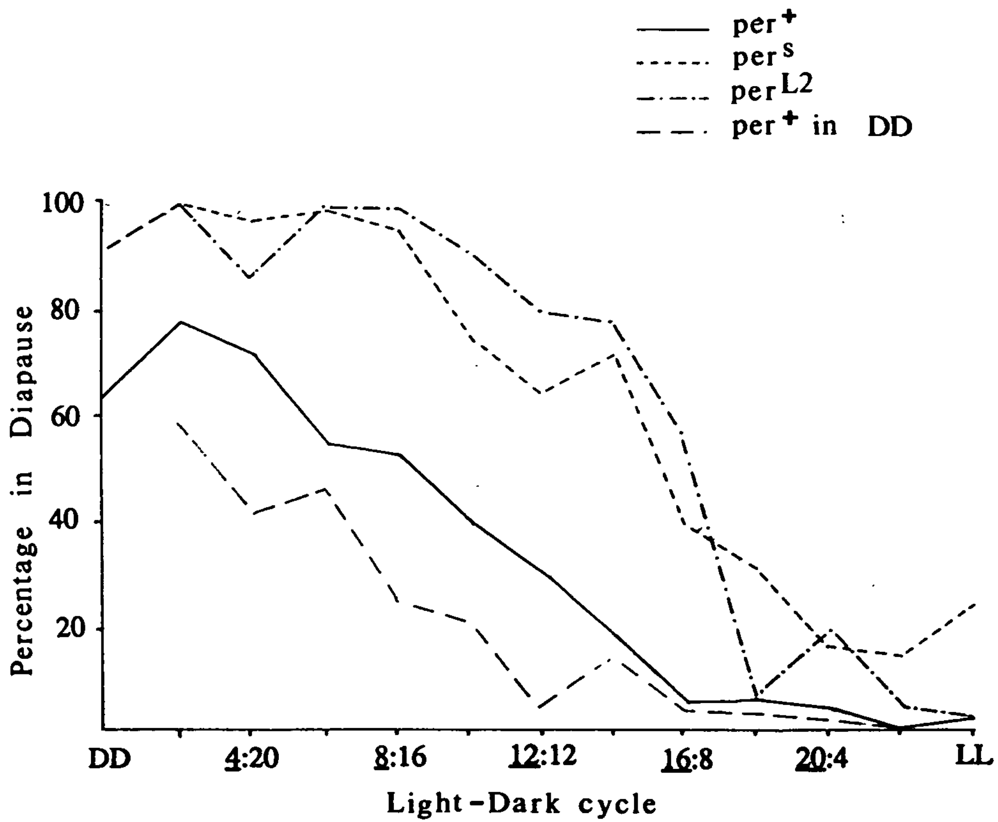


Fig 4.2 Photoperiodic response curves for three strains of *D.melanogaster*, wild-type (*per*$^+$), short period mutant (*per*^{S}) and long period mutant (*per*^{L2}) strains. The curves show a photoperiodic control of diapause where short days induce diapause and long days complete ovarian development. The PPRCs here do not support Saunders (1990) nor do they follow predictions based upon oscillator theory. The fourth curve (*per*$^+$ in DD) plots the diapause response of wild-type flies in constant dark conditions but exposed to the small temperature changes associated with the different light regimens showing that temperature plays a part in diapause development.

highlight a difference in the rate of change from a short day to a long day response. The two mutants have a fairly rapid switch from high to low diapause whilst *per*$^+$ shows a gradual decrease in the diapause response as daylength increases.

The fourth curve shown in Fig 4.2 is the diapause response of *per*$^+$ flies in constant darkness but exposed to temperature cycles in the light boxes. The heat produced by the fluorescent lights clearly effects the diapause response in the wild-type. If temperature had no effect, a constant diapause level of around 60% (the DD response) would be seen irrespective of 'photoperiod'. However, exposure to increasing photoperiods whilst being maintained in constant darkness, *i.e.* a longer duration of heating from the fluorescent bulbs, reduces the level of diapause. Instead of a rapid switch from high to low diapause at the CDL there is a gradual decrease in the incidence of diapause.

Discussion

These results offer only partial support for earlier data (Saunders, 1990). However, they also differ from theoretical predictions based upon entrainment theory. In addition, the data suggest that diapause incidence is being regulated by something in addition to photoperiod. It would appear that raised temperature within the light boxes lowers diapause incidence in the absence of a light dark cycle. This effect of temperature, however, is not a thermoperiodic effect, where the thermoperiod is read by a clock and the duration of the hot (or cold) part of the day (or night) determines seasonally specific development and behaviour. Thermoperiods have been shown to replicate many photoperiodic responses in constant darkness *e.g. Nasonia vitripennis* (Saunders, 1973), *Pieris brassicae* (Dumortier & Brunnarius, 1977) and in *Diatraea grandiosella* (Chippendale *et al*, 1976). However, in all these cases the difference between the high and low temperature of the cycle has been in the order of 10°C, not

the 1 or 2°C difference caused here by the fluorescent tubes. The small rise in temperature experienced in longer photoperiods almost certainly increases the ambient temperature in the boxes above 12°C and causes some flies to break or avoid diapause. This explains the gradual decrease in diapause incidence as photoperiod increases. The photoperiodic response is clearly very sensitive to ambient temperature, an increase of only a few degrees being sufficient to cause a female to break or avoid diapause.

The most obvious conclusion that can be drawn from these experiments is that the light boxes are unsuitable for measuring the photoperiodic response in *Drosophila melanogaster* because these flies are unusually sensitive to the small changes in ambient temperature which are produced in this equipment. This sensitivity is probably due to the "shallow" nature of the response.

For subsequent work the only alternatives to the light boxes were two plant growth cabinets which had very accurate control of temperature but which obviously would take a long time to produce a full PPRC. In contrast, the advantage of using the light boxes was that there was a large number of them. In order to get around this problem, a compromise solution was adopted, in which the accurate temperature cabinets were used to assay the photoperiodic response at different temperatures but only at selected photoperiods. The light boxes were also used to investigate responses to skeleton photoperiods on the assumption that each one hour light pulse used to mark the start and end of a skeleton photoperiod would have very little effect upon the ambient temperature.

Diapause responses of *per*$^+$, *per*^{s} and *per*^{L2} in carefully controlled temperatures

Materials and Methods

Per$^+$, *per*^{s} and *per*^{L2} flies were collected daily from S-Y bottles in the usual way, ensuring that the adults were less than 10 hours old. Collected flies were placed in glass shell vials over 7ml of standard Lewis medium and then placed in plant growth cabinets for 2 weeks before being dissected and scored for diapause. Four experimental conditions were chosen: DD, LD 10:14 (a short day), LD 16:8 (a long day) and LL. The DD maintained flies were kept in glass shell vials wrapped in aluminium foil and placed in constant light at the same time as the LL flies. This was done for two reasons, the first to speed up the experimental procedure, and the second to act as an internal control ensuring that the changes in diapause response were not due to temperature differences. The experimental flies were maintained in constant temperature cabinets with time control of both light and temperature. The cabinets could be set to a given temperature when the lights were on and then reset to the same temperature when the lights were off. This ensured that the temperature within the cabinet was constant irrespective of the heating effects of the light. The three strains of fly were tested three times each in all four conditions at five different temperatures, 11 to 15°C.

The mean arc-sin transformed percentage diapause responses of the strains of flies at all photoperiods at all temperatures were measured and compared. The aim of the experiment was to determine if photoperiodic control of diapause could be observed at temperatures other than 12°C, and to see if the CDL changed in response to temperature in a predictable way in the three strains.

Table 4.1 The mean percent diapause response of females of the three strains of fly tested in four different conditions at 5 different temperatures.

		Temperature /°C				
		11	12	13	14	15
Canton-S						
	DD	37.7	6.5	8.2	0.2	7.6
	10:14	64.9	74.2	63.7	12.4	13.0
	16: 8	72.6	1.4	15.3	0.2	2.9
	LL	64.9	2.4	11.2	7.6	3.0
Per^{S}						
	DD	71.0	41.3	25.0	2.6	
	10:14	99.8	99.5	96.6	18.7	5.4
	16: 8	97.6	7.7	96.3	5.8	6.9
	LL	87.8	26.0	64.3	4.9	7.7
Per^{L2}						
	DD	85.9	3.1	16.7	3.3	33.9
	10:14	98.5	98.9	98.0	4.6	3.6
	16: 8	99.6	4.1	92.3	3.0	0.0
	LL	100.0	27.8	70.2	0.0	3.0

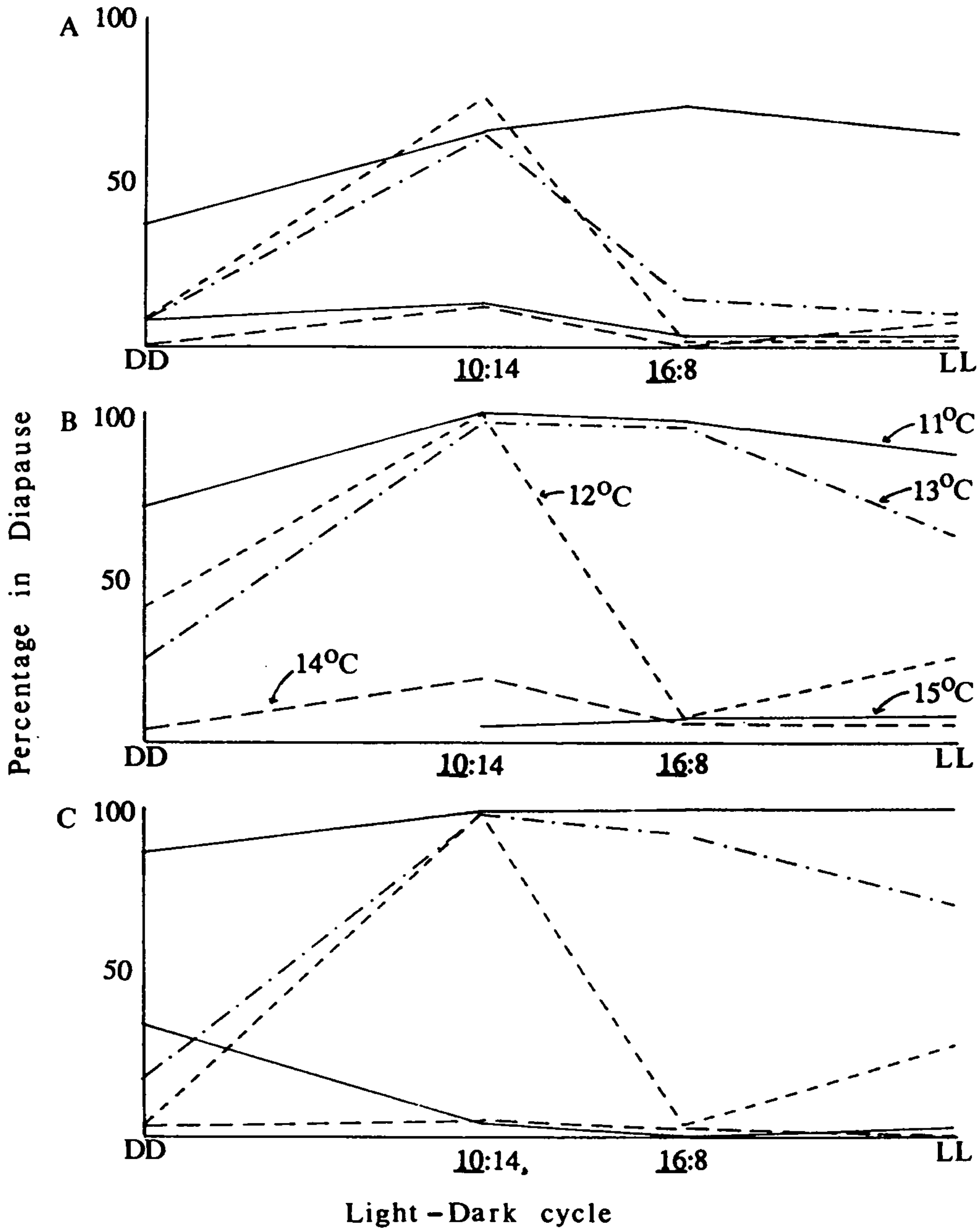


Fig 4.3 Three graphs showing the photoperiodic response of flies to a selection of experimental conditions (DD, LD 10:14, LD 16:8, LL) at five different temperatures ranging from 11°C to 15°C. A) shows the response in wild-type flies, photoperiodic control can only clearly be seen at 12°C and 13°C. B) and C) show the responses of per^S and per^{L2} flies respectively. In both cases photoperiodic control of diapause can only be seen at 12°C.

Results

The diapause responses of the three strains at each temperature are given in Table 4.1 and in Fig 4.3. For all three strains the portion of females entering diapause, in all the photoperiods, at 14°C and 15°C is low. On the other hand, at 11°C the three strains have a high incidence of diapause irrespective of the photoperiod. In addition, *per*^{S} and *per*^{L2} at 13°C appear to demonstrate very little photoperiodic control of diapause in contrast to the wild-type which demonstrates a response at this temperature as well as at 12°C. At 12°C all three strains demonstrate a clear photoperiodic response with a decrease in the incidence of diapause in LD 16:8.

A comparison of the photoperiodic responses of the three strains at 12 and 13°C is shown in Fig 4.4. The three strains respond to the different photoperiods at 12°C in a similar fashion, with the same critical daylength although the level of the response in the two mutants again appears to be higher than in the wild-type when they are exposed to LD 10:14.

The final figure, Fig 4.5, shows the change in diapause at each photoperiod at different temperatures, for *per*$^{+}$, *per*^{S} and *per*^{L2} respectively. In *per*$^{+}$ it appears that diapause incidence is low in DD, LL and LD 16:8 at all temperatures except 11°C. Diapause incidence in LD 10:14 is quite different, rising at 13°C and peaking at 12°C. The response of the two mutant strains, however, is not the same. In *per*^{S}, the incidence of diapause in DD increases with decreasing temperature. The response of flies in LD 10:14 to different temperatures is similar to the response of the wild-type flies with an increase in diapause at 13°C. However, the response in LD 16:8 and LL are surprising: at 13°C there is a rise in diapause which is followed by a sharp decrease at 12°C. The same can be seen in the response of *per*^{L2}. Flies in LD 10:14

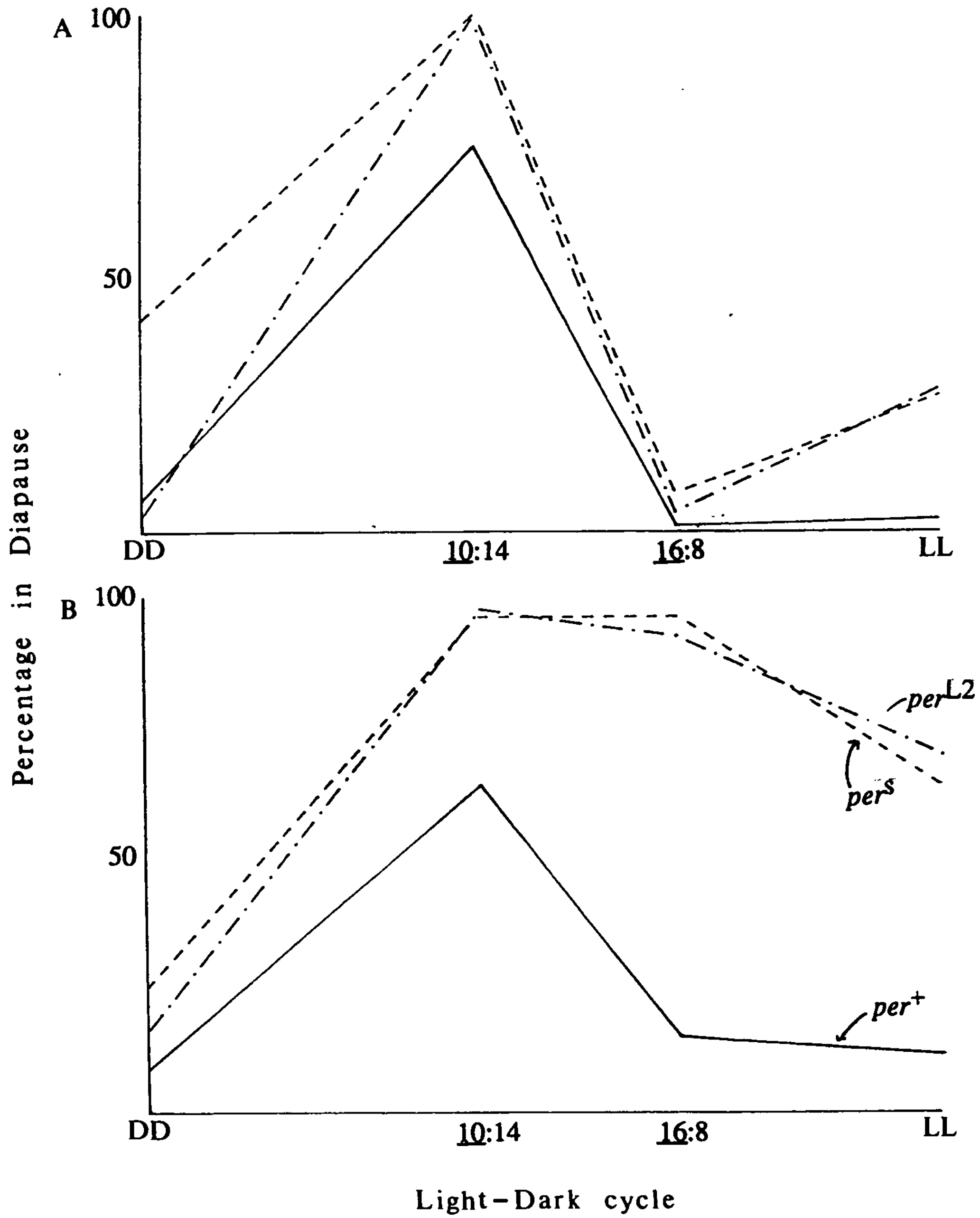


Fig 4.4 A comparison of the data shown above for *per*$^{+}$, *per*^{S} and *per*^{L2} at A) 12°C and B) 13°C. In A) all three strains appear to have a very similar critical daylength of around 13 hours, whilst in B) a critical daylength can only be seen in the wild-type strain.

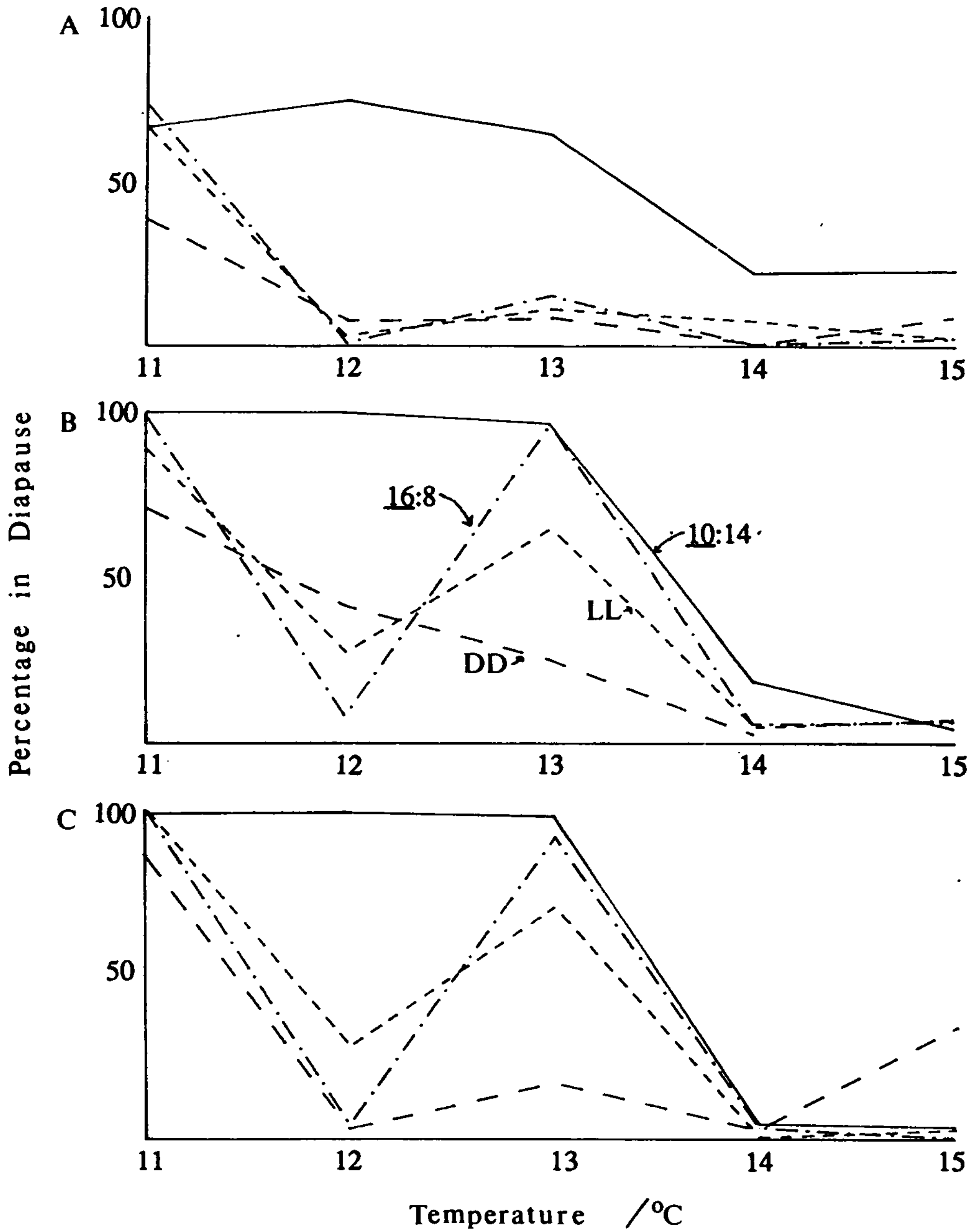


Fig 4.5 Three graphs showing the response of a population of flies to different temperatures in the same lighting conditions. A) shows the high diapause response of *per*$^+$ flies in LD 10:14 beginning at 13°C and peaking at 12°C. B) and C) show the response of *per*^{S} and *per*^{L2} respectively. These two strains have high diapause responses at 13°C in all lighting conditions.

respond in a similar fashion to the wild-type at the different temperatures with an increase in the incidence of diapause at 13°C. The response in DD is also very similar to the response of the wild-type flies with an increase in the response at 11°C. However, the response of *per*^{L2} in LL and LD 16:8 at different temperatures differs from the wild-type with an increase at 13°C but a swift decrease again at 12°C.

Discussion

The diapause response measured in this experiment is the response of the flies to light alone. The plant growth cabinets accurately control the temperature so that there are no large fluctuations in the ambient temperature when the lights go on or off. The procedure ought to remove the differences caused by changing temperature shown in the earlier photoperiodic response curves (Fig 4.1).

Responses of all three strains to the four photoperiods at 11°C are similar and are not a photoperiodic effect. The lack of ovarian development is probably due to quiescence caused by low temperature at which developmental/biochemical processes cannot proceed, or proceed so slowly that it takes more than 14 days for yolk deposition in the oocytes to be detected. Similarly, at 14°C and 15°C the low level of diapause in all three strains is not due to photoperiodic control but to temperature stimulating full ovarian development even in short day conditions. As such, these temperatures mark the boundary of the photoperiodic response in *Drosophila melanogaster*: the photoperiodic system seems to only operate in a very narrow range (12°C to 13°C). This range of temperature is even more restricted in both mutant strains. These results show how important temperature is to the expression of the photoperiodic response, and may highlight the primitive nature of the system (see chapter 5 & 6).

Comparing the photoperiodic responses of the three strains of fly at the different temperatures would seem to indicate that all three strains are similar in their responses at all the temperatures but one (13°C). At 11°C, 14°C and 15°C the three strains have very similar responses which do not involve the photoperiodic system, or at least if the photoperiodic system is involved then its control of ovarian maturation is over-ruled by the ambient temperature. At 12°C the photoperiodic responses are similar with the three strains having similar critical daylengths (CDLs). This result supports Saunders (1990). However, the fact that a PPRC can be produced for the wild-type at 13°C and not for *per*^{S} or *per*^{L2} implies that mutations at the *period* locus plays a modulatory role in the photoperiodic response.

The narrow range of temperatures at which photoperiodism can be observed strengthens the conclusion that the PPRCs produced in the light boxes were influenced by temperature. It would not take a large rise in temperature for the photoperiodic response to be over-ridden by the temperature and obviously the longer photoperiods are more likely to have an increase in ambient temperature than the shorter ones. However, strong evidence for the photoperiodic control of diapause is provided by these experiments with accurately controlled temperatures. In all three strains at both 12°C and 13°C (Fig 4.4) there is a much lower incidence of diapause in constant darkness when compared to the short day condition.

Fig 4.5 plots the change in diapause induction in each photoperiod with temperature for the three strains. The data suggest that the temperature effects on the photoperiodic response at 12°C are due to a reduction in diapause induction in long days. The effects of temperature on the diapause response of all three strains in LD 10:14 are very similar: a rise in diapause incidence at 13°C which reaches a maximum at 12°C. However, in longer photoperiods (LD 16:8 and LL) the loss of a photoperiodically controlled diapause response in *per*^{S} and *per*^{L2} strains at 13°C

appears to be due to a higher level of ovarian quiescence *i.e.* the photoperiodic effect of long days (low diapause) is over-ruled by the low temperature. This suggests that in *D.melanogaster* the photoperiodically important mechanism is a forcing of ovarian development at low temperatures due to long day conditions.

Responses to skeleton photoperiods

Materials and Methods

Two methods were used to investigate responses to skeleton photoperiods. Both methods attempted to synchronize suspected photoperiodic oscillators in the freshly emerged adults so that all flies would experience the first pulse of light at the same phase.

Method A: Freshly emerged adults were collected from S-Y bottles in the usual manner (Chapter 2) ensuring that they were under 10 hours old. These flies were divided into two groups and placed in glass shell vials over standard Lewis medium. Both vials of flies were then placed into a light box in the walk-in constant-temperature room at 12°C. These flies were then exposed to a light pulse which ensured that at 'lights off' all the flies had experienced a 12 hour photophase during the previous 24 hours. After this first 12 hour light pulse, into which the adults emerged and experienced the drop in temperature necessary for photoperiodic control of ovarian maturation, the flies experienced a further 12 hours of darkness. At 'lights on' the following morning the flies were exposed to the first one hour light pulse of the skeleton photoperiod. The two vials of flies collected from each strain were placed into reciprocal skeleton photoperiods (*e.g.* 1:x:1:y or 1:y:1:x) for 14 days before the females were dissected and their diapause status assessed. For each experimental lighting condition three vials of flies from each strain were used to assay the response. The raw data were combined by arc-sin transformation to produce mean diapause responses to each skeleton photoperiod for the three strains.

Method B: The experimental adults were raised from eggs in constant light and collected upon emergence in the usual manner, ensuring that all the experimental

adults were less than 10 hours old. Freshly emerged *per*$^{+}$, *per*^{s} and *per*^{L2} adults were collected daily and divided into two groups, each placed into glass shell vials over standard Lewis medium. The experimental adults were then placed in reciprocal skeleton photoperiods at 12°C experiencing the first dark gap in the skeleton photoperiod at the same time. This protocol was intended to ensure that all the females began measuring nightlength at the same phase because the photoperiodic oscillators would in theory begin at the approximately the same time (around Ct12) at the first light/dark transition (Pittendrigh, 1966). The flies were left in these skeleton photoperiods for 14 days before being dissected and their diapause status assessed. Three separate vials of flies of each strain in each photoperiod were used to assay the photoperiodic response to that photoperiod. The raw data were combined in the way described for method A.

Results

Responses to the symmetrical skeleton photoperiods were produced by both method A and method B (Fig 4.6). This figure shows a difference between the wild-type and *per*^{s} and *per*^{L2} mutants, consistent with earlier results for complete photoperiods. That is, the maximum diapause response of the *per*^{s} and *per*^{L2} flies is higher than the maximum for *per*$^{+}$. It appears that both mutant strains have a greater tendency to enter diapause in diapause inducing conditions than the wild-type.

In Fig 4.6B (method B), aside from this quantitative difference in response, there appears to be no qualitative differences. The response of the flies, in terms of diapause induction, is quite high in these low temperature conditions and not significantly influenced by the skeleton photoperiod.

Method A (Fig 4.6A) shows both a quantitative difference in the response of *per*$^{+}$ and the mutants, and a qualitative difference. Again the *period* mutants tend to

have higher maximum levels of diapause induction. These two strains appear to have elevated diapause responses across the whole of the skeleton range. In the wild-type, however, the response curve has an area in the middle where the diapause response is depressed. Skeleton photoperiods of about 13 hours in duration (*e.g.* LDLD 1:11:1:11) induce a drop in diapause induction, which then quickly rises again. The amount of diapause induced in the skeleton of 12 hours and 15 hours is significantly higher than the level of diapause induced in 13 or 14 hour skeleton photoperiods (P<0.001 and P<0.01 level by Chi Square).

Discussion

The first most obvious difference between the strains in both experimental protocols is the higher maximum diapause response in the two mutants when compared to the wild-type. This difference was also seen in the previous section (p121). Why the *period* mutant strains have this elevated diapause incidence, and whether the difference is due to the *period* mutant *per se*, are questions that need to be addressed. There are two possible explanations for this: (1) that this difference is due to a general similarity in response of all mutant females because of their genetic similarity; or (2) that the two *period* mutants somehow effect the level of response in these strains in the same way perhaps by changing the tendency of the females to respond.

The other major difference between the skeleton PPRCs is the presence of a depressed region of diapause induction in the skeleton PPRC of wild-type when method A was used. This drop in diapause induction has been taken to indicate a circadian basis to photoperiodic time measurement in some organisms because it implies a parallel between the photoperiodic system and overt behavioural rhythms in the zone of bistability (Saunders,1982). This one result suggests that the

photoperiodic time measurment system is controlled by a circadian oscillator which entrains to the skeleton photoperiods in a 'classic' fashion. This is not seen in either mutant strain or in the wild-type when method B is used. The explanation for this is obscure. However, it may lie in one or more of the following: the experimental design in *method B* was inadequate; the presumed constituent oscillators might be excessively damped; or the changed periods of the mutant flies might cause a loss of entrainment to the 24 hour skeleton photocycles.

If the oscillator(s) controlling photoperiodism are damping, it implies that this clock is separate from the locomotor activity rhythm, which is remarkably self-sustained. In other words, the circadian oscillator(s) governing locomotor activity in per^{s} and per^{L2} are self-sustained and show periods of 19 and 29 hours respectively, whereas those involved in photoperiodism may be highly damped with a period of 24 hours.

Summary

1. Wild-type, *per*^{S} and *per*^{L2} strains of flies measure daylength or nightlength in order to determine season and use this to control ovarian development.

2. The measurement of day/nightlength is extremely temperature sensitive in wild-type, *per*^{S} and *per*^{L2} flies.

3. At 12°C the critical daylength of the photoperiodic response in *Drosophila melanogaster* is about 13 hours in the wild-type and in both long and short period mutant strains.

4. Loss of photoperiodic control at low temperatures appears to be due to an inability of the system to overide low temperature-induced quiescence in long day (summer) conditions.

5. Evidence suggests a circadian control of photoperiodic time measurment although the oscillator measuring day/nightlength appears to be highly damped and have a similar period in the wild-type, *per*^{S} and *per*^{L2} flies.

Chapter 5

Modelling the Circadian Photoperiodic Clock in *D.melanogaster*

Introduction

Models in biological research can fall into roughly three categories: descriptive, computer/mathematical, or animal. As a solution to a biological problem models provide a working hypothesis which can successfully summarise all the available data. Any model can be deemed successful only so far as it can explain these data, and as new results show a model to be erroneous then it can either be adapted to account for the new data or replaced by a more effective model. Such models are not intended to be 'true' representations of the underlying biological system but active developing descriptions which help in understanding the possible workings of a biological phenomenon.

A large number of different types of physiological rhythms have been modelled in a large variety of ways. The models used can be generally classified into two types; relaxation oscillator models, or limit cycle oscillations. Of the later class of model there are a number of different mechanisms. The "integrate and fire" models are clearly more straight forward. For example, in the micturation reflex the bladder fills as time passes until eventually micturation takes place, the bladder empties and the process starts anew. In general, such models have a quantity called the *activity* that rises to a threshold and this leads to an event. The activity then instantaneously relaxes back to a second lower threshold from where it begins again to rise. Oscillations in systems associated with limit cycle oscillations are most commonly modelled by non-linear equations. Limit cycle oscillations are ones that are usually re-established after a small perturbation.

The vast number of rhythmic systems modelled and the variety of models used makes it impossible to distil the list into anything but a crude categorization which splits limit cycle oscillators into either pacemaker models, in which a cell or group of cells spontaneously oscillates, or neural network interaction models, in which neural cells produce a rhythmic output as a result of network interactions between the different cells or cell groups of the network. It is now generally accepted that circadian rhythms can be modelled successfully as limit cycle oscillators, and, in fact, phase response curves have been successfully modelled by these and their responses to perturbations (Winfree, 1980).

The modelling of circadian oscillators has itself become very popular, partly due, no doubt, to the fact that very little is known about their general physiology or biochemistry. Models based upon biochemical interactions (Pavlidis & Kauzmann, 1969), time delays (Johnsson & Karlson, 1972; Engelmann *et al*, 1973) and membranes (Njus *et al*, 1974; Njus, 1976) have all been described in the literature. The variety of different models reflects the relative ease with which simple oscillators can be produced which in turn may be reflected *in vivo* in the variety of different oscillatory systems developed through natural selection. Selective pressures upon organisms will affect the accuracy and reliability of the biological timing mechanism but not necessarily their underlying structure. Different oscillatory systems may have evolved several times in different groups of organisms and convergent evolutionary forces acted on these systems resulting in a seemingly "universal" biological clock.

Such simple dynamic models that have been described in the literature appear initially to be very successful models of biological data; however, nearly all of them have a common criticism. They only appear to be able to model 'free-run period lability', that is, such things as spontaneous change in the period of the free-running rhythm or rhythm splitting, when random variations in the parameters, noise or an

increase in the number of parameters are incorporated into the model. Inclusion of such processes are biologically feasible but they no longer leave models simple to use or understand. Pavlidis (1969) first suggested that the coupling of individual 'pacers' to produce a composite clock enables the more aberrant behaviour seen *in vivo* to be observed in the models.

Many of the ideas behind models of circadian oscillations have been used to describe the *period* mutants' involvement in a circadian oscillation and experimental evidence has been sought, and found, to support these. One of the first models was a membrane model proposed by Konopka & Orr (1980). Evidence for this model came from two sources. The first was from analysis of the amino-acid sequence of the *period* protein product which appeared to contain a Threonine-Glycine repeat sequence similar to those found in transmembrane proteins. In this model Konopka & Orr proposed that the basic oscillation controlling locomotor activity and eclosion consisted of the establishment of an ion gradient across a membrane. The *period* gene product (PER) is an ATP-requiring ion pump which during the subjective day establishes an ion gradient. During the subjective night a second transmembrane protein which is photosensitive opens and allows the ion gradient to deplete. Mutations at the *period* locus would, theoretically, alter the duration of the subjective day so, for example, per^{S} ought to have a short τ because the duration of the subjective day, α, is shortened, whilst the subjective night, ρ, remains the same as in the wild-type. This has been shown to be the case (Konopka & Orr, 1980; chapter 3). However, the model also predicts that the increase in τ seen in per^{L2} ought to be due to an increase in the duration of α with ρ remaining the same as in the wild-type. This is not supported by the data in chapter 3.

Another model for *period*'s involvement in the production and maintenance of a circadian oscillation comes from observations in the larval salivary glands of

Drosophila melanogaster in which *period* gene product was seen to be localised at cell membranes, and appeared to control cell-cell interactions by acting at gap junctions (Bargiello *et al*, 1987). In preparations of larval tissue *period* seemed to modulate gap junction channels so that their conductance varied inversely with the period of the behavioural rhythm in the mutants. It was proposed that a similar effect on junctional communication in the nervous system may explain how *period* influences behavioural rhythms. This idea seemed to fit exceptionally well with MESA analysis of the locomotor activity rhythms of per^0 flies which appeared to exhibit ultradian rhythmicity within ostensibly arrhythmic records. It also agreed with the Pavlidis (1969) model of ultradian pacer interaction. Cells within the adult nervous system could spontaneously oscillate with ultradian periods and *period* could influence the coupling between such cells allowing them to form composite clocks of different periodicities. However, this model has lost favour for a combination of reasons, including an inability to replicate the evidence from salivary glands and evidence that far from being localised at cell boundaries in the adult nervous system the *period* gene product is nuclear (Liu *et al*, 1992) and the *per* mRNA and PER have been shown to cycle with a circadian period in the wild-type (Hardin *et al*, 1990).

Molecular analysis of the *period* gene function has more recently suggested that the PER product is a DNA binding protein involved in sequestration of a transcriptional factor (Rosbash, *pers comm*). There are four pieces of evidence which Rosbash claims indicate this: the *period* gene product is predominantely nuclear; it influences its own circadian transcription; PER bears a family resemblance to other DNA binding proteins like SIM (protein product of *single-minded* in *D.melanogaster*), ARNT and AHR (human gene products involved in DNA binding and sequestration of transcription factors); and finally there appears to be no association of PER with chromatin.

Modelling has also been used by Lewis & Saunders (1987) to provide a simulation of Bunning's original (1936) hypothesis that the circadian system provides the clockwork for photoperiodic time measurement. This means that a circadian oscillator is used to determine daylength, and daylength is used as an indicator of season. The Lewis & Saunders model assumed an external coincidence model of photoperiodic induction in which light illuminating a photoinducible phase during steady state entrainment causes non-diapause development, whereas, when this phase is not illuminated, diapause supervenes.

The computer model was based upon a control system feedback model for the locomotor activity rhythm in *Hemideina thoracica* (Gander & Lewis, 1979; Christensen *et al*, 1984). The single oscillator involved depends upon the interaction of a temperature dependent synthesis of an oscillating chemical, *c*, and its temperature independent loss. The synthesis of the oscillating chemical *c* is controlled by the difference between an arbitrary reference concentration ($conc_{ref}$) and time delayed levels of *c*. Each time *c* is synthesised the amount produced is added to the existing concentration of it and simultaneously a portion of *c* is lost from the system. The amount of *c* that degrades is determined by two processes, the first light independent and the second light dependent. The light independent loss is determined by a 'loss' function such that a proportion of the concentration of *c* degrades. If, however, the system is exposed to light then a further amount of *c* is destroyed by the light. The amount destroyed by this process is determined by the strength of the light. Synthesis and loss are thus major components in a negative feedback system in which time delay is a prerequisite for the oscillation.

The photoinducible phase (Φ_i) is incorporated into this circadian oscillation through the use of a threshold. In the model Φ_i is the point on the oscillation when the level of *c* falls below the threshold. If this point occurs in the dark then a

"diapause titre" (INDSUM) is synthesised in a temperature dependent way. If, on the other hand, this point is illuminated then this titre is broken down. The titre is synthesised only during a temperature dependent "sensitive period", at the end of which the total amount of titre synthesised is taken to be an indication of the population diapause response.

When the parameters of this model were changed, simulated photoperiodic response curves were produced which were remarkably similar to experimentally produced PPRCs in a variety of insects (Saunders & Lewis, 1987a). Specifically, changing time-delay alters the free-running period of the oscillation which in turn changes the critical daylength, all other parameters remaining equal. A large time-delay, and consequently a long free-running period, produces a PPRC with a short critical daylength and *vice versa.*

The use of time delay in this model to control the period of the circadian oscillator underlying photoperiodism is appropriate for two reasons. The first is that in modelling the photoperiodic system it is not necessary to accurately model the oscillatory mechanism. The data show that the *period* mutants are involved in the production and/or maintenance of a central pacemaker, and the aim is to model how this oscillator can be used to measure season. The negative feedback system is a convenient tool for producing oscillations with 19, 24 and 29 hour periods. Secondly, however, the time delay and negative feedback may also be an appropriate model for central pacemaker function. The molecular biology data discussed above appear to require some form of post-transcriptional control which will incorporate a time delay.

Lewis & Saunders' model (1987), however, will clearly not model the photoperiodic response in *Drosophila melanogaster* as their work showed that when the oscillator controlling overt rhythmicity also controls PPTM then the alteration of

the period of the oscillator controlling overt rhythmicity had predictable effects upon the critical daylength of the photoperiodic response; namely a short τ resulted in a long CDL and a long τ resulted in a short CDL. This is not what is observed in this fly. This chapter therefore aims to model the photoperiodic response in two ways. Using the light PRCs produced in chapter 3 it is possible to predict the phase relationship between oscillator and light during steady state entrainment. This can then be used for wild-type, *per*^{S} and *per*^{L2} to predict either CDL or the photoinducible phase, assuming that the oscillator controlling locomotor activity and incorporating *period* is the same one that measures nightlength in the photoperiodic system. Secondly the Lewis & Saunders' model will be adapted to account for the results of chapter 4, *i.e.* that *period* mutations have no causal effect upon CDL, and also results reported in other drosophilids.

Modelling the photoperiodic response using PRC data from the locomotor activity rhythm

Materials and Methods

For equivalent photoinducible phases in the three strains, wild-type, *per*^{S} and *per*^{L2}, their critical daylengths can be predicted, or, for similar CDLs the photoinducible phases of the three strains can be predicted. This relies upon two assumptions; the first is that photoperiodic time measurement can be adequately described using the external coincidence model; the second, that one long light pulse can be conveniently modelled by a series of shorter ones end to end (Johnsson & Karlsson, 1972).

Using the one hour and six hour PRCs for *per*$^{+}$, *per*^{S} and *per*^{L2} a computer program was written in BBC BASIC (Appendix iii) to calculate the circadian phase of the locomotor activity rhythm first illuminated by the light during steady state entrainment. Light pulses other than one and six hours in duration were modelled by adding different light pulses together, so entrainment to LD 8:16 could be modelled by assuming the light pulse consisted of eight one hour pulses one after the other with no dark gap in between or, alternatively, one six hour pulse followed by two one hour pulses. Assuming a one hour light pulse initially falls at ct12 of the wild-type free-running rhythm, the PTC (Fig 3.15) can be used to predict the phase of the rhythm after the light pulse due to the phase shift induced by the light pulse and the subsequent continuation of the rhythm during the one hour of light to ct8.7. A second one hour light pulse immediately after this first (to produce a composite two hour light pulse) then falls at this new phase of the rhythm, resulting in the oscillator entering darkness at ct 7.2. After a further 22 hours of darkness the next one hour light pulse illuminates the rhythm (T=24; LD 2:22) at ct5.5 (ct7.2 + 22.2circadian hours of

darkness - 24 = 5.5). The first of the two light pulses in the next light-dark cycle falls at ct5.5 shifting the rhythm to ct5.9, the second pulse shifts the rhythm to 6.1 and then 22 hours of darkness (= 22.2 circadian hours in the wild-type) means that the next light pulse illuminates ct4.3. This process is continued until steady state entrainment is observed when the light pulses illuminate the same phase of the rhythm over number of consecutive cycles. Statistical comparison of the two methods of modelling long light pulses (using only one hour light pulses or using six hour and one hour light pulses) for the three strains showed no significant differences and therefore, for consistency, only the one hour light PRCs were used to model the photoperiodic system (Mann Whitney $U = 39, n_1 = 9, n_2 = 9$).

Results

The model used the PRC data to predict the phase of the rhythm first illuminated by light during steady state entrainment and these values are shown in Table 5.1. The critical daylength of the three strains of flies was around 14 hours (see chapter 4). When these flies were entrained to LD 14:10 different phases of the locomotor activity rhythm were first illuminated by the light. In *per*$^+$ it was ct16.51, in *per*^{s} it was ct19.61 and in *per*^{L2} it was ct21.53, *i.e.* the photoinducible phase in the three strains is not the same. If on the other hand we assume that the photoinducible phase is the same in the mutants as it is in the wild-type (ct16.5) then we can plot the position of the photoinducible phase as a function of the entraining light dark cycle (Fig 5.1).

Discussion

This figure (5.1) clearly demonstrates that the critical daylength (the point when the photoinducible phase is first illuminated) would not be the same in the three

Table 5.1 **Predicted phase of rhythm first illuminated by light during steady state entrainment using experimentally measured one hour light PRCs**

Duration of Light in 24 hour light cycle*	Phase of rhythm first illuminated for		
	per^{+}	*per*^{S}	*per*^{L2}
2	3.41	9.89	
4	1.35	7.79	
6	23.82	5.35	3.86
8	22.35	2.91	2.46
10	20.49	0.48	0.69
12	18.51	22.04	23.18
14	16.51	19.61	21.53
16	14.49	17.17	19.88
18	12.47	14.73	18.23
20	10.44	12.30	16.58
22	8.42	9.86	14.93

* calculated by summating the phase shifting effects of consecutive one hour light pulses.

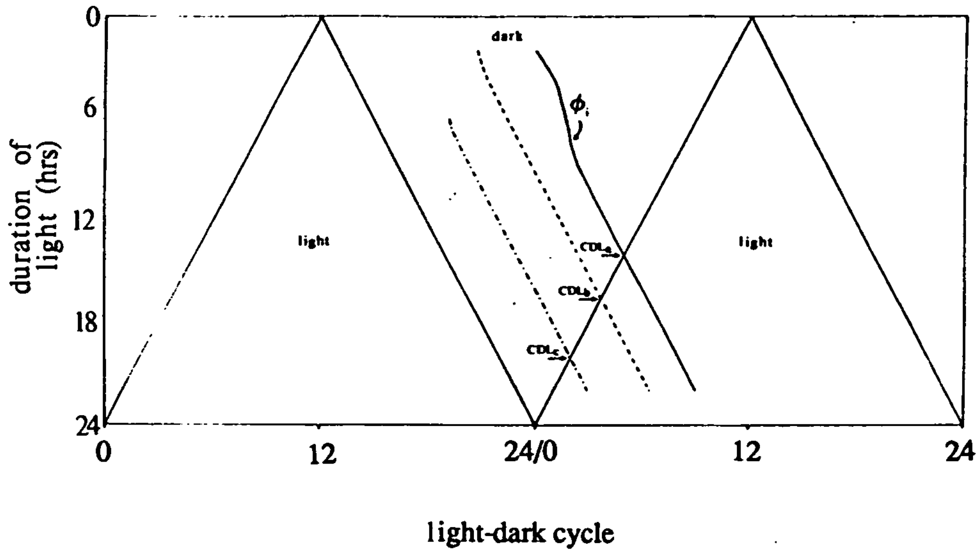


Fig 5.1 Model predictions of the phase relationship between the locomotor activity rhythm in *per*$^+$, *per*^{S} and *per*^{L2} and various light-dark cycles. The phase marker is a hypothetical photoinducible phase, Φ_i, (assuming that the oscillator controlling locomotor activity also measures nightlength in the photoperiodic control of diapause) of the wild-type response producing a critical daylength of 14 hours (CDL_a). Using the light PRC data collected in chapter 3 predictions for the CDL of *per*^{S} (CDL_b) and *per*^{L2} (CDL_c) show that if the oscillator controlling locomotor activity also measures nightlength then one could predict different CDLs in the three strains.

strains of fly if the oscillator controlling the locomotor activity rhythm also contained the photoinducible phase at CT 16.5 in all strains. The figure actually predicts that *per*^{L2} would have the longest CDL at 20 hours, *per*^{S} the next longest CDL at 16.6 hours and the wild-type the shortest CDL at 14 hours. Clearly this is not what is shown in Fig 4.2

The other explanation for similar CDLs is that the photoinducible phase is different in the three strains. However, for CDL to be the same in the three strains then the photoinducible phase would have to be at CT 19.6 and CT 21.5 for *per*^{S} and *per*^{L2} respectively. This is not supported by the evidence in chapter 3. In the case of the short period mutant the change in the photoinducible phase is from CT 16.5 (wild-type) to CT 19.6. Whilst this cannot be ruled out the evidence in chapter 3 shows that the *per*^{S} mutation appears only to effect the subjective day, and does so by reducing it in duration from 12 to 7 hours, a decrease of 5 hours. This figure of five hours is not compatible with the three hour change in position of the photoinducible phase during the subjective night. The modelling of the CDL of the photoperiodic response using the light PRCs of the locomotor activity rhythm does not produce data consistent with experimental results.

This work implies that the oscillation controlling the locomotor activity rhythm in *D.melanogaster* is not the same oscillator as that involved in photoperiodic time measurement, a conclusion also reached by Saunders (1990) when he compared the photoperiodic response curves and resonance responses of the *period* mutant flies. This work leads to the conclusion that whilst *period* may be involved in photoperiodic time measurement at *some* level, it is not *causally* involved, and therefore, by extension, neither is the oscillation controlling the locomotor activity rhythm. In other words, although the locomotor activity rhythm and photoperiodic time measurement are both based on circadian rhythmicity, they do not have a common pacemaker.

Modelling the photoperiodic response in *Drosophila melanogaster*

Materials and methods

The success of the original damped oscillator model (Lewis & Saunders 1987), and its ability to describe a range of experimental situations (*i.e.* the PPRCs of a number of insects) cannot easily be dismissed. The model proposed here, therefore, builds upon it, and involves two circadian oscillators, a 'pacemaker' and a 'slave'. The pacemaker is self-sustained in constant darkness, light entrainable but without a photoinducible phase. It is regulated by the *period* gene and as such can have a free-running period of 19, 24 or 29 hours. A second oscillator (the 'slave'), which does not involve *period* and is therefore the same in all three strains, contains the photoinducible phase which determines whether the ovaries develop along a diapause or non-diapause pathway. This oscillation (the slave) is the same as that described in Lewis & Saunders (1987); it is damped and light entrainable, but also entrained by the first oscillation, or pacemaker. The level of entrainment from the pacemaker is determined by the strength of a coupling factor (CF). The modelling program was written in BBC BASIC and is given in Appendix iii. The control system for this model is shown in Fig 5.2. The following description can be applied equally to the production of the 'pacemaker' or 'slave' oscillators, except that, as was mentioned above, the 'slave' has an additional input from the 'pacemaker' through the 'coupling factor' (CF). A reference concentration of the oscillating chemical *c* is compared with the concentration of *c* a fixed amount of time in the past, governed by the 'time delay' (TD), a large TD results in an oscillation witha long period and a small TD results in a short period oscillation. The difference between the reference *c* and the time-delayed *c* is used to determine the amount of *c* synthesised. The rate of synthesis (SR) can be altered with a high SR producing a self-sustained oscillation, a low SR producing a damped oscillation. A percentage of *c*, determined by a 'LOSS' function, is lost and

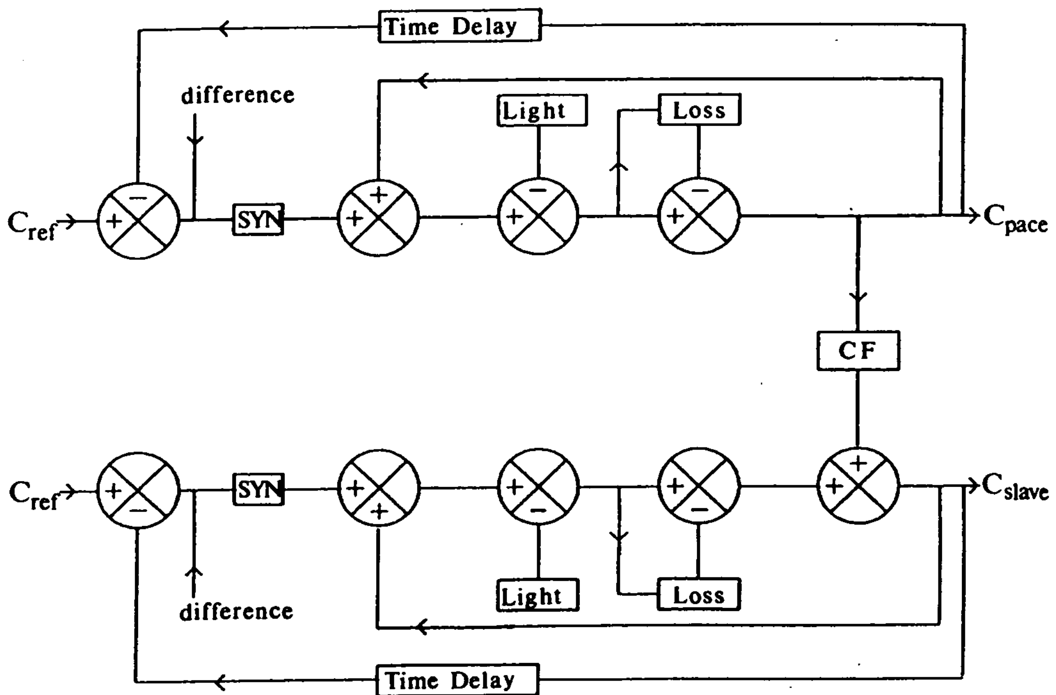


Fig 5.2 Control systems diagram for the photoperiodic determination of diapause in *Drosophila melanogaster* using a self-sustained pacemaker oscillation and a damped slave, both of which are light sensitive. The slave oscillation is entrained by both the pacemaker through a variable coupling factor and the external light-dark cycle. Both oscillations are produced because synthesis and loss of a substrate are interlinked by a time delay factor.

light degrades *c* at a rate proportional to the intensity of the light. The oscillating chemical (*c*) has a 'threshold' level which determines the phase of Φ_i. When the concentration of *c* falls below the 'threshold' the system determines whether it is illuminated or not and this signal is used to determine whether diapause ensues or not through a 'counter' system.

Computer simulations

The parameters were set for the pacemaker oscillation so that it was self-sustained (SR=0.14)b and ran with a free-running period of either 19.5 hours (TD = 23; P_s equivalent to *per*^{S}) or 29.4 hours (TD = 37; P_l equivalent to *per*^{L2}). The parameters for the slave oscillation were set so that this damped out in constant conditions (SR=0.10)and had a period length of 23.4 hours (TD = 28). The threshold value was set so that the photoinducible phase was in the late subjective night/early subjective day and the critical daylength (CDL) of the slave oscillation (with no influence from the pacemaker) was 14.5 hours, similar to the CDL of wild type *D.melanogaster*. The other parameters are given in the program listed in Appendix iii.

The model was used to simulate PPRCs for coupled systems with different levels of the coupling factor. The aim was to show how CDL can be conserved in the three strains of fly with radically different pacemaker periods, and how CDL can change without necessitating major changes in the circadian system. The results of the computer simulations are shown in Fig 5.3. The PPRC for the slave oscillation alone is shown in the bottom panel with a critical daylength of 14.5 hours. The series of PPRCs on the left show the effect of increasing coupling strength on CDL and PPRC shape when the slave is coupled to a short period pacemaker, P_s. Increasing the coupling factor leads to an increase in CDL. However, a CF of 2 (weak) produces a PPRC very similar in shape to the slave PPRC with a CDL of 15.5 hours. The right

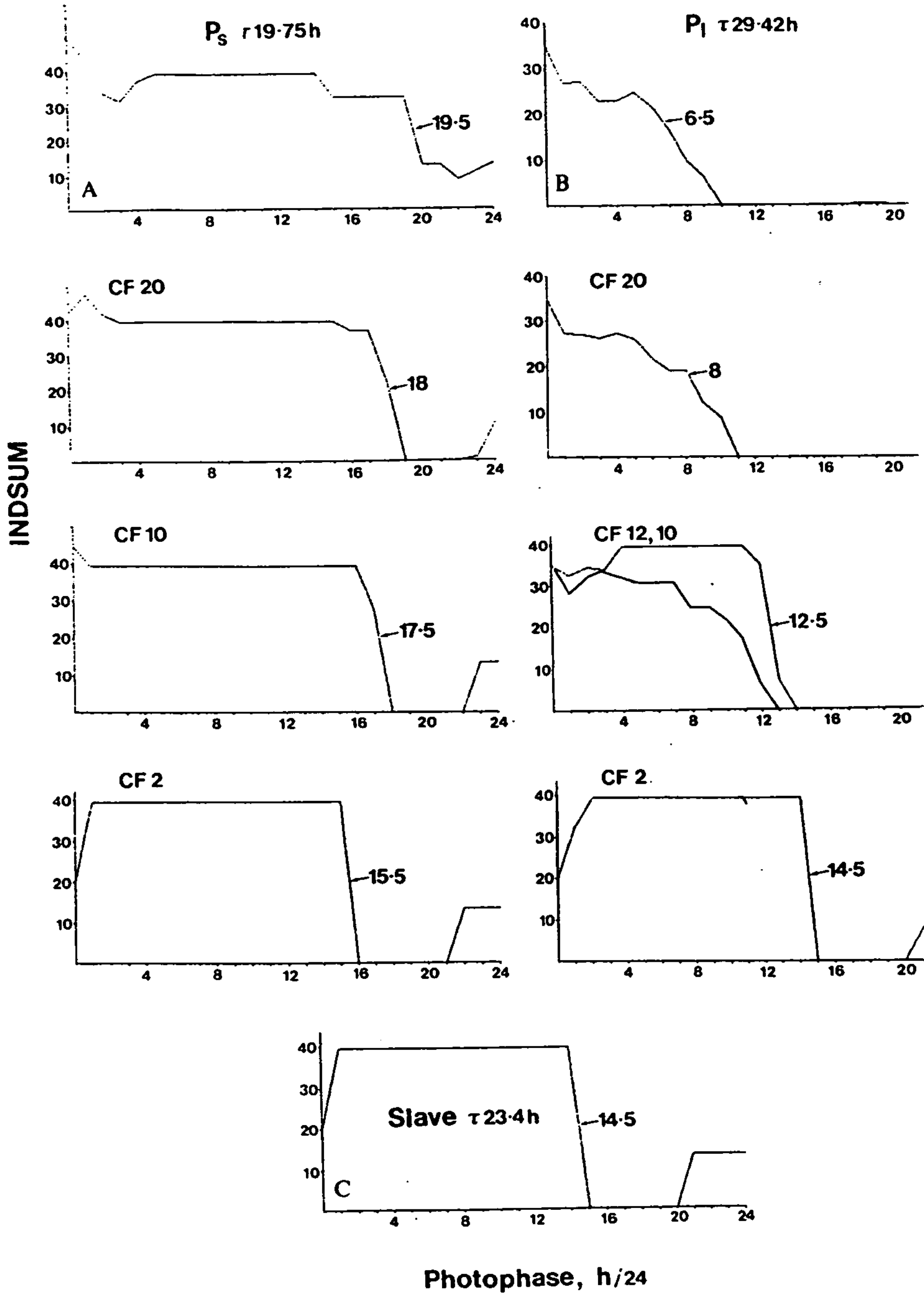


Fig 5.3 Computed photoperiodic response curves (PPRCs) for pacemakers, slave and coupled pacemaker-slave systems. A) PPRC for a hypothetical short period pacemaker in isolation, period 19.7 hours. B) PPRC for a hypothetical long period pacemaker in isolation, period 29.4 hours. C) PPRC for the uncoupled light entrainable slave oscillation, period 23.4 hours. The other panels show PPRCs computed for various coupling strengths (CF 2 to CF 20) between the pacemaker and the slave. With increasing CF the critical daylength approaches that of the pacemaker.

hand panel shows the change in PPRC shape and CDL for the photoperiodic system due to increased coupling strength when the slave is coupled to a long period pacemaker, P_l. Increasing CF leads to a decrease in CDL, but with a CF of 2 this system produces a PPRC with a CDL of 14.5 hours, the same as the slave.

Discussion

The coupled pacemaker-slave model described above provides a theoretical explanation of the photoperiodic responses in the wild-type and *period* mutant strains of *Drosophila melanogaster.* With a weak coupling between pacemaker and slave the two oscillations, and the rhythms they control, can act virtually independently. This is unusual because most experimental work has assumed that the locomotor activity (and eclosion) rhythm provide an accurate measure of the phase of the photoperiodic clock. This assumption, that the locomotor (and eclosion) rhythm and the photoperiodic oscillator are 'hands' of the same underlying 'clock', has in some insects, like *Sarcophaga argyrostoma,* been used to great effect. In *Sarcophaga argyrostoma* the eclosion rhythm does appear to provide an accurate marker of the phase of the photoperiodic clock. The model proposed here does not preclude this possibility. With increasing coupling strength the slave oscillation will be increasingly controlled/entrained by the pacemaker oscillation leading to a slave oscillation that is not independent of the pacemaker.

In other drosophilid species there is also evidence that the two rhythms of locomotor activity or eclosion, and photoperiodic time measurement are not closely linked. In *D.auraria* the photoperiodic response and circadian system of populations from a geographic cline, were investigated by Pittendrigh & Takamura (1987). It was found that changes in the phase relationship of eclosion as a function of photoperiod did not support the hypothesis that changes in CDL are caused by changes in an

underlying circadian system controlling eclosion/locomotor activity *and* photoperiodism. The hypothesis that the eclosion rhythm may not provide an adequate marker for the behaviour of a separate 'photoperiodic pacemaker' is rejected by Pittendrigh & Takamura. The explanation provided by Pittendrigh & Takamura (1987) for the changes in CDL expressed in the latitudinal cline relies upon changes in the level of response at all photoperiods. For example, the reduction in CDL in a southern strain compared to a northern strain is due to a general reduction in the level of diapause at all photoperiods. In the *D.melanogaster* results reported here a similar difference in the general diapause response can be seen in the three strains, but this difference is not accompanied by a difference in CDL.

The independence of a photoperiodic oscillation from a pacemaker controlling locomotion and eclosion is further supported by genetic evidence from *Drosophila littoralis* (Lankinen, 1986; 1992). Original experiments involving crosses between northern and southern strains of this species revealed that lengthening of CDL in more northerly populations was associated with a decrease in the free-running period of the eclosion rhythm. However, this relationship is not causal: rather the correlations observed were associative and linked to similar effects of changing latitude on both the overt rhythm and photoperiodism. Further work on these original crosses has provided evidence that the two systems are separate through complete genetic segregation of one system from the other. Strains have been produced which express entrainment behaviour typical of a population from the far north but which expresses CDLs expected from a population from the southerly end of the cline and *vice versa*. Clearly this genetic independence may be reflected in the virtual independence of the two oscillators in the model proposed here.

The present model adequately describes the shape of the PPRC in *Drosophila melanogaster*, wild-type and *period* mutants, and in a more general way reflects

photoperiodic responses in other drosophilids. The model may also be effective for other insect species which seem to express linked locomotor rhythms and photoperiodism (when the coupling strength is increased, the independence of the photoperiodic oscillation is lost) and can explain how natural selection may act to produce latitudinal clines in CDL without disturbing the circadian system in general. This is important because the circadian system has to control a whole series of rhythms, each of which needs to be correctly phase set to the external light dark cycle (Pittendrigh, 1981b).

Reliance upon changing the central pacemaker in order to alter CDL could have disastrous effects upon the phase relationship of other rhythms to the light dark cycle and to each other. The present model allows the changes in CDL to be brought about by changes in coupling strength between a pacemaker and slave with different τs. However, correlations between pacemaker period and CDL will be produced in large geographic clines for two reasons. Firstly, the coupling strength will not be able to change CDL to extreme values because it is restricted ultimately by the period of the pacemaker, and any gross changes in CDL may possibly require changes in the pacemaker period. Secondly, the pacemaker period will vary latitudinally because the entrainment behaviour of the whole circadian system will need to change with latitude. So there will be two factors controlling CDL in a population of insects with a large latitudinal range. Gross changes in entrainment behaviour and CDL can be achieved simultaneously by alterations in the period of the central pacemaker, whilst more precise local variations in CDL can be produced by changes in coupling strength between the pacemaker and slave. This latter variation will also have the advantage that it will be more responsive to selection because there will be few potential drawbacks to changing CDL associated with changes in the central pacemaker.

The model is useful as it not only replicates the photoperiodic response curves of wild-type and *period* mutant strains but it can also be applied to a range of other animals like the spider mite, *Tetranychus urticae*, (Vaz Nunes *et al*, 1991a, b). As such, it is not a specific representation of the photoperiodic system in *Drosophila melanogaster*.

The model is not so successful in modelling the responses to skeleton photoperiods shown in chapter 4. However, with a few minor adjustments it could probably model the response but at the expense of losing its general applicability. For example, *D.melanogaster* is known to lack a sensitive period as such. The decision to enter diapause is made possible by exposure to a low temperature within the first ten hours after eclosion. Subsequent entrainment to light-dark cycles can break this diapause response in long days. It would seem that this exposure to low temperatures primes a proportion of the population to enter diapause, exposure then to short days may increase the numbers of flies exhibiting a diapause state whilst long days break diapause in those already primed.

In constant darkness 50% of the emerging adults may be primed by the temperature to enter ovarian diapause. The coupling between pacemaker and slave is weak in this system allowing the two oscillators to act almost independently. Consequently the oscillation measuring nightlength quickly damps out within a few cycles and not many more flies are stimulated into diapause. In a short photoperiod (*i.e.* LD 10:14) the light pulses entrain the oscillation preventing it from damping below threshold, and in addition to the original 50% of flies that were primed to remain pre-vitellogenic, exposure of the photoinducible phase to darkness in each cycle induces a proportion of the remainder to enter a photoperiodically controlled diapause. When the same population of flies is exposed to long day conditions (*i.e.* LD 18:6), entrainment results in the photoinducible phase being illuminated in each

cycle which induces flies predetermined to remain pre-vitellogenic by low temperature to undergo ovarian maturation. In constant light the oscillation again damps below threshold effectively annihilating the photoinducible phase before diapause is induced. In this specific model both temperature and photoperiod interact. Initial low temperature (about 12°C) arrests the onset of vitellogenesis. Short days then maintain this previtellogenic state (diapause) whereas long days allow a slow cycle of yolk accumulation to begin. Fig 5.4 is a diagram representing the photoperiodic system determining diapause in *Drosophila melanogaster* as described above. This system has not been modelled on the computer (although this would be relatively straight-forward) because a specific explanation of diapause induction in such a model would be gained at the expense of a more widespread applicability of the model to Diptera and other insects.

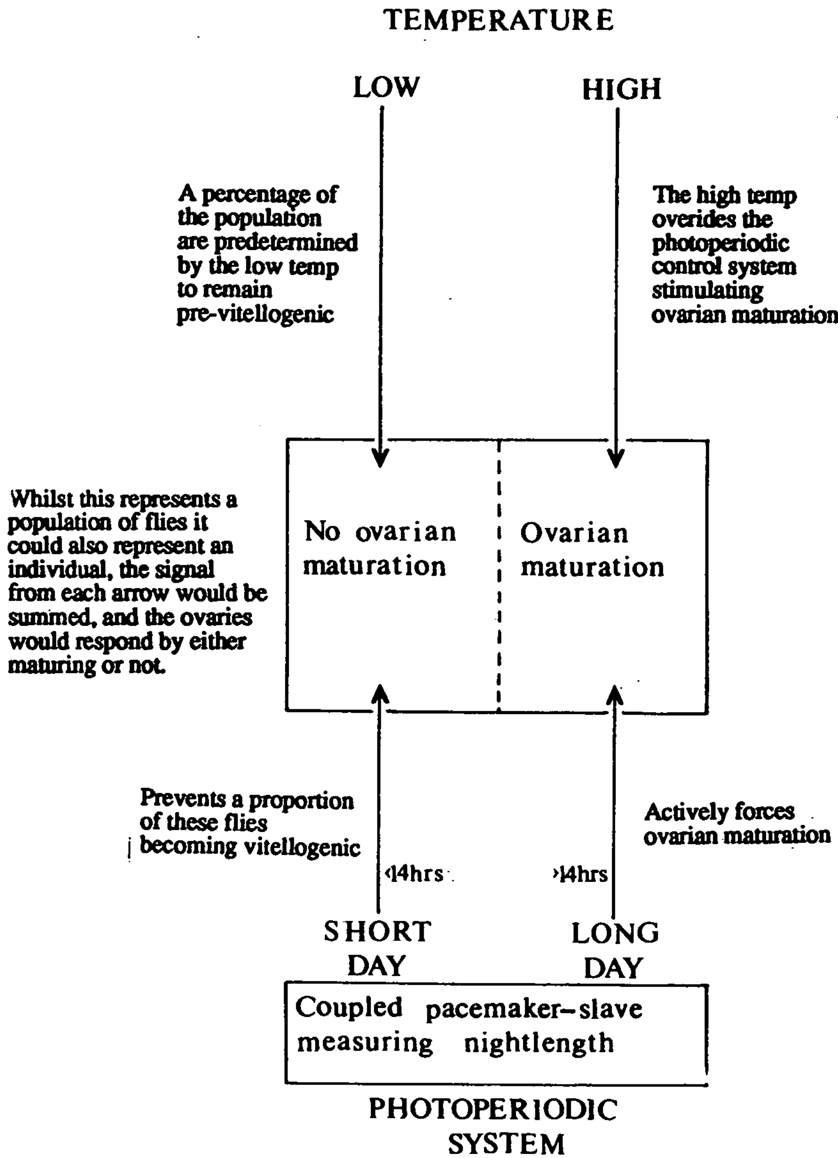


Fig 5.4 Diagram representing the interaction between temperature and photoperiod in the diapause control system of *D.melanogaster*. Low temperatures predetermine a proportion of the emerging adults to remain pre-vitellogenic, daylength can then adjust this proportion through a coupled pacemaker-slave system. The major component of this is the forcing of ovarian maturation at low temperatures in the presence of long days.

Chapter 6

General Discussion

Throughout this thesis the relationship between circadian rhythms and photoperiodism has been discussed. Particular attention being paid to the role of circadian rhythms in measuring daylength and using this information to determine season. This idea is not recent. In 1936 Bunning first suggested that the circadian system could be used to determine seasonality. Since then work has attempted to show, in various organisms, that the daily biological clock controlling many overt circadian rhythms is also used to control seasonally appropriate behaviour or development. This often involved comparing the responses of circadian rhythms and the photoperiodic system in similar lighting regimens. Comparable responses were used to argue that just as circadian oscillators lay behind the behavioural daily rhythms so they also were involved in photoperiodic time measurement (PPTM).

In some organisms both circadian rhythms and photoperiodism have been shown to have very similar underlying circadian oscillators. In these cases both overt behavioural rhythms and the photoperiodic response could be considered as "hands" of the same circadian clock. However in *Sarcophaga argyrostoma* the period of the endogenous eclosion rhythm has been shown to be about 23.8 hours and self-sustained whereas the rhythm underlying PPTM has a period of about 25 hours and appears to damp (Saunders, 1986) and in *Calliphora vicina* there is a broad parallel between locomotion and PPTM but these have different τs and damping coefficients (Kenny & Saunders, 1991). Whilst in detail the nature of oscillators controlling locomotor activity and photoperiodism appear to be different, in many cases one (locomotor activity) can be used as an accurate marker of the phase of the oscillator controlling the other (photoperiodism).

The isolation in 1971 of *Drosophila melanogaster* mutants with considerably different overt rhythms from the wild-type (Konopka, 1971) and the discovery of a photoperiodically controlled diapause (Saunders *et al*, 1989) seemed to provide an ideal situation in which to test Bunning's hypothesis. If the oscillator controlling the locomotor activity rhythm in this insect also controls photoperiodic time measurement then mutations altering the period of the former ought to produce clear changes in the response of the latter.

In *Drosophila melanogaster* it has become clear that both the locomotor activity rhythm and PPTM are governed by the circadian system. The evidence for this claim comes from work covered in the preceding chapters and previously published experiments (Saunders, 1990). The locomotor activity rhythm in *D.melanogaster* can be seen to free-run in constant darkness with a period of about 24 hours. This rhythm can also be entrained by the external light-dark cycle and experiments have produced several phase response curves which are typical of overt rhythms controlled by circadian oscillators (chapter 3). These PRCs can be used to predict entrainment behaviour of the locomotor activity rhythm by light-dark cycles (p147 and Fig 5.1).

The *period* mutants also show free-running locomotor activity rhythms, with endogenous periods that are different from each other and the wild-type. Effects of the *period* mutations upon the locomotor activity rhythm are parallelled by similar effects upon the eclosion rhythm in these strains. The one allele can apparently cause alterations in the period of a number of overt rhythms and it is this evidence that suggests that the *period* gene and gene product are somehow involved in the maintenance and/or production of a central pacemaker. This central pacemaker is believed to be the primary oscillator which entrains to the external light-dark cycle and internalises this signal. In turn, the pacemaker may entrain a whole series of

hierarchically-arranged slave oscillators. It is one of these slave oscillators which is believed to measure daylength in the photoperiodic time measuring system, and to determine the onset of diapause or non-diapause development.

Circadian involvement in PPTM has been demonstrated in *D.melanogaster*. Resonance experiments performed on the wild-type flies have shown approximately 24 hour peaks in diapause induction (Saunders, 1990). Such an experiment, where groups of flies are exposed to the same light pulse embedded in light-dark cycles of different durations, implies that the underlying mechanism in PPTM has to be circadian because the period of the light-dark cycle changes and not the duration of the light pulse. The same duration of light can elicit either a long day (summer) or a short day(winter) response depending upon the period of the light-dark cycle.

The response of PPTM on exposure to skeleton photoperiods (p134) also suggests a circadian involvement, although only a weak one. If the circadian system controls PPTM then one would predict that the photoperiodic response to skeleton photoperiods (where a photoperiod is replicated by two short light pulses representing the start and end of a complete long light pulse) would be similar to the entrainment response of the overt rhythms to such skeletons. Typically, such a response involves (1) entrainment of the rhythm to the shortest gap between the two light pulses and (2) a 'zone of bistability' when the gap is close to $\tau/2$. For the photoperiodic system results show that the incidence of diapause within the zone of bistability is variable. In the experiments covered in chapter 4, the wild-type has a high diapause (short day) response when exposed to skeleton photoperiods from LD 1:1:1:21 to 1:7:1:15 or from 1:15:1:7 to 1:21:1:1. In these skeletons the circadian system is assumed to take the shorter gap as 'day' and therefore produce a high incidence of diapause. Within the zone of bistability, LD 1:8:1:14 to 1:14:1:8, however, diapause incidence may be

reduced depending upon the phase of the circadian rhythm first illuminated by the one hour light pulse and the duration of the first dark gap.

Whilst the evidence suggests that both locomotor activity and PPTM are controlled by a circadian oscillator, both systems appear to have different properties. The oscillator controlling locomotor activity is, like a large number of behavioural oscillators, remarkably self-sustained. In constant conditions the rhythm free-runs with a period of approximately 24 hours in the wild-type. In *per*^{s} and *per*^{L2} mutants a free-running rhythm is still observed although with drastically altered periods. The locomotor activity rhythm does not appear to damp out in these strains. This is typical of a self-sustained rhythm which in other organisms can be seen to free-run for a considerable length of time (Fig 1.2). In the arrhythmic *per*^{0} and *per*$^{-}$ strains, release into constant darkness results in an arrhythmic locomotor activity pattern. The implication of this is that *period* plays some part in maintaining the rhythm contributing to its self-sustained nature. The oscillator governing PPTM in wild-type, *per*^{s} and *per*^{L2} mutants, on the other hand, appears to be highly damped. The evidence for this comes from resonance experiments (p24) (Saunders, 1990) and the weak responses to skeleton photoperiods given in chapter 4 (p137). Clearly the fact that the oscillatory systems controlling locomotor activity and PPTM have different properties is strong evidence that they are different.

However, there is further evidence that the two systems are governed by different oscillators. The locomotor activity rhythm has a period which is somehow *per* encoded. The period of the rhythm is clearly effected by the *period* allele, with mutations producing both short and long period locomotor activity rhythms. In the preceding chapters, rhythms in locomotor activity have been seen with periods of about 19 hours, 24 hours and 29 hours as well as the two arrhythmic strains which may or may not express ultradian rhythms. However, *period* mutants have now been

produced with locomotor activity rhythms which extend from 16 hours up to 40 hours (Hall, *pers comm*). In the case of the 19 and 29 hour *period* mutants (*per*^{S} and *per*^{L2}) the mutations have separate effects upon different sections of the locomotor activity rhythm, *per*^{S} shortens α whilst *per*^{L2} lengthens ρ (p68 and Fig 3.3). The changes in the period of the rhythm are also reflected in changes in the *shape* of the PRCs. *per*^{S} appears to be more light sensitive than *per*$^{+}$ which, in turn, is more light sensitive than *per*^{L2}. The evidence clearly points to *period* playing some sort of role in the production and maintenance of the oscillatory rhythm governing activity and eclosion.

In PPTM it appears that the period of the oscillator is not *per* encoded. The similarity in critical daylength (CDL) in the wild-type, *per*^{S} and *per*^{L2} strains and the similar resonance experiment results suggests that *period* is not directly involved in the production of the oscillator measuring daylength in the photoperiodic response. Similar CDLs imply that the oscillators have similar periods and the results of resonance experiments show all three strains (*per*$^{+}$, *per*^{S} and *per*^{L2}) have a rhythm underlying PPTM with an endogenous period of about 24 hours. Evidence from *per*^{0} and *per*$^{-}$ (where CDLs are 3 and 5 hours shorter than wild-type respectively) suggest that *period* does play a modulatory role in PPTM (Saunders, 1990), as do the responses to skeleton photoperiods, where the wild-type has a different response from the two mutants. However, since *per*$^{-}$ flies, with the *period* locus deleted, can distinguish long from short days in a photoperiodic response, then *period* cannot be essential to PPTM.

One problem with this argument is that the two systems, photoperiodism and locomotor activity, have been analysed at different temperatures (12°C and 20°C respectively). Normally, circadian rhythms have been shown to be temperature-compensated and so this difference in temperature would have little effect. In the wild-type temperature-compensation of the period of the locomotor

activity rhythm has been demonstrated (Konopka *et al*, 1989). However, in *per*^{s} and *per*^{L2} there is evidence that temperature compensation is altered. The period of the locomotor activity rhythm in *per*^{s} lengthens as temperature decreases and the period of *per*^{L2} shortens as temperature decreases (Konopka *et al*, 1989). At 12°C it is not inconceivable that the oscillators governing locomotor activity in all these strains might have the same period, and this oscillator could therefore control PPTM producing similar CDLs for all three. The evidence that photoperiodic time measurement also occurs in *per*^{0} and *per*$^{-}$ (Saunders, 1990) does not help to solve this dilemma either, as the locomotor activity rhythm of both these strains can be organised by light-dark cycles. If the light-dark cycle forces an underlying highly damped "oscillatory system" which cannot free-run but does entrain in contiguous light-dark cycles, in theory, such a rhythm may also measure daylength.

It has proved impossible to resolve this problem by measuring the CDL of the photoperiodic response of *D.melanogaster* mutants at 20°C, because diapause does not occur at this temperature.

However, the one difference between the two systems which cannot easily be put down to temperature differences is the level of supposed damping in locomotor activity and PPTM. Analysis of the free-running nature of the locomotor activity rhythm at 12°C could prove useful. However, at such low temperatures the recording system used here is not appropriate because the flies become almost totally inactive. In the paper by Konopka *et al* (1989) no mention is made of a change in the self-sustained nature of the locomotor activity rhythm with changing temperature. It may be fair to assume that such changes do not take place and, therefore, we can conclude that the oscillator governing the locomotor activity rhythm is not the same as the one serving photoperiodism.

A model has been proposed which can demonstrate how the self-sustained, *period* encoded oscillator underlying the locomotor activity rhythm can influence a second damped oscillator which does not involve *period* to produce photoperiodic response curves resembling those found in *Drosophila melanogaster* and the *period* mutants (Gillanders & Saunders, 1992). This model not only explains the data in this species but can also be used to explain the photoperiodic responses of other insects (p151).

The diapause response can only be observed when females are exposed to a low temperature, of about 12°C, within the first ten hours after eclosion. This low temperature signal somehow allows the diapause system to determine ovarian development. Wild-type flies never respond to light-dark cycles with a 100% population *diapause* response (as opposed to a 100% quiescence which does not rely upon daylength). The switch from high to low diapause around the critical daylength is also quite protracted and the temperature range in which photoperiodic control of diapause can be observed is very narrow. All of these imply (1) that ovarian diapause in *Drosophila melanogaster* is comparatively shallow; (2) that the photoperiodic system is evolutionarily quite novel and, (3) that the overwintering strategy of this species cannot rely upon this system to any great extent outside the protection of the human environment. Nevertheless, the recently evolved photoperiodic diapause in *D.melanogaster* (see below) must offer a distinct selective advantage over mere quiescence.

Analysis of the photoperiodic response at carefully controlled temperatures clearly shows the shallow nature of the photoperiodic response. At 11°C a switch in development due to daylength cannot be observed because this temperature causes a quiescent ovarian state (*i.e.* the ovaries remain pre-vitellogenic irrespective of daylength). At 14°C and above the 'high' temperatures appear to override any

photoperiodic response, forcing ovarian development, again irrespective of daylength. It is only at 12°C and 13°C (in the wild-type) that a clear photoperiodic control of diapause can be observed.

A comparison of the response of *per*$^{+}$, *per*^{s} and *per*^{L2} flies at 13°C to different photoperiods highlights a difference which implies that *period* might play some modulatory role in diapause determination. Namely there is a photoperiodic control mechanism in *per*$^{+}$ but not in *per*^{s} or *per*^{L2}. Furthermore, the lack of photoperiodic control at this temperature in the two *period* mutants appears to be due to a loss of the ability of long days to reduce diapause induction (Fig 4.5). Put more simply, at 13°C the *period* mutants have lost the tendency for long days to force ovarian maturation.

Because the photoperiodic control mechanism in *D.melanogaster* appears so shallow it leads to speculation about the evolution of photoperiodically controlled diapause development in this and other species. The *melanogaster* group is tropical in origin (Lemeunier *et al*, 1986). However, whilst tropical environments do have seasons, the variation in the duration of the light throughout a year is not marked. The changes in season cannot be 'tied' to changes in daylength through a photoperiodic control mechanism because changes in daylength are not large enough. Therefore it is a fair assumption that tropical drosophilids do not have a highly developed photoperiodic control mechanism, if any at all. Any weak photoperiodic response present can only be assumed to be the remnants of an ancestral system. Spread of the *melanogaster* group northwards has meant that individuals have experienced more seasonally changeable environmental conditions and larger seasonal changes in daylength; these have presumably resulted in natural selection adapting the PPTM of this group to suit their environment.

Drosophila melanogaster has spread northwards as a commensal with humans and as such it has been able to avoid most of the harsh environmental conditions associated with different seasons at higher latitudes. As a commensal, flies would overwinter in and around buildings and this has probably relaxed the selection pressures on the species which would normally lead to a highly developed photoperiodic control system. Because the extreme effects of winter conditions at higher latitudes could be cushioned by the living conditions of humans, any developing photoperiodic system can be considered to be evolutionarily novel and as such may provide insight into PPTM by determining how it may have evolved.

Eighteen other drosophild species have been shown to express a photoperiodically controlled diapause. Of these, 17 have an ovarian diapause and most of these tend to express a fairly strong response to changing daylength. A review of the most highly studied of these, *D.phalerata* (Geyspits & Simonenko, 1970; Tyshchenko, 1972), *D.littoralis* (Lumme *et al*, 1974; Lankinen, 1986), and *D.auraria* (Iwao *et al*, 1980; Pittendrigh & Takamura, 1987), has been given in chapter 1. It is clear that the diapause control mechanism in these three species has evolved to be stronger than the response seen in *D.melanogaster*, and any explanation of the evolution of the photoperiodic response in *melanogaster* must be extended to incorporate further development to evolve along the lines seen in other drosophilids.

Obviously the evolution of the photoperiodic response in *D.melanogaster* must begin from the passive developmental quiescence induced by low temperatures. This process does not take into account daylength but is an immediate response to low temperature, which stops or slows down physiological processes, reducing the uptake of yolk-polypeptides by the ovaries and leaving them pre-vitellogenic. Quiescence is an immediate response, and cannot take account of 'expected' seasonal temperatures. So a cold day in early spring will have the same effect upon ovarian development as a

similar day in autumn despite the difference in future 'expected' environmental conditions. Such a response to temperature cannot be too complex and cannot involve developmental changes which require a relatively long period of time. One of the main selective advantages of photoperiodic control of diapause lies in the ability to disassociate the immediate environmental conditions from the developmental response, and enable complex overwintering processes to protect individuals from harsh environmental conditions to come.

The means by which a photoperiodic control of development can be linked to quiescence could be two-fold. At temperatures higher than those which induce quiescence a daylength measuring system can determine short daylengths (winter conditions) and inhibit full ovarian maturation. Or in long days (summer conditions) at temperatures below which quiescence normally prevents ovarian development, this could be over-ridden. Which of these mechanisms is initially incorporated into the life cycle depends upon which developmental process (entering diapause or avoiding diapause) provides the most appropriate control. In *Drosophila melanogaster* experiments have suggested that the diapause response is due to a forcing of ovarian maturation in long days at temperatures which would otherwise results in quiescence (p125).

Such an initial control mechanism would seem intuitively more appropriate as it enables individuals to maximise their reproductive ability once summer conditions arise by linking ovarian development with daylength and not local temperature. Presumably, if inhibition of ovarian development was a developmentally more 'difficult' task and had to be accurately timed then photoperiodic control would evolve with short days preventing ovarian maturation at temperatures which normally cause normal vitellogenic development.

As the photoperiodic system develops and the advantages of a diapause stage rely less heavily upon temperature (or the necessity of diapause induction is required at higher temperatures) the drosophilid system would begin to incorporate short-day induced diapause development as well as long-day induced ovarian development at low temperatures. Clearly the further evolution of PPTM in drosophilids relies upon the response occurring over larger temperature ranges and then some form of temperature-compensation of the photoperiodic response to be incorporated.

Bunning (1936) suggested that the control mechanism governing photoperiodic time measurement is based upon a circadian oscillator. However, this oscillator is distinct from the circadian oscillator(s) controlling overt rhythmicity and involving *period*. The model proposed to explain *period*'s modulatory role upon the photoperiodic response involving two oscillators weakly coupled can also be usefully adapted for other drosophilds and other species (Vaz Nunes *et al*, 1991). However, whilst *Drosophila melanogaster* is an ideal organism for genetic analysis of the circadian system, and molecular and genetic analysis of the *period* locus is beginning to indicate a molecular mechanism for circadian rhythm generation, the photoperiodic response appears rather too shallow for extensive analysis of the photoperiodic time measuring mechanism. Hopefully, future research may circumvent the shallow nature of the response seen in Canton-S wild-type by either analysing photoperiodic responses of more northerly wild-type strains with more robust photoperiodic responses, or by attempting to identify and mutate *period* homologues in other drosophilids with more robust and less temperature sensitive photoperiodic responses like, for example, *Drosophila littoralis*.

References

Allemand, R., (1974) Importance evolutive du comportement de ponte chez les insectes: comparaison du rythme circadien d'oviposition chez les six especes de *Drosophila* du sous-groupe *melanogaster*. *C.R. Acad. Sci. Paris, D*, **279**, 2075-2077

Allemand, R., (1976a) Les rythmes de vitellogenese et d'ovulation en photoperiode LD 12:12 de *Drosophila melanogaster*. *J. Insect Physiol.*, **22**, 1031-1035

Allemand, R., (1976b) Influence de modifications des conditions lumineuses sur les rhythmes circadiens de vitellogenese et d'ovulation chez *Drosophila melanogaster*. *J. Insect Physiol.*, **22**, 1075-1080

Allemand, R., (1976c) Importance adaptive du rythme circadien de ponte chez les drosophilides: comparaison de huit especes de genre *Zaprionus*. *C.R. Acad. Sci. Paris, D*, **282**, 85-88

Allemand, R., (1977) Influence de l'intensite d'eclairement sur l'expression du rythme journalier d'oviposition de *Drosophila melanogaster* en conditions lumineuses LD 12:12. *C.R. Acad. Sci. Paris, D*, **284**, 1553-1556

Aschoff, J., (1960) Exogenous and endogenous components in circadian rhythms. *Cold Spring Harb. Symp. Quant. Biol.*, **25**, 11-28

Aschoff, J., (1969) Desynchronization and resynchronization of human circadian rhythms. *Aerospace Medecine*, **40**, 844-849

Aschoff, J., Gerecke, U., Kureck, A., Pohl, H., Rieger, P., Saint Paul, U. von and Wever, R., (1971) Interdependent parameters of circadian activity rhythms in birds and man. In *Biochronometry* (Ed. Menaker, M.), *Natl. Acad. Sci.*, Washington, p3-24.

Ashburner, M., (1989) *Drosophila*, a laboratory handbook. *Cold Spring Harb. Laboratory Press*, USA

Atwal, A.S., (1955) Influence of temperature, photoperiod, and food on the speed of development, longevity, fecundity, and other qualities of the diamond backed moth *Plutella maculipennis* (Curtis) (Tineidae, Lepidoptera). *Australian J. Zoology*, **3**, 165-221

Baker, F.C., (1935) The effect of photoperiodism on resting, tree hole, mosquito larvae. *Canadian Entomologist*, **67**, 149-153

Bargiello, T.A. & Young, M.W., (1984) Molecular genetics of a biological clock in *Drosophila*. *Proc. Natl Acad. Sci. USA*, **81**, 2142-2146

Bargiello, T.A., Saez, L., Baylies, M.K., Gasic, G., Young, M. & Spray, D.C., (1987) The *Drosophila* clock gene *per* affects intercellular junctional communication. *Nature*, **328**, 686-691

Basden, E.B., (1954) Diapause in *Drosophila* (Diptera: Drosophilidae). *Proc. R. ent. Soc. Lond. (A)*, **29**, 114-118

Baylies, M.K., Bargiello, T.A., Jackson, F.R. & Young, M.W., (1987) Changes in abundance and structure of the *per* gene product can alter periodicity of the *Drosophila* clock. *Nature*, **326**, 390-392

Beach, R.F. & Craig, G.B.Jr, (1977) Nightlength measurement by the circadian clock controlling diapause induction in the mosquito *Aedes atropalpus*. *J. Insect Physiol.*, **23**, 865-870

Beck, S.D., (1974a) Photoperiodic determination of insect development and diapause. I. Oscillators, hourglasses and a determination model. *J. Comp Physiol.*, **90**, 275-295

Beck, S.D., (1974b) Photoperiodic determination of insect development and diapause. II. The determination gate in a theoretical model. *J. Comp Physiol.*, **90**, 297-310

Beck, S.D., (1980) Insect Photoperiodism. 2nd Edition, *Academic Press*, New York

Beling, I., (1923) Uber das Zeitgedachtnis der Bienen. *Zeitschrift fur vergleichende Physiologie*, **9**, 259-338

Brady, J., (1974) The physiology of insect circadian rhythms. *Adv. Insect Physiol.*, **10**, 1-115

Brett, W.J., (1955) Persistent diurnal rhythmicity in *Drosophila* emergence. *Ann. ent. Soc. Am.*, **48**, 119-131

Bunning, E., (1935) Zur Kenntnis der endogonen Tagesrhythmik bei Insekten und Pflanzen. *Ber. dt. Bot. Ges.*, **53**, 594-623

Bunning, E., (1936) Die endogene Tagesrhythmik als Grundlage der Photoperiodischen Reaktion. *Ber. dt. bot. Ges.*, **54**, 590-607

Chandrashekeran, M.K., (1967) Studies on phase shifts in endogenous rhythms. I. Effects of light pulses on the eclosion rhythm in *Drosophila pseudoobscura*. *Z. vergl. Physiol.*, **56**, 154-162

Chippendale, G.M., Reddy, A.S. & Catt, C.L., (1976) Photoperiodic and thermoperiodic interactions in the regulation of the larval diapause of *Diatraea grandiosella*. *J. Insect Physiol.*, **22**, 823-828

Christensen, N.D., Lewis, R.D. & Gander, P.H., (1984) Properties of a feedback model for the circadian clock of *Hemideina thoracica* (Orthoptera, steropelmatidae). *Biol. Cybern.*, **51**, 87-92

Citri, Y., Colot, H.V., Jacquier, A.C., Yu, Q., Hall, J.C., Baltimore, D., & Rosbash, M., (1987) A family of unusually spliced and biologically active transcripts encoded by a *Drosophila* clock gene. *Nature*, **326**, 42-47

Claret, J., Dumortier, B. & Brunnarius, J., (1981) Mise en evidence d'une composante circadienne dans l'horloge biologique de *Pieris brassicae* (Lepidotera), lors de l'induction photoperiodique de la diapause. *C. R. Acad. Sci. Paris*, **292**, 427-430

Clayton, D.L. & Paietta, J.V., (1972) Selection for eclosion time in *Drosophila melanogaster*. *Science*, **178**, 994-995

Crews, S.T., Thomas, J.B. & Goodman, C.S., (1988) The *Drosophila* single-minded gene encodes a nuclear protein with a sequence similarity to the *per* gene product. *Cell*, **52**, 143-151

Daan, S. & Pittendrigh, C.S., (1976) A functional analysis of circadian pacemakers in nocturnal rodents II. The variability of phase response curves. *J. Comp. Physiol.*, **106**, 253-266

David, J. & Fouillet, P., (1973) Enregistrement continue de la ponte chez *Droscphila melanogaster* et importance des conditions experimentales pour l'etude du rythme circadien d'ovipositio. *Rev. comp. Anim.*, **7**, 197-202

Dingle, H., (1974) The experimental analysis of migration and life history strategies in insects. In *'Experimental analysis of insect behaviour'* (Ed. Barton Browne, L.), *Springer-Verlag*, Berlin p329-342

Dowse, H.B., Hall, J.C. & Ringo, J.M., (1987) Circadian and ultradian rhythms in *period* mutants of *Drosophila melanogaster*. *Behav. Genet.*, **17**, 19-35

Dowse, H.B. & Ringo, J.M., (1989) Rearing Drosophila in constant darkness produces phenocopies of *period* circadian clock mutants. *Physiol. Zool.*, **62**, 785-803

Dummortier, B. & Brunnarius, J., (1977) L'information thermoperiodique et l'induction de la diapause chez *Pieris brassicae*. *C.R. Acad. Sci. Paris, D.*, **284**, 957-960

Dummortier, B. & Brunnarius, J., (1989) Diet dependent switch from circadian to hourglass-like operation of an insect photoperiodic clock. *J.Biol. Rhythms*, **4**, 481-490

Dyson-Hudson, V.R.D., (1956) The daily activity rhythm of *Drosophila pseudoobscura* and *D. obscura*. *Ecology*, **37**, 562-567

Edmunds, L.N., (1971) Persistent circadian rhythm of cell division in *Euglena*: Some theoretical considerations and the problem of intercellular communication. In *'Biochronometry'* (Ed. Menaker, M.), *Natl Acad. Sci*, Washington, p594-611

Engelmann, W., (1969) Phase shifting of eclosion in *Drosophila pseudoobscura* as a function of energy of light pulse. *Z. vergl. Physiol.*, **64**, 111-117

Engelmann, W., Karlsson, H.G. & Johnsson, A., (1973) Phase shifts in the *kalanchoe* petal rhythm caused by light pulses of different durations. *International Journal of Chronobiology*, **1**, 147-156

Engelmann, W. & Mack, J., (1978) Different oscillators control the circadian rhythm of eclosion and activity in *Drosophila*. *J. comp. Physiol.*, **127**, 229-237

Ewer, J., Rosbash, M. & Hall, J.C., (1988) An inducible promotor fused to the *period* gene in *Drosophila* conditionally rescues *per*-mutant arrhythmicity. *Nature*, **333**, 82-84

Ewer, J., Hamblen-Coyle, M., Rosbash, M. & Hall, J.C., (1990) Requirement for *period* gene expression in the adult and not during development for locomotor activity rhythms of imaginal *Drosophila melanogaster*. *J. Neurogenet.*, **7**, 31-73

Fernandez, A.T. & Randolph, N.M., (1966) The susceptibility of houseflies reared under various photoperiods to insecticide residues. *J. econ. Ent.*, **59**, 37-39

Fleugel, W., (1978) Oviposition rhythm of individual *Drosophila melanogaster*. *Experentia*, **34**, 65-66

Gander, P.H. & Lewis, R.D., (1979) The circadian locomotor activity rhythm of *Hemideina thoracica* (Orthoptera): A feedback model for the underlying clock oscillation. *Int. J. Chronobiol.*, **6**, 263-280

Geyspits, K.F. & Simonenko, N.P., (1970) *Ent. Obozr.*, **49**, 83-96 (In Russian)

Gillanders, S.W. & Saunders, D.S., (1992) A coupled pacemaker-slave model for the insect photoperiodic clock: interpretation of ovarian diapause datain *Drosophila melanogaster*. *Biol. Cybern.*, **67**, 451-459

Gruwez, G., Hoste, C. Lints, C.V. & Lints, F.A., (1972) Oviposition rhythm in *Drosophila melanogaster* and its alteration by a change in the photoperiodicity. *Experentia*, **27**, 1414-1416

Hackman, W., (1969) A new *Drosophila* species from northern Fennoscandia (Diptera). *Notube entomol.*, **49**, 69-72

Hall, J.C., (1990) Genetics of circadian rhythms. *Ann. Rev. Genet.*, **24**, 659-697

Hall, J.C. & Rosbash, M., (1987) Genes and biological rhythms. *TIG*, **3**, 185-191

Hall, J.C. & Kyriacou, C.P., (1990) Genetics of biological rhythms in *Drosophila*. *Advances Insect Physiol.*, **22**, 221-298

Hamblen, M., Zehring, W.A., Kyriacou, C.P., Reddy, P., Yu, Q., Wheeler, D.A., Zwiebel, L.J., Konopka, R.J., Rosbash, M. & Hall, J.C., (1986) Germ line transformation involving DNA from the *period* locus in *Drosophila melanogaster*: overlapping genomic fragments that restore circadian and ultradian rhythmicity to per^{0} and per^{-} mutants. *J. Neurogenet.*, **3**, 249-291

Hamblen-Coyle, M., Konopka, R.J., Zwiebel, L.J., Colot, H.V., Dowse, H.B., Rosbash, M. & Hall, J.C., (1989) A new mutation at the *period* locus with some novel effects on circadian rhythms. *J. Neurogenet.*, **5**, 229-256

Hamblen-Coyle, M., Wheeler, D.A., Rutila, J.E., Rosbash, M. & Hall, J.C., (1992) Behaviour of period-altered circadian rhythm mutants of *Drosophila* in light:dark cycles (Diptera: Drosophilidae). *J. Insect Behav.*, **5**, 417-446

Handler, A.M. & Konopka, R.J., (1979) Transplantation of a ciracadian pacemaker in *Drosophila*. *Nature*, **279**, 236-238

Hardeland, R. & Stange, G., (1973) Comparative studies on the circadian rhythms of locomotor activity of 40 drosophila species. *J. interdiscipl. Cycle Res.*, **4**, 353-359

Hardeland, R. & Stange, G., (1971) Einflusse von Geschlecht und Alter auf die lokomotorische Aktivitat von *Drosophila*. *J. Insect Physiol.*, **17**, 427-434

Hardin, P.E., Hall, J.C. & Rosbash, M., (1990) Feedback of the *Drosophila period* gene product on circadian cycling of its messenger RNA levels. *Nature*, **343**, 536-540

Hastings, J.W., (1960) Biochemical aspects of rhythms: Phase shifting by chemicals. *Cold Spring Harb. Symp. Quant. Biol.*, **25**, 131-143

Helfrich, C. & Engelmann, W., (1987) Evidence of circadian rhythmicity in the per^{0} mutant of *Drosophila melanogaster*. *Z. naturforsch.*, **42c**, 1335-1338

Hoelscher, C.E. & Vinson, S.B., (1971) The sex ratio of a hymenopterous parasitoid, *Campoletis perdistinctus*, as affected by photoperiod, mating and temperature. 1971, *Annals Entomological Soc America*, **64**, 1373-1376

Hoffman, K., (1960) Experimental manipulation of the orientational clock in birds. *Cold Spring Harbor Symposia in Quantitative Biology*, **25**, 370-388

Hoffmann, K., (1965) Overt circadian frequencies and circadian rule. In *'Circadian Clocks'* (Ed. Aschoff,J.), *North-Holland*, Amsterdam, p87-94

Huang, T-C., Jenn, T., Chow, T-J. & Chen, T-H., (1990) Circadian rhythm of the prokaryote *Synechococcus* species Rf-1. Plant Physiology, **92**, 531-533

Huang, T-C., Lay, K-C. & Tong S-R., (1991) Resetting the endogenous circadian nitrogen fixing rhythm of the prokaryote *Synechococcus* RF-1. *Botanical Bulletin of the Acadamy of Singapore*, **32**, 129-134

Iwao. Y., Kimura, M.T., Minami, N. & Watabe, H., (1980) Bionomics of Drosophilidae (Diptera) in Hokkaido, Japan. *Kontyu*, **48**, 160-168

Jackson, F.R., (1983) The isolation of biological rhythm mutations on the autosomes of *Drosophila melanogaster*. *J. Neurogenetics*, **1**, 3-15

Jackson, F.R., Bargiello, T.A., Yun, S.-H. & Young, M., (1986) Product of *per* locus of Drosophila shares homology with proteoglycans. *Nature*, *320*, 185-188

Johnsson, A. & Karlesson, H.G., (1972) The *Drosophila* eclosion rhythm, the transformation method, and the fixed point theorem. Report 2/1972, Dept of Electrical Measurements, Lund Institute of Technology

Kenny, N.A.P., (1989) Locomotor activity rhythms and photoperiodic time-measurementin the Blow-fly *Calliphora vicina* R-D. PhD Thesis

Kenny, N.A.P. & Saunders, D.S., (1991) Adult locomotor rhythmicity as "hands" of the maternal photoperiodic clock regulating larval diapause in the blowfly, *Calliphora vicina*. *J. Biol. Rhythms*, **6**, 217-234

King, R.C., (1970) Ovarian development in *Drosophila melanogaster*. *Academic Press*, New York

Koehler, W.K. & Fleissner, G. (1978) Internal desynchronization of bilaterally organized circadian oscillatorsin the visual system of insects. *Nature*, **274**, 708-710

Konopka, R.J., (1987) Neurogenetics of *Drosophila* circadian rhythms. In *'Evolutionary genetics of invertebrate behaviour'* (Ed. Huettel,M.D.), *Plenum Press*, New York, p215-221.

Konopka, R.J. & Benzer, S., (1971) Clock muta
nts of *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA*, **68**, 2112-2116

Konopka, R.J. & Orr, D., (1980) Effects of a clock mutation on the subjective day - implications for a membrane model of the *Drosophila* circadian clock. In *'Development and Neurobiology of Drosophila'* (Ed.Siddiqi,O., Babu,P., Hall,L.M. & Hall,J.C.), *Plenum Press*, New York, p409-416.

Konopka, R.J., Pittendrigh, C.S. & Orr, D., (1989) Reciprocal behaviour associated with altered homeostasis and photosensitivity of *Drosophila* clock mutants. *J. Neorogenet.*, **6**, 1-10

Kram, K.R., (1980) Why circadian rhythms. *American Naturalist*, **116**, 452-453

Lakovarra, S., Saura, A., (1971) Genetic variation in natural populations of *Drosophila obscura. Genetics*, **69**, 377-384

Lakovarra, S., Saura, A., Koref-Santibanez, S. & Ehrman, L., (1972) Aspects of diapause in northern drosophilids. *Hereditas*, **70**, 89-96

Lamprecht, G. & Weber, F., (1977) Die Lichtempfindlichkeit der Circadianen rhythmik dreier Hohlenkafer arten der Gattung *Laemostenus*. *J. Insect Physiol*, **23**, 445-452

Lankinen, P., (1985) Genetic variation of circadian eclosion rhythm, and its relation to photoperiodism in *Drosophila littoralis*. PhD Thesis, University of Oulu, Finland

Lankinen, P., (1986) Geographical variation in circadian eclosion rhythm and photoperiodic adult diapause in *Drosophila littoralis. J. Comp. Physiol.*, **159**, 123-142

Lankinen, P., (pre-print) North-South differences of the circadian eclosion rhythm in European populations of *Drosophila subobscura. Heredity*

Lankinen, P. & Lumme, J., (1984) Genetic analysis of geographical variation in photoperiodic diapause and pupal eclosion rhythm in *Drosophila littoralis*. In Photoperiodic regulation of insect and molluscan hormones, *Ciba Foundation Symposium*, **104**, 97-114, Pitman, London

Lees, A.D., (1973) Photoperiodic time measurement in the aphid *Megoura viciae. J. Insect Physiol.*, **19**, 2279-2316

Lemeunier, F., David, J.R., Tsacas, L. & Ashburner, M. (1986) XX In '*The Genetics and Biology of Drosophila*' (Eds Ashburner, M., Caraon, H.L. & Thompson, J.N.Jnr), Academic Press, New York, Vol 3c p148-256

Lewis, R.D. & Saunders, D.S., (1987) A damped circadian oscillator model of an insect photoperiodic clock I. description of the model based on a feedback control system. *J. Theor. Biol.*, **128**, 47-59

Lewis, T. & Taylor, L.R., (1965) Diurnal periodicity of flight by insects. *Tans R. ent. Soc. Lond.*, **116**, 393-479

Liu, X., Zwiebel, L.J., Hinton, D., Benzer, S., Hall, J.C. & Rosbash, M., (1992) The *period* gene encodes a predominantly nuclear protein in adult *Drosophila. J. Neurosci.*, **12**, 2735-2744

Lumme, J., Oikarinen, H., Lakovaara, S., & Alatalo, R., (1974) The environmental regulation of adult diapause in *Drosophila littoralis. J. Insect Physiol.*, **18**, 2043-2053

MacLeod, E.G., (1967) Experimental induction and elimination of adult diapause and autumnal coloration in *Chrysopa carnea* (Neuroptera). *J. Insect Physiol*, **13**, 1343-1349

Maniatis, T., Fritsch, E.F. & Sambrook, J., (1982) Molecular cloning: A laboratory manual. *Cold Spring Harbor Laboratories*, Cold Spring Harbor, New York

Marcovitch, S., (1923) Plant lice and light exposure. *Science*, **58**, 537-538

McClung, C.R., Fox, B.A. & Dunlap, J.C., (1989) *frequency*, a clock gene in *Neurospora*, shares a sequence element with the *Drosophila* clock gene *period*. *Nature*, **339**, 558-562

Njus, D., (1976) Experimental approaches to membrane models. In *'The Molecular Basis of Circadian Rhythms'* (Ed. Hastings, J.W. & Schweiger, H.G.), *Dahlem Konferenzen*, Berlin, p283-294

Njus, D., Sulzman, F.M. & Hastings, J.W., (1974) Membrane model for the circadian clock. *Nature*, **248**, 116-120

Partridge, L., Green, A. & Fowler, K., (1987) Effects of egg production and exposure to males on female survival in *Drosophila melanogaster*. *J. Insect Physiol.*, **33**, 745-749

Pavlidis, T., (1969) Populations of interacting oscillators and circadian rhythms. *J. Theoretical Biol.*, **22**, 418-436

Pavlidis, T. & Kauzmann, W., (1969) Toward a quantitative biochemical model for circadian oscillators. *Archives of Biochemistry and Biophysics*, **132**, 338-348

Perez, Y., Verdier, M., & Pener, M.P., (1971) The effect of photoperiod on male sexual behaviour in a north adriatic strain of the migratory locust. *Entomologia experimentalis et applicata*, **14**, 245-250

Pittendrigh, C.S., (1954) On temperature independence in the clock controlling emergence time in *Drosophila*. *Proc. Natl. Acad. Sci. USA*, **40**, 1018-1029

Pittendrigh, C.S., (1960) Circadian rhythms and the circadian organization of living systems. *Cold Spring Harb. Symp. Quant. Biol.*, **25**, 159-184

Pittendrigh, C.S., (1961) On temporal organization in living systems. *Harvey Lectures Series*, **56**, 93-125

Pittendrigh, C.S., (1965) On the mechanism of entrainment of a circadian rhythm by light cycles. In *'Circadian Clocks'* (Ed. Aschoff, J.), *North-Holland*, Amsterdam, p277-297

Pittendrigh, C.S., (1966) The circadian oscillation in *Drosophila pseudoobscura* pupae: a model for the photoperiodic clock. *Z. pflanzenphysiol.*, **54**, 275-307

Pittendrigh, C.S., (1967) Circadian rhythms, space research and manned space flight. In *'Life science and space research Vol 5'*, *North Holland PUblishing Company*, Amsterdam, p122-134

Pittendrigh, C.S., (1972) Circadian surfaces and the diversity of possible roles of circadian organization in photoperiodic induction. *Proc. Natl. Acad Sci. USA*, **69**, 2734-2737

Pittendrigh, C.S., (1980) Some functional aspects of circadian pacemakers. In *'Biological rhythms and their central mechanism'* (Ed. M.Suda, O.Hayaishi & H.Nakagawa), *Elsevier Press*, New York, p3-12

Pittendrigh, C.S., (1981a) Circadian Systems: General Perspectives. In *'Handbook of Behavioural Neurobiology'* (Ed. Aschoff, J.), *Plenum Press*, New York, p57-95

Pittendrigh, C.S., (1981b) Circadian Systems: Entrainment. In *'Handbook of Behavioural Neurobiology'* (Ed. Aschoff, J.), *Plenum Press*, New York, p95-124

Pittendrigh, C.S. & Daan, S., (1977) A functional analysis of circadian pacemakers in nocturnal rodents IV. Entrainment: pacemaker as clock. *J. Comp. Physiol.*, **106**, 291-331

Pittendrigh, C.S., Kyner, W.T. & Takamura, T., (1991) The amplitude of circadian oscillations: temperature dependence, latitudinal clines, and the photoperiodic time measurement. *J. Biol. Rhythms*, **6**, 229-313

Pittendrigh, C.S. & Minis, D.H., (1964) The entrainment of circadian oscillations by light and their role as photoperiodic clocks. *American Naturalist*, **98**, 261-294

Pittendrigh, C.S. & Takamura, T., (1987) Temperature dependence and evolutionary adjustment of critical night length in insect photoperiodism. *Proc. Natl Acad. Sci. USA*, **84**, 7169-7173

Puchalski, W. & Lynch, G.R., (1992) Relationship between phase resetting and the free-running period in Djungarian hamsters. *J. Biol. Rhythms*, **7**, 75-83

Reddy, P., Zehring, W.A., Wheeler, D.A., Pirrotta, V., Hadfield, C., Hall, J.C. & Rosbash, M., (1984) Molecular analysis of the *period* locus in *Drosophila melanogaster* and identification of a transcript involved in biological rhythms. *Cell*, **38**, 701-710

Reddy, P., Jacquier, A.C., Abovich, N., Petersen, G. & Rosbash, M., (1986) The *period* clock locus of *D.melanogaster* codes for a proteoglycan. *Cell*, **46**, 53-61

Rensing, L. & Hardeland, R., (1967) Zur Wirkung der circadianen Rhythmik auf die Entwicklung von *Drosophila*. *J. Insect Physiol.*, **13**, 1547-1568

Roberts, S.K. de F., (1956) *'Clock'* controlled activity rhythm in the fruit fly. *Science*, **124**, 172

Roberts, S.K. de F., (1960) Circadian activity in cockroaches.I. The freerunning rhythm in steady-state. *J. Cell. Comp. Physiol*, **55**, 99-110

Rosbash, M. & Hall, J.C., (1989) The molecular biology of circadian rhythms. *Neuron*, **3**, 387-398

Rutila, J.E., Edery, I., Hall, J.C. & Rosbash, M., (1992) The analysis of new short-period circadian rhythm mutants suggests features of *D.melanogaster period* gene function. *J. Neurogenet.*, ????

Saez, L., Young, M.W., Baylies, M.K., Gasic, G., Bargiello, T.A. & Spray, D.C., (1992) *Per* - no link to gap junctions. *Nature*, **360**, 542

Saunders, D.S.. (1970) Circadian clock in insect photoperiodism. *Science*, **168**, 601-603

Saunders, D.S., (1972) Circadian control of larval growth rates in *Sarcophaga argyrostoma. Proc. Natl. Acad. Sci USA*, **69**, 2738-2740

Saunders, D.S., (1973) Thermoperiodic control of diapause in an insect: theory of internal coincidence. *Science*, *181*, 358-360

Saunders, D.S., (1975) 'Skeleton' photoperiods and the control of diapause and development in the flesh-fly, *Sarcophaga argyrostoma. J. comp. Physiol.*, **97**, 97-112

Saunders, D.S., (1976) The circadian eclosion rhythm in *Sarcophaga argyrostoma*: some comparisons with the photoperiodic "clock". *J. Comp. Physiol.*, **110**, 111-133

Saunders, D.S., (1978) An experimental and theoretical analysis of photoperiodic induction in the flesh-fly *Sarcophaga argyrostoma. J. comp. Physiol.*, **124**, 75-95

Saunders, D.S., (1979) The circadian eclosion rhythm in *Sarcophaga argyrostoma*: delineation of the responsive period for entrainment. *Physiol. Ent.*, **4**, 263-274

Saunders, D.S., (1981) Insect Photoperiodism. In *'Handbook of Behavioural Neurobiology'* (Ed. Aschoff, J.), *Plenum Press*, New York, p411-447

Saunders, D.S., (1982) Insect clocks. *Pergamon Press*. UK

Saunders, D.S., (1990) The circadian basis of ovarian diapause regulation in *Drosophila melanogaster*: Is the *period* gene causally invovled in photoperiodic time measurement? *J. Biological Rhythms*, **5**, 315-331

Saunders, D.S. & Gilbert, L.I., (1990) Regulation of ovarian diapause in *Drosophila melanogaster* by photoperiod and moderately low temperature. *J. Insect Physiol.*, **36**, 195-200

Saunders, D.S., Henrich, V.C. & Gilbert, L.I., (1989) Induction of diapause in *Drosophila melanogaster*: Photoperiodic regulation and the impact of arrhythmic clock mutations on time measurement. *Proc. Natl Acad. Sci. USA*, **86**, 3748-3752

Saunders, D.S. & Lewis, R.D., (1987a) A damped circadian oscillator model of an insect photoperiodic clock II. Simulations of the shapes of the photoperiodic reponse curves. *J. Theor. Biol.*, **128**, 61-71

Saunders, D.S. & Lewis, R.D., (1987b) A damped circadian oscillator model of an insect photoperiodic clock III. Circadian and "hourglass" responses. *J. Theor. Biol.*, **128**, 73-85

Saunders, D.S., Richards, D.S., Applebaum, S.W., Ma, M., & Gilbert, L.I., (1990) Photoperiodic diapause in *Drosophila melanogaster* involves a block to juvenile hormone regulation of ovarian maturation. *Gen. Comp. Endocrinology*, **79**, 174-184

Saunders, D.S. & Thompson, E.J., (1977) 'Strong' phase response curve for the rhythm of locomotor activity in a cockroach (*Nauphoeta cibnerea*). *Nature*, *270*, 242-243

Silyn-Roberts, H., Engelmann, W. & Grell, F.G., (1986) *Thalassomyxa australis* rhythmicity I. Temperature dependence. *J. Interdiscipl. Cycle Res.*, **17**, 181-187

Siwicki, K.K., Strack, S., Rosbash, M., Hall, J.C. and Jacklet, J.W., (1989) An antibody to the *Drosophila period* protein recognizes circadian pacemaker neurons in *Aplysia* and *Bulla*. *Neuron*, **3**, 51-58

Siwicki, K.K., Schwartz, W.J. & Hall, J.C., (1992) An antibody to the *Drosophila period* protein labels antigens in the suprachiasmatic nucleus of the rat. *J. Neurogenet.*, **8**, 33-42

Smith, R.F. & Konopka, R.J., (1981) Circadian clock phenotypes of chromosome aberrations with a breakpoint at the *per* locus. *Mol. Gen. Genet.*, **183**, 243-251

Smith, R.F. & Konopka, R.J., (1982) Effects of dosage alterations at the *per* locus on the period of the circadian clock in *Drosophila*. *Mol. Gen. Genet.*, **185**, 243-251

Stellar, H., Fischbach, K.F. & Rubin, G.M., (1987) *disconnected*: a locus required for neuronal pathway formation in the visual system of *Drosophila*. *Cell*, **50**, 1139-1153

Sweeney, B.M., (1969) Rhythmic phenomena in Plants. *Academic Press*, New York

Szabo, I., Kovats, T.G. & Halberg, F., (1978) Circadian rhythm in murine reticuloendothelial function. *Chronobiologia*, **5**, 137-143

Truman, J.W., (1971) Circadian rhythms and physiology with special reference to neuroendocrine processes in insects. *Proc. Int. Symp. Circadian Rhythmicity* (Wageningen), p111-135

Truman, J.W., (1972) *Z. Vergl. Physiologie* **76**, 32-40

Tyshchenko, V.P., Goryshin, N.I., & Azaryan, A.G., (1972) The role of circadian processes in insect photoperiodism. *Zhur.obshch. Biol.*, **33**, 21-31 (In Russian)

Vaz Nunes, M., (1981) A 'simple clock' approach of circadian rhythms: an easy way to predict the clock's singularity. *J. Theor. Biol.*, **92**, 227-239

Vaz Nunes, M., Kenny, N.A.P. & Saunders, D.S., (1990) The photoperiodic clock in the blowfly *Calliphora vicina*. *J. Insect Physiol.*, **36**, 61-67

Vaz Nunes, M., Saunders, D.S. & Lewis, R.D., (1991a) A coupled oscillator feedback system as a model for the photoperiodic clock in insects and mites I. The basic control system as a model for circadian rhythms. *J. Theor. Biol.*, **??**

Vaz Nunes, M., Saunders, D.S. & Lewis, R.D., (1991b) A coupled oscillator feedback system as a model for the photoperiodic clock in insects and mites II. Simulations of photoperiodic responses. *J. Theor. Biol.*, **??**

Vaz Nunes, M. & Veerman, A., (1979a) Photoperiodic time measurement in spider mites. I. Development of a two interval timers model. *J. Comp. Physiol.*, **134**, 203-217

Vaz Nunes, M. & Veerman, A., (1979b) Photoperiodic time measurement in spider mites. II. Effects of skeleton photoperiods. *J. Comp. Physiol.*, **134**, 219-226

Vaz Nunes, M. & Veerman, A., (1982) External coincidence and photoperiodic time measurement in the spider mite *Tetranychus urticae*. *J. Insect Physiol.*, **28**, 143-154

Wahl, O., (1932) Neue Untersuchungen uber das Zetgedachtnis der Bienen. *Zeitschrift fur vergleichende Physiologie*, **16**, 529-589

Wiedenmann, G., (1977) Weak and strong phase shifting in the activity rhythm of *Leucophaea maderae* (Blaberidae) after light pulses of high intensity. *Z. Naturforsch.*, **32C**, 464-465

Winfree, A.T., (1970a) The temporal morphology of a biological clock. In '*Lectures on Mathmatics in the Life Sciences*' (Ed. Gerstenhaber, M.), *American Math. Soc.*, Providence, p111-150

Winfree, A.T., (1970b) Integrated view of resetting a circadian clock. *J. Theoret. Biol.*, **28**, 327-374

Winfee, A.T., (1980) The geometry of biological time. *Springer-Verlag*, New York

Winfree, A.T. & Gordon, H., (1977) The photosensitivity of a mutant circadian clock. *J. Comp. Physiol.*, **122**, 87-109

Wobus, V., (1966) Der Einfluss Lichtintensitat auf die Resynchronisation der circadianen Laufaktivitat der Schabe *Blaberus craniifer* Burm.(Insecta: Blattidae). *Z. vergl. Physiol.*, **52**, 276-289

Yu, Q., Jacquier, A.C., Citri, Y., Hamblen, M., Hall, J.C. & Rosbash, M.. (1987) Molecular mapping of point mutations in the *period* gene that stop or speed up biological clocks in *Drosophila melanogaster*. *Proc. Natl. Acad. Sc. USA*, **84**, 784-788

Zerr, D.M., Rosbash, M., Hall, J.C. & Siwicki, K.K., (1990) Circadian fluctuations of period protein immunoreactivity in the CNS and the visual system of *Drosophila*. *J. Neurogenet.*, **10**, 2749-2762

Zimmerman, W.F., (1969) On the absence of circadian rhythmicity in *Drosophila pseudoobscura* pupae. *Biol. Bull. Mar. Biol. Lab., Woods Hole*, **136**, 494-500

Zimmerman, W.F. & Ives, D., (1971) Some photophysiological aspects of circadian rhythmicity in *Drosophila*. In '*Biochronometry*' (Ed. Menaker, M.), *Natl. Acad Sci.*, Washington, p381-391

Zimmerman, W.F., Pittendrigh, C.S, & Pavlidis, T., (1968) Temperature compensation of the circadian oscillation in *Drosophila pseudoobscura* and its entrainment by temperature cycles. *J. Insect Physiol.*, **14**, 669-684

Zwiebel, L.J., Hardin, P.E., Liu, X., Hall, J.C. & Rosbash, M., (1991) A post-transcriptional mechanism contributes to circadian cycling of *per* B-galactosidase fusion protein. *Proc. Natl. Acad. Sci. USA.*, **88**, 3882-3886

Acknowledgements

I would like to thank Prof. D.S.Saunders for his help and advice throughout my post-graduate stay at Edinburgh University. I would also like to thank the staff at Edinburgh University for the help they have given and to SERC without which this would not have been possible. A special thanks go to Katie for putting up with my unpredictable hours which often changed at short notice.

The following lists the computer programs used and listed in Appendix i to iii, describing their major PROCedures.

Record:-

PROCinit, issues instructions to run the program, sets the dimensions for the various variables and inputs the filename on which the data will be stored.

PROCbox, draws on the screen the rectangular boxes in which the data will be displayed.

PROClog, for each of seven days, the program constantly checks the state of each of the 32 infra-red receivers in turn. The number of times each beam is broken in a ten minute 'bin' is counted.

PROCdisplay, the number of times each channels beam is broken in the ten minute bin is displayed in the appropriate box produced in PROCbox on the screen.

PROCstore, for each channel the number of times the beam is broken is converted to a string variable and added together to produce a long string of data listing consecutively the number of times each channel recorded a beam being broken in a ten minute bin.

PROClog to PROCstore is repeated for each of 144 ten minute bins. Each of the strings of data are added together so that at the end of a day there is a long string of data which records the data for channel 1 for the first ten minute bin, followed by the data far channel 2, to channel 32, followed by the data for channel 1 for the second ten minute bin e.t.c.The following lists the computer programs used and listed in Appendix i to iii, describing their major PROCedures.

Record:-

PROCinit, issues instructions to run the program, sets the dimensions for the various variables and inputs the filename on which the data will be stored.

PROCbox, draws on the screen the rectangular boxes in which the data will be displayed.

PROClog, for each of seven days, the program constantly checks the state of each of the 32 infra-red receivers in turn. The number of times each beam is broken in a ten minute 'bin' is counted.

PROCdisplay, the number of times each channels beam is broken in the ten minute bin is displayed in the appropriate box produced in PROCbox on the screen.

PROCstore, for each channel the number of times the beam is broken is converted to a string variable and added together to produce a long string of data listing consecutively the number of times each channel recorded a beam being broken in a ten minute bin.

PROClog to PROCstore is repeated for each of 144 ten minute bins. Each of the strings of data are added together so that at the end of a day there is a long string of data which records the data for channel 1 for the first ten minute bin, followed by the data far channel 2, to channel 32, followed by the data for channel 1 for the second ten minute bin e.t.c.

PROCsave, the filename given in PROCinit is opened up and the string of data collected is recorded onto floppy disc.

PROClog to PROCsave is repeated for each of seven days.

Recov32:-

PROCinit, issues user instruction, inputs the name of the file holding data to be separated, the numbers of the channels to be recovered and the number of days of data recorded.

PROCfrom_disc, for each channel for the requested number of days of data a pointer is moved through the recorded data file selecting the sequence of data points relevant to each fly. The data collected by the pointer is stored in a second data string.

PROCto_disc, the data strings produced in PROCfrom_disc are stored on floppy disc.

PROCdisplay, a representation of the data recovered is shown on the screen.

Join32F:-

PROCinit, sets the dimensions of a number of variables, inputs the names of the files to be joined together and the name of the final joined file.

PROCread, opens up the first data file to be joined and reads the data strings into a series of variables, opens up the second data file and reads he data strings in this into a consecutive series of variables.

PROCwrite, opens up the data file to store the joined data and reads the complete series of string variables into this file.

Join32(21):-

This file follows the same procedures as Join32F except one fourteen day file and a second seven day file are joined together.

Ctwork:-

PROCinit, inputs the name of the data file to be printed, the number of days in this data file, the start time of the experiment and the time of the light pulse. Then this procedure sets the dimensions of the variables.

PROCread, opens up the data file and reads the data into a series of string variables.

PROCdisplay, via PROCbox and PROChisto a double plotted actogram is displayed on the screen showing the activity of the individual fly.

PROCmouse, using the mouse this allows sections of the actogram to be selected. The duration of each section of activity is calculated and a mean duration for a number of selected sections calculated. The actual number of times the infra-red beam was broken during the sections of activity is calculated and a mean value calculated for a number of different sections selected. The midpoint of each selected section of the activity rhythm is calculated and a regression line calculated through the midpoint of a number of subsequent days.

PROCagain, a second regression line can be calculated, or the actogram with one regression line can be printed.

PROCphshift, the subjected phase of the activity rhythm illuminated by the light pulse is calculated with reference to the first regression line calculated. This process is repeated, calculating the phase of the light pulse with reference to the second regression line. The phase shift due to the light pulse illuminating the free-running rhythm is calculated by subtracting one value from the other.

Periodogram:-

The initial portion of the program issues instructions initialises variables and loads the data to be analysed.

PROCrandomize, pseudo-randomly reorganizes the data from the file into a random order.

PROCsetscanspeed, inputs the periods to be tested for in the data.

PROCformestimate, statistically 'fits' rhythms over the randomised data to produce confidence limits with which to compare the analysis of the actual data.

PROCfindvarmax, attempts to calculate maximum level of activity.

PROCdrawpergram, draws the periodogram of the data shoeing the variance in the data from each of the input periods to be tested. This variance demonstrates the likely periodicity, if any, present in the data. A more thorough explanation of the mathematical basis behind periodogram analysis is not appropriate here.

Formest:-

This program works in almost the same way as Periodogram except that no periodogram calculations are formed, instead the average formestimate for a number of individuals is superimposed to give a group form estimate.

Entper:-

PROCinit, inputs the strain of fly to be modelled, the light regimen under which it is to be modelled and sets the dimensions of a number of females.

PROCdata, lists the circadian time resulting from a light pulse of given duration falling at a given phase of a free-running rhythm. This data is taken from the experimental work undertaken in the thesis.

PROCcalc, calculates the phase of the phase shifts induced in the free-running rhythm and repeats this for a number of pulses to show steady state entrainment between them.

PROCdisplay, displays the model entrained rhythm on a stylised fictional actogram.

Appendix i

Program Name : Record
Aim: To record the raw data, 32 flies can be monitored at a time in the holders, the number of times each fly breaks the infra-red beam in a ten minute period is stored on floppy disc for later analysis.

```
 10 ON ERROR PROCerror
 20 MODE 128
 30 REM Fly Counter Program
 40 REM Dave Saunders/Niall Kenny
 50 PROCinit
 60 PROClog
 70 END
 80 DEF PROClog
 90 FOR day%=1 TO 7
 100 FOR ch%= 1 TO 32
 110 res$(ch%)=""
 120 NEXT ch%
 130 FOR iter%=1 TO 144
 140 TIME=0
 150 REPEAT
 160 FOR ch%=1 TO 32:?&FE60=(ch%-1 AND &3F):x%=(?&FE60 DIV64) AND 1:IF x%=1 THEN
f%(ch%)=1 ELSE IF f%(ch%)=1 THEN f%(ch%)=0: d%(ch%)=d%(ch%)+1
 170 NEXT ch%
 180 UNTIL TIME>59740
 190 PROCdisplay
 200 PROCstore
 210 NEXT iter%
 220 PROCsave
 230 NEXT day%
 240 ENDPROC
 250 END
 260 :
 270 DEF PROCinit
 280PRINTTAB(2,2)"This program is RECORD"
 290PRINTTAB(2,3)"______________________"
 300PRINTTAB(2,5)"The function of this program is to record the data as it comes
straight"TAB(2,7)"from the infrared sensors. Before this can be done you should take this"
 310PRINTTAB(2,9)"disc out and turn the computer off and then on again. Now push down
the"TAB(2,11)"switch at the back of the disc drive (changing the machine from 80 to"
 320PRINTTAB(2,13)"40 track) and place in drive 0 the disc marked 'ACORN 64K SIDEWAYS
RAM"TAB(2,15)"SUPPORT DISC'. Type in the command *BAS128, press return and fairly"
 330PRINTTAB(2,17)"quickly two lines of text will appear in the top left-hand corner of
the"TAB(2,19)"screen. If this does not happen and an error message appears turn
the"TAB(2,21)"computer off and start again. Once the command has been"
 340PRINTTAB(2,21)"computer off and start again. Once the command has been obeyed take this"
 350PRINTTAB(2,23)"disc out and (NB.!!) push up the switch at the back of the disc drive
to"TAB(2,25)"return it to 80 track. Now you can load RECORD."
 360PRINTTAB(2,27)"The display will show all 32 channels at once: the top row being,
from"TAB(2,29)"left to right, ch1-8; the second row ch9-16 etc."
 370PRINTTAB(20,31)"PRESS SPACE BAR WHEN READY"
 380REPEAT UNTIL GET=32
 390 CLS
 400 ?&FE62=&BF
 410 DIM res$(32),d%(32),f%(32)
 420 p%=0
 430 INPUT"Name of file",n$
 440 PROCbox
 450 ENDPROC
 460 :
 470 DEF PROCsave
 480 A=OPENUP(n$)
 490 PTR#A=p%
 500 FOR ch%= 1 TO 32
 510 PRINT#A,res$(ch%)
```

```
520 res$(ch%)=""
530 NEXT ch%
540 p%=PTR#A
550 CLOSE#A
560 ENDPROC
570 :
580 DEF PROCbox
590 CLS
600 h%=1000
610 FOR i%=0 TO 7
620 x%=(i%*160)+2
630 MOVE x%,h%
640 DRAW x%,h%-224
650 MOVE x%,h%-232
660 DRAW x%,h%-456
670 MOVE x%,h%-464
680 DRAW x%,h%-688
690 MOVE x%,h%-696
700 DRAW x%,h%-920
710 x%=x%+144
720 MOVE x%,h%
730 DRAW x%,h%-224
740 MOVE x%,h%-232
750 DRAW x%,h%-456
760 MOVE x%,h%-464
770 DRAW x%,h%-688
780 MOVE x%,h%-696
790 DRAW x%,h%-920
800 DRAW x%,h%-914
810 NEXT i%
820 ENDPROC
830 :
840 DEF PROChisto
850 h%=h%-(day%*32)
860 FOR i%=0 TO 7
870 MOVE (i%*160)+2+iter%,h%
880 DRAW (i%*160)+2+iter%,h%+SQR(d%(i%+ch%))
890 NEXT i%
900 ENDPROC
910 :
920 DEF PROCdisplay
930 h%=1000
940 ch%=1
950 PROChisto
960 h%=768
970 ch%=9
980 PROChisto
990 h%=536
1000 ch%=17
1010 PROChisto
1020 h%=304
1030 ch%=25
1040 PROChisto
1050 ENDPROC
1060 :
1070 DEF PROCstore
1080 FOR ch%=1 TO 32
1090 IF d%(ch%)>254 THEN d%(ch%)=254
1100 res$(ch%)=res$(ch%)+CHR$(d%(ch%))
1110 d%(ch%)=0
1120 NEXT ch%
1130 ENDPROC
1140 :
1150 DEF PROCerror
1160 CLOSE#0
1170 REPORT
1180 PRINT ERL
```

```
1190 END
1200 ENDPROC
1210 :
```

Program Name : Recov32
Aim: To convert the data file originally recorded on the BBC into 32 seperate files, each one representing an individual fly, each one 7 containing 7 days data and stored on in a format suitable for analysis on the Archimedes.

```
10 ON ERROR PROCerror
20REM PROGRAM RECOVER32
30MODE 128
40PROCinit
50FOR ch%=a% TO chs%
60PROCfrom_disc
70PROCto_disc
80PROCdisplay
90NEXT ch%
100CLOSE#0
110END
120DEF PROCinit
130PRINTTAB(2,2)"This program is RECOV32 for the ARCHIMEDES"
140PRINTTAB(2,3)"________________________________________"
150PRINTTAB(2,6)"This program processes the raw data file that has come from the
computer"TAB(2,8)"and separates out the data for each channel.The name for each new"
160PRINTTAB(2,10)"file incorporates the raw data file-name plus the number of
that"TAB(2,12)"channel.Therefore it is important that the raw data file name is no longer
"TAB(2,14)"than 5 numbers or letters."
170PRINTTAB(2,16)"The raw data file must have been copied to an ARCHIMEDES
disc.The"TAB(2,18)"new files will also be stored on this file."
190PRINTTAB(15,30)"PRESS SPACE BAR WHEN READY"
200REPEAT UNTIL GET=32
210CLS:PRINT"Place disc in ARCHIMEDES DRIVE 0"
220 PROCpause
240*.
250INPUT"Name of recorded data file?"n$
260INPUT"First channel to be recovered"a%
265INPUT"Last channel to be recovered"chs%
266INPUT"Number of days to recover?"days%
270DIM res$(days%)
280A=OPENUP(n$)
290ENDPROC
300:
310DEF PROCfrom_disc
330FOR day%= 1 TO days%
340PTR#A=((ch%-1)*146)+(4672*(day%-1))
350INPUT#A,x$
360res$(day%)=x$
370NEXT day%
380ENDPROC
390:
400DEF PROCto_disc
420chn$=n$+STR$(ch%)
430CLS:PRINT"Channel ";ch%;" ";chn$;" "
440B=OPENOUT(chn$)
450FOR day%= 1 TO days%
460PRINT#B,res$(day%)
470NEXT day%
480CLOSE#B
490ENDPROC
500:
510DEF PROCdisplay
520 height%=900
530FOR day%= 1 TO days%
540d$=res$(day%)
550 FOR iter%= 1 TO 144
```

```
560i$=MID$(d$,iter%,1)
570n%=ASC(i$)
580MOVE iter%+20,height%-(day%*32)
590DRAW iter%+20,height%-(day%*32)+SQR(n%)
600NEXT iter%
610NEXT day%
620ENDPROC
630:
640DEF PROCerror
650 CLOSE#0
660REPORT:PRINT ERL:PRINT ERR
670END
680ENDPROC
690:
700DEF PROCpause
710PRINT"Press SPACE BAR when ready"
720REPEAT UNTIL GET=32
730ENDPROC
```

Program Name : Join32F
Aim: To join together two 7 day data files for an individual fly recorded on subsequent days to produce a data file containing all 14 days of data which can be used to produce 14 day actograms.

```
10REM PROGRAM JOIN32F
20 MODE 128
30ON ERROR PROCerror
40REMPROCinfo
50PROCinit
60PROCjoin
70END
80:
90DEF PROCinit
100DIM join%(32)
110DIM res$(14)
120CLS
250PRINTTAB(2,2)"Insert file containing";TAB(2,4)"data to be joined."
260PROCpause
270INPUT"Stem name of first files? "stem1$
280INPUT"Stem name of second files? "stem2$
290INPUT"Stem name of final files? "stemf$
300INPUT"No. of files to be joined? "files%
305 INPUT"No. of days recorded in second file? "secdays%
310CLS
320FOR file%=1 TO files%
330CLS:INPUT"File nos. to be joined? "join%(file%)
340NEXT file%
350ENDPROC
360:
370DEF PROCread
390f$=stem1$+STR$(join%(file%))
400B=OPENIN(f$)
410FOR day%=1 TO 7
420INPUT#B,res$(day%)
430NEXT day%
440CLOSE#B
460s$=stem2$+STR$(join%(file%))
470B=OPENIN(s$)
480FOR day%=8 TO (7+secdays%)
490INPUT#B,res$(day%)
500NEXT day%
510CLOSE#B
520ENDPROC
530:
540DEF PROCwrite
560n$=stemf$+STR$(join%(file%))
```

```
570A=OPENOUT(n$)
580FOR day%=1 TO (7+secdays%)
590PRINT#A,res$(day%)
600NEXT day%
605CLOSE#A
610ENDPROC
620:
630DEF PROCpause
640PRINT TAB(2,20);"PRESS SPACE BAR WHEN READY"
650REPEAT UNTIL GET=32
660ENDPROC
670:
680DEF PROCerror
690CLOSE#0
700REPORT:PRINT ERL
710END
720ENDPROC
730:
740DEF PROCinfo
750CLS
760PRINT TAB(2,2)"This program is JOIN32F"TAB(2,3)"____________________"
765PRINT TAB(2,6);"This program joins files from";TAB(2,8);"two original files."
770PRINT TAB(2,10);"Place disc with the first";TAB(2,12);"files in Drive 2, the second files";TAB(2,14);"in Drive 3, and a clear disc in";TAB(2,16);"Drive 0."
780PROCpause
790ENDPROC
800:
810DEF PROCjoin
820FOR file%=1 TO files%
825 *DR.0
830PROCread
835 *DR.0
840PROCwrite
841IF file%=files% THEN GOTO 855
847ENDIF
850NEXT file%
855ENDIF:CLS:PRINTTAB(15,10)"THE END"
860ENDPROC
```

Program Name : Join32(21)
Aim: To join together the data recorded for an individual fly over a fourteen day period with data recorded for the same fly over a subsequent 7 day period to produce a data file which can be used to produce 21 day actograms.

```
10REM PROGRAM JOIN32(21)
20MODE 128
30ON ERROR PROCerror
40REMPROCinfo
50PROCinit
60PROCjoin
70END
80:
90DEF PROCinit
100DIM join%(32)
110DIM res$(21)
120CLS
250PRINTTAB(2,2)"Insert file containing";TAB(2,4)"data to be joined."
260PROCpause
270INPUT"Stem name of first files? "stem1$
280INPUT"Stem name of second files? "stem2$
290INPUT"Stem name of final files? "stemf$
300INPUT"No. of files to be joined? "files%
310CLS
320FOR file%=1 TO files%
330CLS:INPUT"File nos. to be joined? "join%(file%)
340NEXT file%
```

```
350ENDPROC
360:
370DEF PROCread
390f$=stem1$+STR$(join%(file%))
400B=OPENIN(f$)
410FOR day%=1 TO 14
420INPUT#B,res$(day%)
430NEXT day%
440CLOSE#B
460s$=stem2$+STR$(join%(file%))
470B=OPENIN(s$)
480FOR day%=15 TO 21
490INPUT#B,res$(day%)
500NEXT day%
510CLOSE#B
520ENDPROC
530:
540DEF PROCwrite
560n$=stemf$+STR$(join%(file%))
570A=OPENOUT(n$)
580FOR day%=1 TO 21
590PRINT#A,res$(day%)
600NEXT day%
605CLOSE#A
610ENDPROC
620:
630DEF PROCpause
640PRINT TAB(2,20);"PRESS SPACE BAR WHEN READY"
650REPEAT UNTIL GET=32
660ENDPROC
670:
680DEF PROCerror
690CLOSE#0
700REPORT:PRINT ERL
710END
720ENDPROC
730:
740DEF PROCinfo
750CLS
760PRINT TAB(2,2)"This program is JOIN32F"TAB(2,3)"____________________"
765PRINT TAB(2,6);"This program joins files from";TAB(2,8);"two original files."
770PRINT TAB(2,10);"Place disc with the first";TAB(2,12);"files in Drive 2, the second
files";TAB(2,14);"in Drive 3, and a clear disc in";TAB(2,16);"Drive 0."
780PROCpause
790ENDPROC
800:
810DEF PROCjoin
820FOR file%=1 TO files%
825 *DR.0
830PROCread
835 *DR.0
840PROCwrite
841IF file%=files% THEN GOTO 855
847ENDIF
850NEXT file%
855ENDIF:CLS:PRINTTAB(15,10)"THE END"
860ENDPROC
```

Program Name : Ctwork

Aim: To firstly plot an actogram from the data recorded for an individual fly, then by using the mouse to highlight the start and end of the activity band a regression line is calculated through the midpoint of activity and the period of the rhythm calculated from this line. The mean duration of α is calculted and the mean amount of activity found within α. The program can by calculating two regression lines, one before a disturbing pulse and one after a pulse, calculate the phase shift in circadian hours produced by the disturbance in the rhythm.

```
10REM DISPLAY 32
20 ON ERROR PROCerror
30 MODE 128
40 PROCinit
50 PROCread
60 PROCtitle
70 PROCdisplay
80 n=1
90 PROCmouse
100 PROCagain
110 IF n=2 THEN PROCphshift
120 PROCdata
129 INPUT TAB(30,30) "PRESS P TO PRINT";C$
130 PRINT TAB(30,30) "                    "
131 IF C$ = "P" THEN PROCprint
140 MOUSE OFF
150 END
160 :
170 DEF PROCinit
180 CLS:INPUT"Name of file to be displayed? "n$
190 INPUT "Start time of experiment? " start
200 INPUT"Total number of days? " days%
210 INPUT"Time of light pulse? "hpulse
220 IF hpulse < start THEN hpulse = hpulse + 24
230 INPUT"Day of light pulse? "dpulse%
240 DIM res$(days%+1):DIM dstart%(days%+2):DIM istart%(days%+2)
250 DIM dfin%(days%+2):DIM ifin%(days%+2):DIM tau(2)
260 DIM tot%(days%+1):DIM yd(days%+1):DIM xi(days%+1)
270 DIM time%(days%+1):DIM mac(2):DIM mt(2):DIM b(2):DIM a(2)
280 DIM xstart(days%+1):DIM ystart(days%+1):DIM xend(days%+1)
290 DIM yend(days%+1):size=1
300 no% = 1
310 ENDPROC
320 :
330 DEF PROCtitle
340 CLS:PRINT TAB(0,0); "Display for ";n$
350 PRINT TAB(57,0); "Start time ";start
360 ENDPROC
370 :
380 DEF PROCread
390 A=OPENUP(n$)
400 FOR day%= 1 TO days%
410 INPUT#A,res$(day%)
420 NEXT day%
430 CLOSE#A
440 ENDPROC
450 :
460 DEF PROCdisplay
470 PROCbox
480 PROChisto
490 ENDPROC
500 :
510 DEF PROCbox
520 x%=80
530 FOR y%=896 TO (896-(64*days%)) STEP-64
540 MOVE x%,y%:DRAW x%+1152,y%
550 NEXT y%
560 y%=896
570 FOR x%=80 TO 1239 STEP 576
580 MOVE x%,y%:DRAW x%,y%-(64*days%)
590 NEXT x%
600 FOR x%= 80 TO 1232 STEP 48
610 MOVE x%,y%:DRAW x%,y%+16
620 NEXT x%
630 ENDPROC
640 :
```

```
650 DEF PROCpause
660 REPEAT UNTIL GET=32
670 ENDPROC
680 :
690 DEF PROChisto
700 height%=896
710 FOR day%= 1 TO days%
720 y%=height%-(64*day%)
730 x%=80:right% = 0
740 PROCdraw
750 x% = 656:right% = 1
760 PROCdraw
770 NEXT day%
780 INPUT TAB(2,2) "PRESS B=INCREASE SCALE, C=CONTINUE P=PRINT, S=DECREASE
SIZE";C$
790 PRINT TAB(2,2) "                                        "
800 IF C$ = "B" THEN size = size + 1: GOTO 60
810 IF C$ = "C" THEN GOTO 820
811 IF C$ = "P" THEN PROCprint
812 IF C$ = "S" THEN size = size/2: GOTO 60
820 ENDPROC
830 :
840 DEF PROCdraw
850 d$=res$(day% + right%)
860 FOR iter% = 1 TO 144
870 MOVE 4*iter%+x%,y%
880 i$=MID$(d$,iter%,1)
890 n%=ASC(i$)
900 IFn%>(60/size) THEN n%=(60/size)
910 DRAW 4*iter%+x%,y%+(n%*size)
920 NEXT iter%
930 LOX = 80+((hpulse-start)*24):LOY = 896 - (dpulse%*64)
940 MOVE LOX,(LOY-5):DRAW LOX,(LOY+69)
950 ENDPROC
960 :
970 DEF PROCmouse
980 *POINTER
990 MOUSE ON
1000 REPEAT
1010 MOUSE xm%,ym%,b%
1020 CASE b% OF
1030 WHEN 4:b%=0:PROCstcalc
1040 WHEN 2:b%=0:PROCencalc:no% +=1
1050 ENDCASE
1060 UNTIL b%=1
1070 FOR runs%  = 1 TO (no%-1)
1080 d$ = res$(dstart%(runs%))
1090 IF dfin%(runs%)>dstart%(runs%) THEN PROCtwodays
1100 FOR i% = istart%(runs%) TO  ifin%(runs%)
1110 i$ = MID$(d$, i%, 1)
1120 n% = ASC(i$)
1130 tot%(runs%) = tot%(runs%) + n%
1140 time%(runs%) = time%(runs%) + 1
1150 NEXT i%
1160 act = act +tot%(runs%)
1170 ti = ti +time%(runs%)
1180 NEXT runs%
1190 mac(n) = act/(no%-1)
1200 mt(n) = (ti/(no%-1))/6
1210 FOR runs% = 1 TO (no%-1)
1220 xi(runs%) = ((xend(runs%)-xstart(runs%))/2)+xstart(runs%)
1230 yd(runs%) = (ystart(runs%)DIV64)*64
1240 sxi = sxi + xi(runs%)
1250 syd = syd + yd(runs%)
1260 smult = smult + (xi(runs%)*yd(runs%))
1270 sxisq = sxisq + (xi(runs%)*xi(runs%))
1280 sydsq = sydsq + (yd(runs%)*yd(runs%))
```

```
1290 MOVE xi(runs%),yd(runs%):DRAW xi(runs%),yd(runs%)-15
1300 NEXT runs%
1310 Bxy = (((no%-1)*smult)-(sxi*syd))/(((no%-1)*sydsq)-(syd*syd))
1320 Axy = (sxi/(no%-1))-((syd/(no%-1))*Bxy)
1330 Xo = (Bxy*(((ystart(1)+64)DIV64)*64))+Axy
1340 MOVE Xo,(((ystart(1)+64)DIV64)*64)
1350 Xl = (Bxy*((yd(no%-1)-64)))+Axy
1360 DRAW Xl,((yd(no%-1)-64))
1370 MOUSE OFF
1380 ENDPROC
1390 :
1400 DEF PROCstcalc
1410 xstart(no%) = xm%:ystart(no%) = ym%
1420 IF xstart(no%) < 656 THEN GOTO 1440
1430 xm% -=656:ym% -= 64
1440 istart%(no%) = (xm%-80)/4
1450 dstart%(no%) = ((896-ym%)/64)+1:b%=0
1460 FOR A = 1 TO 7000
1470 NEXT A
1480 ENDPROC
1490 :
1500 DEF PROCencalc
1510 xend(no%) = xm%:yend(no%) = ym%
1520 IF xend(no%) < 656 THEN GOTO 1540
1530 xm%-=656:ym%-=64
1540 dfin%(no%) = ((896-ym%) / 64)+1
1550 ifin%(no%) = (xm%-80)/4
1560 b%=0
1570 FOR A = 1 TO 7000
1580 NEXT A
1590 ENDPROC
1600 :
1610 DEF PROCtwodays
1620 FOR i = istart%(runs%) TO 144
1630 i$ = MID$(d$, i, 1)
1640 n% = ASC(i$)
1650 tot%(runs%) = tot%(runs%) + n%
1660 NEXT i
1670 time%(runs%) = 144 - istart%(runs%)
1680 istart%(runs%) = 0:d$ = res$(dstart%(runs%)+1)
1690 ENDPROC
1700 :
1710 DEF PROCagain
1711 INPUT TAB(2,2) "PRESS P TO PRINT, A TO DRAW ANOTHER LINE";C$
1712 PRINT TAB(2,2) "                                        "
1720 IF C$ = "A" THEN GOTO 1750
1730 IF C$ = "P" THEN PROCprint
1740 GOTO 1890
1750 b(n) = Bxy
1760 a(n) = Axy
1770 sxi=0:syd=0:smult=0:sxisq=0:sydsq=0
1780 FOR runs% = 1 TO no%
1790 tot%(runs%) = 0:time%(runs%) = 0
1800 NEXT runs%
1810 no% = 1
1820 act=0:ti=0
1830 xb = (b(n)*896)+a(n)
1840 xa = (b(n)*832)+a(n)
1850 tau(n) = (144-((xb-xa)/4))/6
1860 n+=1
1870 PROCmouse
1880 INPUT TAB(2,2) "PRESS P TO PRINT";C$
1881 PRINT TAB(2,2) "                "
1883 IF C$ = "P" THEN PROCprint
1890 b(n) = Bxy
1900 a(n) = Axy
1910 xb = (b(n)*896)+a(n)
```

```
1920 xa = (b(n)*832)+a(n)
1930 tau(n) = (144-((xb-xa)/4))/6
1940 ENDPROC
1950 :
1960 DEF PROCdata
1970 n=1
1980 CLS
1990 PRINT
2000 PRINT
2010 PRINT "BEFORE DISTURBING PULSE"
2020 PRINT "MEAN AMOUNT OF ACTIVITY = ";mac(n)
2030 PRINT "MEAN LENGTH OF 'ALPHA' = ";mt(n)
2040 PRINT "TAU FR0M REGRESSI0N LINE = ";tau(1)
2050 n=2
2060 PRINT
2070 IF mac(n) = 0 THEN GOTO 2180
2080 PRINT "AFTER DISTURBING PULSE"
2090 PRINT "MEAN AMOUNT OF ACTIVITY = ";mac(n)
2100 PRINT "MEAN LENGTH OF 'ALPHA' = ";mt(n)
2110 PRINT "TAU FR0M REGRESSI0N LINE = ";tau(2)
2120 PRINT
2130 IF n = 1 THEN GOTO @@@@
2140 PRINT "Circadian time of pulse (before pulse) is ";ctlipulse
2150 PRINT "Circadian time of pulse (after pulse) is ";ctlipulseaft
2160
2170 PRINT "Phase shift = ";phaseshift;" hours"
2180 ENDPROC
2190 IF GET$ = "P" THEN PROCprint
2200 :
2210 DEF PROCphshift
2220 ct6bef = (b(1)*(896-(dpulse%*64)))+a(1)
2230 ct6aft = (b(2)*(896-(dpulse%*64)))+a(2)
2240 ct6bef = ((ct6bef-80)/4)
2250 ct6aft = ((ct6aft-80)/4)
2260 lix = (hpulse-start)*6
2270 IF lix >ct6bef THEN ctlibef=6+(((lix-ct6bef)/6)*(24/tau(1))) ELSE ctlibef = 30-(((ct6bef-
lix)/6)*(24/tau(1)))
2280 IF lix >ct6aft THEN ctliaft=6+(((lix-ct6aft)/6)*(24/tau(2))) ELSE ctliaft = 30-(((ct6aft-
lix)/6)*(24/tau(2)))
2290 phaseshift = (ctliaft-ctlibef)
2300 IF phaseshift > 12 THEN phaseshift = phaseshift - 24
2310 IF phaseshift <-12 THEN phaseshift = phaseshift + 24
2320 ctlipulse = ctlibef
2330 ctlipulseaft = ctliaft
2331 IF ctlipulse < 0 THEN ctlipulse = ctlipulse+24
2340 IF ctlipulse > 24 THEN ctlipulse = ctlipulse-24
2341 IF ctlipulseaft < 0 THEN ctlipulseaft = ctlipulseaft+24
2342 IF ctlipulseaft > 24 THEN ctlipulseaft = ctlipulseaft-24
2350 ENDPROC
2360 :
2370 DEF PROCprint
2380 VDU2
2390 *HARDCOPYMX
2400 VDU3
2410 ENDPROC
2420 :
2430 DEF PROCerror
2440 CLOSE#0
2450 REPORT: PRINT ERL:PRINT ERR
2460 STOP
2470 ENDPROC
2480 :
```

Program Name : Periodogram analysis

Aim: To determine whether the locomotor activity of an individual exhibits a rhythmic pattern, to highlight the period of this rhythm and then plot a form estimate for the rhythm with this period

```
10REM PERIODOGRAM ANALYSIS
20REMPROCScreenDumpAssemble
30REM USE CALL &A00 FOR SCDUMP
40
60MODE128
150CLS:INPUT"Name of file to be analysed? "n$
160INPUT"Number of days?"days%
170INPUT"Start iter no?" start%
180 INPUT"End iter no?" end%
190DIM D%(3024)
200DIM res$(days%+1)
210PROCrandomize
220PROCsetscanspeed
230CLS
240pass=0
250REPEAT
260pass=pass+1
270IF pass=1 GOTO420
280CLS:PRINTTAB(3,0)"Hello, at this point the actual data are being loaded in order to be analysed."
290 A=OPENIN(n$)
300FOR day%=1 TO days%
310 INPUT#A,res$(day%)
320NEXT day%
330CLOSE#A
340FOR day%=1 TO days%
350d$=res$(day%)
360FOR iter%=1 TO 144
370int%=(day%*144)-144+iter%
380i$=MID$(d$,iter%,1)
390D%(int%)=ASC(i$)
400NEXT iter%
410NEXT day%
420 FOR P=B TO E
430FOR I=1 TO P:F(I)=0:NEXT
440PROCformestimate
450IF speed=1 THEN 490
460PROCdrawformest
470IF GET=32 THEN PROCdavid
480PROCprintformest
490V(P)=V
500IF pass=1 THEN V1(P)=V(P)
510NEXT P
520UNTIL pass=2
530PROCfindvarmax
540PROCconflimits
550pass=0
560MODE128
570REPEAT
580pass=pass+1
590IF pass=1 GOTO 610
600FOR I=B TO E:V1(I)=V(I):NEXT I
610IF E-B=0 THEN 650
620PROCdrawpergram
630IF GET=32 THEN PROCdavid
640PROCprintperio
650UNTIL pass=2
660P=permax:speed=2
670PROCformestimate
680PROCdrawformest
690IF GET=32 THEN PROCdavid
700PROCprintformest
710CLOSE#0
720END
```

```
730:
1010DEF PROCsetscanspeed
1020CLS
1040PRINT"What is the shortest period to test for "
1050INPUT B
1060PRINT"What is the longest period to test for "
1070INPUT E
1080DIM F(E),V(E),V1(E)
1090PRINT"Select 1 for Fast,2 for Slow route.":speed=GET:speed=speed-48:PRINT speed
1100ENDPROC
1110:
1120DEF PROCformestimate
1130CLS:PRINTTAB(0,0)"Calculating periodicity=";P
1140FOR U=1 TO P
1150FOR Y=U TO (end%-start%) STEP P
1160X=D%(Y+ start%)
1170F(U)=F(U)+X
1180NEXT
1190NEXT
1200reps=(end%-start%) DIV P:rem=(end%-start%) MOD P
1210FOR I=1 TO rem
1220F(I)=F(I)/(reps+1)
1230NEXT I
1240FOR I=rem+1 TO P:F(I)=F(I)/reps:NEXT
1250REM Select Highest F(I)
1260IF speed=1 THEN 1320
1270Z=F(1)
1280FOR I=2 TO P
1290X=F(I)
1300IF X>Z THEN Z=X
1310NEXT I
1320REM CALC OF MEAN AND VARIANCE
1330M=0
1340FOR I=1 TO P:M=M+F(I):NEXT I
1350M=M/P
1360V=0
1370FOR I=1 TO P:V=V+((F(I)-M)^2):NEXT I
1380V=V/(P-1)
1390ENDPROC
1400:
1410DEF PROCrandomize
1420CLS
1450PRINT"At this point the data is being loaded to be randomised in order to produce the confidence
limits on the periodogram."
1460A=OPENIN(n$)
1470FOR day%=1 TO days%
1480INPUT#A,res$(day%)
1490NEXT day%
1500CLOSE#A
1510FOR day%=1 TO days%
1520d$=res$(day%)
1530FOR iter%=1 TO 144
1540int%=(day%*144)-144+iter%
1550i$=MID$(d$,iter%,1)
1560D%(int%)=ASC(i$)
1570NEXT iter%
1580NEXT day%
1590FOR iter%=1 TO (days%*144)-2
1600Y%=D%(iter%)
1610X%=iter%+RND((days%*144)-iter%)
1620D%(iter%)=D%(X%)
1630D%(X%)=Y%
1640NEXT iter%
1650ENDPROC
1660:
1670DEF PROCdrawformest
1680CLS
```

? ?

```
1690MOVE 100,100
1700DRAW 100,924:DRAW 1180,924:DRAW 1180,100:DRAW 100,100
1710MOVE 100,100+(F(P)*840/Z)
1720FOR I=1 TO P:GOSUB2000:DRAW X,Y:NEXT I
1730average=100+(M*824/Z):MOVE 100,average:PLOT 21,1180,average
1740VDU5
1750IF P>50 step=5 ELSE step=2
1760 FOR I=0 TO P STEP step
1770GOSUB2000
1780MOVE X,80:DRAW X,100
1790MOVE X-16,76
1800L$=STR$(INT(((I/10-INT(I/10))*10)+.5))
1810PRINT L$
1820NEXT
1830MOVE 400,40:PRINT"PERIOD PHASE"
1840MOVE 100,970:PRINT"FORM ESTIMATE,P= ";P;"  from "
1850IF pass<>1 THEN 1870
1860MOVE 900,970:PRINT"RANDOMIZED"
1870FOR I=0 TO Z STEP2
1880Y=100+(I*824/Z)
1890MOVE 80,Y:DRAW100,Y
1900NEXT
1910A$="AMPLITUDE"
1920FOR I=1 TO 9
1930MOVE 10,I*50+300:PRINT MID$(A$,10-I,1)
1940NEXT
1950mean$=STR$((INT((M)*100))/100)
1960MOVE 1200,average+16:PRINT LEFT$(mean$,4)
1970VDU4
1980ENDPROC
1990:
2000Y=100+(F(I)*824/Z):X=100+(I*1080/P)
2010RETURN
2020:
2030DEF PROCprintformest
2040CLS
2050PRINT"PRINT OUT FORM ESTIMATE VALUES? (Y/N) "
2060INPUTA$:IF A$="N" THEN ENDPROC
2070PRINT;"PHASE","REPL.","AMPL."
2080FOR I=1 TO P:PRINT;I,STR$(reps+1),LEFT$(STR$((INT(F(I)*100))/100),4):NEXT
2090PRINT"PERIOD=";P;"MEAN=";M;"VARIANCE=";V
2100IF GET=32 THEN PROCdavid
2110ENDPROC
2120:
2130DEF PROCfindvarmax
2140varmax=0
2150FOR I=B TO E
2160IF V(I)>varmax THEN  varmax=V(I)
2170IF V(I)=varmax THEN permax=I
2180NEXT I
2190ENDPROC
2200:
2210DEF PROCdrawpergram
2220CLS
2230MOVE 100,100:DRAW 100,924:DRAW 1180,924:DRAW 1180,100:DRAW 100,100
2240I=B
2250GOSUB2560
2260MOVE X,Y
2270FOR I=B+1 TO E:GOSUB2560:DRAW X,Y:NEXT
2280VDU5
2290step=1:IF E-B>10 THEN step=2:IF E-B>20 THEN step=5:IF E-B>50 THEN step=10
2300FOR I=B TO E STEP step:GOSUB2560:MOVE X,80:DRAW X,100:MOVE X-26,76:PRINT
STR$(I):NEXT
2310FOR I=0 TO varmax STEP 2
2320Y=100+(I*824/varmax)
2330MOVE 80,Y:DRAW 100,Y
2340NEXT
```

```
2350MOVE 20,Y+16:PRINT STR$(I-2)
2360A$="VARIANCE "
2370FOR I=1 TO 9
2380MOVE 10,I*50+300:PRINT MID$(A$,10-I,1)
2390NEXT
2400MOVE 100,970:PRINT"PERIODOGRAM,P=";B;" TO ";E
2410MOVE 400,45:PRINT"PERIOD LENGTH IN TEN MINUTE INTERVALS"
2420IF pass<>1 THEN 2440
2430MOVE 900,970:PRINT"RANDOMIZED"
2440REM IF pass<>2 THEN 2660
2450y1=100+(Y1*824/varmax)
2460y2=100+(Y2*824/varmax)
2470y3=100+(Y3*824/varmax)
2480y4=100+(Y4*824/varmax)
2490MOVE 100,y1
2500PLOT 21,1180,y2:PRINT"95%"
2510MOVE 100,y3:PLOT 21,1180,y4
2520VDU4
2530SOUND 1,-15,90,20
2540ENDPROC
2550:
2560Y=100+(V1(I)*824/varmax)
2570IF pass=1 AND Y>924 THEN Y=924
2580X=100+((I-B)*1080/(E-B))
2590RETURN
2600:
2610DEF PROCprintperio
2620CLS
2630PRINT"PRINT OUT PERIODOGRAM VALUES? (Y/N) "
2640INPUTA$:IF A$="N" THEN ENDPROC
2650PRINT"PERIOD","VARIANCE"
2660FOR I=B TO E:PRINT;I,(INT(V1(I)*100))/100:NEXT
2670IF GET=32 THEN PROCdavid
2680ENDPROC
2690:
2700DEF PROCconflimits
2710sumx=0:sumy=0:ssx=0:ssy=0:sxy=0:n=(E-B)+1
2720FOR I=B TO E
2730sumx=sumx+I
2740sumy=sumy+V1(I)
2750ssx=ssx+I^2
2760ssy=ssy+V1(I)^2
2770sxy=sxy+V1(I)*I
2780NEXT I
2790mnx=sumx/n:mny=sumy/n
2800cxx=ssx-sumx^2/n
2810cyy=ssy-sumy^2/n
2820cxy=sxy-(sumx*sumy)/n
2830b=cxy/cxx
2840a=mny-b*mnx
2850resss=cyy-cxy^2/cxx
2860resms=resss/(n-2)
2870IF n=3 T=12.706
2880IF n=4 T=4.303
2890IF n=5 T=3.182
2900IF n=6 T=2.776
2910IF n=7 T=2.571
2920IF n=8 T=2.447
2930IF n=9 T=2.365
2940IF n=10 T=2.306
2950IF n=11 T=2.262
2960IF n=12 T=2.228
2970IF n>12 T=2.179
2980IF n>14 T=2.145
2990IF n>16 T=2.120
3000IF n>18 T=2.101
3010IF n>20 T=2.086
```

```
3020IF n>22 T=2.064
3030IF n>26 T=2.042
3040IF n>32 T=2.021
3050d=T*SQR(resms)
3060Y1=(a+d)+b*B
3070Y2=(a+d)+b*E
3080Y3=a+b*B
3090Y4=a+b*E
3100ENDPROC
3110:
3120DEFPROCdavid
3130VDU2
3140*HARDCOPYMX
3150VDU3
3160ENDPROC
```

Appendix ii

Program Name : Formest
Aim: To produce a population mean form estimate by calculating the shape of a number of form estimates for individuals from a given population and 'adding' them together

```
10REM PROGRAM TO PRODUCE MEAN FORM ESTIMATE FROM  NUMBER OF FLIES
20REM ENTRAINED TO LIGHT DARK CYCLE
30MODE128
40CLS:INPUT"Number of data files to produce mean form estimate from? "N
50INPUT"What period do you want to test for? "P
60INPUT"At what iteration does light come on? "LON
70INPUT"At what iteration does light go off? "LOF
80INPUT"Number of days?"days%
90DIM res$(days%+1)
100DIM D%(3024):DIM F(P):DIM V(P):DIM A(P)
110 FOR I=1 TO P:A(I)=0:NEXT I
120FOR runs= 1 TO N
130INPUT"Name of file? "n$
140INPUT"Start iter no?" start%
150 INPUT"End iter no?" end%
160CLS
170 A=OPENIN(n$)
180FOR day%=1 TO days%
190 INPUT#A,res$(day%)
200NEXT day%
210CLOSE#A
220FOR day%=1 TO days%
230d$=res$(day%)
240FOR iter%=1 TO 144
250int%=(day%*144)-144+iter%
260i$=MID$(d$,iter%,1)
270D%(int%)=ASC(i$)
280NEXT iter%
290NEXT day%
300FOR I=1 TO P:F(I)=0:NEXT
310PROCformestimate
320NEXT runs
330FOR I = 1 TO P:tot=tot+A(I):NEXT I:M=tot/P
340PROCdrawformest
350IF GET=32 THEN PROCdraw
360END
370:
380DEF PROCformestimate
390FOR U=1 TO P
400FOR Y=U TO (end%-start%) STEP P
410X=D%(Y+ start%)
420F(U)=F(U)+X
430NEXT
440NEXT
450reps=(end%-start%) DIV P:rem=(end%-start%) MOD P
460FOR I=1 TO rem
470F(I)=F(I)/(reps+1)
480NEXT I
490FOR I=rem+1 TO P:F(I)=F(I)/reps:NEXT
500Z=F(1)
510FOR I=2 TO P
520X=F(I)
530IF X>Z THEN Z=X
540NEXT I
550FOR I=1 TO P:A(I)=A(I)+(F(I)/Z):NEXT I
560ENDPROC
570:
580DEF PROCdrawformest
590CLS
```

```
600INPUT"Strain of flies? "s$
610INPUT"T-cycle? "t$
620CLS
630PRINT TAB(10,1) s$:PRINT TAB(25,1) t$:PRINT TAB(30,1) N
640MOVE 100,100
650DRAW 100,924:DRAW 1180,924:DRAW 1180,100:DRAW 100,100
660MOVE 100,100+(A(P)*840/N)
670FOR I=1 TO P:GOSUB800:DRAW X,Y:NEXT I
680average=100+(M*824/Z):MOVE 100,average:PLOT 21,1180,average
690VDU5
700mean$=STR$((INT((M)*100))/100)
710MOVE 1200,average+16:PRINT LEFT$(mean$,4)
720VDU4
730X=100+(LON*1080/P)
740MOVE X,60:DRAW X,924
750X=100+(LOF*1080/P)
760MOVE X,60:DRAW X,924
770X=100:Y=100:MOVE X,Y:DRAW X,60:DRAW 1180,60:DRAW 1180,100
780ENDPROC
790:
800Y=100+(A(I)*824/N):X=100+(I*1080/P)
810RETURN
820:
830DEFPROCdraw
840VDU2
850*HARDCOPYMX
860VDU3
870ENDPROC
```

Appendix iii

Program Name : Entper
Aim: To predict the phase relationship between various light-dark cycles and the circadian oscillator controlling locomotor activity using the experimentally acquired PRC data.

```
 100 ON ERROR PROCerror
 200 REM ENTRAINMENT PROGRAM
 300 REM PER-S 1, 6, 10 HOUR LIGHT PULSE
 400 PROCinput
 500 PROCdata
 600 PROCcalc
 650 PROCpause
 700 PROCdisplay
 800 IF GET=32 THEN PROCprint
 900 PROCformest
1000 IF GET=32 THEN PROCprint
1100 END
1200 :
1300 DEF PROCinput
1400 INPUT "Which strain, pers = 1; per+ = 2; perL2 = 3?";strain
1500 INPUT "How many pulses of light in an L:D cycle ";p
1600 DIM dp(p+1):DIM lp(p+1):DIM CT(41,p+1)
1700 FOR n = 1 TO p
1800 INPUT "Length of light pulse ";lp(n)
1900 INPUT "Length of dark gap ";dp(n)
2000 IF n=p THEN GOTO 2200
2100 NEXT n
2200 INPUT "Circadian time of first pulse ";CT(1,1)
2300 lp(0)=0:CASE strain OF
2400   WHEN 1: dp(0)=CT(1,1)/1.2183
2500   WHEN 2: dp(0)=CT(1,1)/1.0118
2600   WHEN 3: dp(0)=CT(1,1)/0.8247
2700 ENDCASE
2800 DIM T(3,10,25)
2900 ENDPROC
3000 :
3100 DEF PROCdata
3200 T(1,1,0)=3.8
3300 T(1,1,1)=3.7
3400 T(1,1,2)=4.0
3500 T(1,1,3)=4.5
3600 T(1,1,4)=5.2
3700 T(1,1,5)=6.2
3800 T(1,1,6)=6.9
3900 T(1,1,7)=7.3
4000 T(1,1,8)=7.6
4100 T(1,1,9)=7.2
4200 T(1,1,10)=6.4
4300 T(1,1,11)=5.6
4400 T(1,1,12)=4.7
4500 T(1,1,13)=3.6
4600 T(1,1,14)=3.3
4700 T(1,1,15)=3.7
4800 T(1,1,16)=10.6
4900 T(1,1,17)=18.2
5000 T(1,1,18)=22.8
5100 T(1,1,19)=0.9
5200 T(1,1,20)=2.3
5300 T(1,1,21)=3.2
5400 T(1,1,22)=3.6
5500 T(1,1,23)=3.3
5600 T(1,1,24)=3.8
5700 T(1,6,0)=9.4
5800 T(1,6,1)=9.1
```

```
5900 T(1,6,2)=9.0
6000 T(1,6,3)=8.9
6100 T(1,6,4)=8.7
6200 T(1,6,5)=8.5
6300 T(1,6,6)=8.3
6400 T(1,6,7)=8.2
6500 T(1,6,8)=8.1
6600 T(1,6,9)=8.0
6700 T(1,6,10)=7.8
6800 T(1,6,11)=7.6
6900 T(1,6,12)=7.4
7000 T(1,6,13)=7.5
7100 T(1,6,14)=7.6
7200 T(1,6,15)=7.7
7300 T(1,6,16)=7.9
7400 T(1,6,17)=8.0
7500 T(1,6,18)=8.2
7600 T(1,6,19)=8.3
7700 T(1,6,20)=8.6
7800 T(1,6,21)=8.6
7900 T(1,6,22)=8.9
8000 T(1,6,23)=9.1
8100 T(1,6,24)=9.4
8200 T(2,1,0)=1.3
8300 T(2,1,1)=2.0
8400 T(2,1,2)=3.0
8500 T(2,1,3)=4.0
8600 T(2,1,4)=4.9
8700 T(2,1,5)=5.6
8800 T(2,1,6)=6.2
8900 T(2,1,7)=6.7
9000 T(2,1,8)=7.0
9100 T(2,1,9)=7.3
9200 T(2,1,10)=7.7
9300 T(2,1,11)=8.0
9400 T(2,1,12)=8.7
9500 T(2,1,13)=9.5
9600 T(2,1,14)=10.5
9700 T(2,1,15)=11.8
9800 T(2,1,16)=13.7
9900 T(2,1,17)=16.5
10000 T(2,1,18)=20.2
10100 T(2,1,19)=22.9
10200 T(2,1,20)=0.3
10300 T(2,1,21)=1.1
10400 T(2,1,22)=1.3
10500 T(2,1,23)=0.7
10600 T(2,1,24)=1.3
10700 T(2,6,0)=8.3
10800 T(2,6,1)=8.2
10900 T(2,6,2)=8.2
11000 T(2,6,3)=8.2
11100 T(2,6,4)=8.1
11200 T(2,6,5)=8.3
11300 T(2,6,6)=8.3
11400 T(2,6,7)=8.2
11500 T(2,6,8)=8.2
11600 T(2,6,9)=8.2
11700 T(2,6,10)=8.2
11800 T(2,6,11)=8.2
11900 T(2,6,12)=8.2
12000 T(2,6,13)=8.2
12100 T(2,6,14)=8.2
12200 T(2,6,15)=8.0
12300 T(2,6,16)=8.0
12400 T(2,6,17)=8.0
12500 T(2,6,18)=8.0
```

```
12600 T(2,6,19)=8.0
12700 T(2,6,20)=8.0
12800 T(2,6,21)=8.1
12900 T(2,6,22)=8.1
13000 T(2,6,23)=8.2
13100 T(2,6,24)=8.3
13101 T(3,1,0)=3.9
13102 T(3,1,1)=8.1
13103 T(3,1,2)=10.5
13104 T(3,1,3)=11.3
13105 T(3,1,4)=10.2
13106 T(3,1,5)=8.5
13107 T(3,1,6)=8.0
13108 T(3,1,7)=8.4
13109 T(3,1,8)=9.0
13110 T(3,1,9)=9.8
13111 T(3,1,10)=10.8
13112 T(3,1,11)=11.7
13113 T(3,1,12)=12.5
13114 T(3,1,13)=13.2
13115 T(3,1,14)=13.5
13116 T(3,1,15)=13.7
13117 T(3,1,16)=13.6
13118 T(3,1,17)=13.2
13119 T(3,1,18)=12.6
13120 T(3,1,19)=12.7
13121 T(3,1,20)=13.5
13122 T(3,1,21)=14.7
13123 T(3,1,22)=16.4
13124 T(3,1,23)=19.3
13125 T(3,1,24)=3.9
13126 T(3,6,0)=12.7
13127 T(3,6,1)=13.0
13128 T(3,6,2)=12.8
13129 T(3,6,3)=12.7
13130 T(3,6,4)=12.7
13131 T(3,6,5)=12.6
13132 T(3,6,6)=12.6
13133 T(3,6,7)=12.5
13134 T(3,6,8)=12.6
13135 T(3,6,9)=12.5
13136 T(3,6,10)=12.4
13137 T(3,6,11)=12.5
13138 T(3,6,12)=12.5
13139 T(3,6,13)=12.4
13140 T(3,6,14)=12.4
13141 T(3,6,15)=12.4
13142 T(3,6,16)=12.4
13143 T(3,6,17)=12.4
13144 T(3,6,18)=12.4
13145 T(3,6,19)=12.4
13146 T(3,6,20)=12.3
13147 T(3,6,21)=12.3
13148 T(3,6,22)=12.4
13149 T(3,6,23)=12.5
13150 T(3,6,24)=12.7
13200 ENDPROC
13300 :
13400 DEF PROCcalc
13500 CLS
13600 FOR N=1 TO 21
13700 FOR n = 1 TO p
13800 i=INT(CT(N,n))
13900 f=CT(N,n)-i
14000 ct=T(strain,lp(n),i):cn=T(strain,lp(n),(i+1))
14100 diff=ct-cn
14200 CASE strain OF
```

```
14300    WHEN 1:CT(N,n+1)=ct-(diff*f)+(dp(n)*1.2183)
14400    WHEN 2:CT(N,n+1)=ct-(diff*f)+(dp(n)*1.0118)
14500    WHEN 3:CT(N,n+1)=ct-(diff*f)+(dp(n)*0.8247)
14600 ENDCASE
14700 REPEAT
14800 IF CT(N,n+1)>24 THEN CT(N,n+1)=CT(N,n+1)-24
14900 UNTIL CT(N,n+1)<=24
15000 NEXT n
15010 PRINT "CT AT START OF PULSE ";CT(N,1)
15100 CT(N+1,1)=CT(N,p+1)
15200 CT(N,1)=(INT(CT(N,1)*100))/100
15300 CT(N+1,1)=(INT(CT(N+1,1)*100))/100
15400 NEXT N
15500 ENDPROC
15600 :
15700 DEF PROCpause
15800 REPEAT UNTIL GET=32
15900 ENDPROC
16000 :
16100 DEF PROCdisplay
16200 CLS:MODE128
16300 PROCbox
16400 PROCdraw
16500 ENDPROC
16600 :
16700 DEF PROCbox
16800 x%=40
16900 FOR y%=40 TO 880 STEP 40
17000 MOVE x%,y%:DRAW x%+1200,y%
17100 NEXT y%
17200 RECTANGLE 40,40,1200,840
17300 y%=880
17400 FOR x%=40 TO 1240 STEP 50
17500 MOVE x%,y%:DRAW x%,y%+7
17600 NEXT x%
17700 ENDPROC
17800 :
17900 DEF PROCdraw
18000 D=1
18100 CASE strain OF
18200   WHEN 1: x%=40+((CT(1,1)/1.2183)*50)
18300   WHEN 2: x%=40+((CT(1,1)/1.0118)*50)
18400   WHEN 3: x%=40+((CT(1,1)/0.8247)*50)
18500 ENDCASE
18600 FOR N=1 TO 21
18700 ast%=0
18800 FOR n=1 TO p
18900 z%=lp(n)*50
19000 IF x%>1240 THEN PROCnew
19100 IF x%>(1240-(lp(n)*50)) THEN GOTO 19900
19200 y%=880-(40*D)
19300 PROCast
19400 RECTANGLE x%,y%,z%,40
19500 x%=x%+(lp(n)*50)+(dp(n)*50)
19600 NEXT n
19700 NEXT N
19800 ENDPROC
19900 w%=1240-x%
20000 y%=880-(40*D)
20100 PROCast
20200 RECTANGLE x%,y%,w%,40
20300 D=D+1
20400 IF D>14 THEN ENDPROC
20500 z%=(lp(n)*50)-w%
20600 w%=40
20700 y%=880-(40*D)
20800 RECTANGLE w%,y%,z%,40
```

```
20900 D=D-1
21000 GOTO 19500
21100 :
21200 DEF PROCnew
21300 x%=x%-1200
21400 D=D+1:d=d+1
21500 ENDPROC
21600 :
21700 DEF PROCsplit
21800 w%=1240-x%
21900 y%=880-(40*D)
22000 RECTANGLE x%,y%,w%,40
22100 D=D+1
22200 z%=(lp(n)*50)-w%
22300 x%=40
22400 ENDPROC
22500 :
22600 DEF PROCast
22700 IF CT(N,n)<6 AND n<>p THEN ENDPROC
22800 IF ast%>=1 THEN GOTO 26100
22900 d=D
23000 CASE strain OF
23100   WHEN 1: dist=(6-(CT(N,n))/1.2183)*50
23200   WHEN 2: dist=(6-(CT(N,n))/1.0118)*50
23300   WHEN 3: dist=(6-(CT(N,n))/0.8247)*50
23400 ENDCASE
23500 X%=x%+dist
23600 IF X%<40 THEN d=d-1
23700 IF X%>1240 THEN PROCnewast
23800 Y%=916-(40*d)
23900 VDU5
24000 MOVE X%,Y%:PRINT"*"
24100 VDU4
24200 dlo=D:dhi=D
24300 CASE strain OF
24400  WHEN 1: halfalpha = 212
24500  WHEN 2: halfalpha = 304
24600  WHEN 3: halfalpha = 325
24700 ENDCASE
24800 XLO%=(x%+dist)-halfalpha
24900 XHI%=(x%+dist)+halfalpha
25000 IF XLO% < 40 THEN dlo = D-1
25100 IF XHI% > 1240 THEN dhi = D+1
25200 IF XLO% < 40 THEN XLO% = XLO% +1240
25300 IF XHI% > 1240 THEN XHI% = XHI% -1200
25400 YLO% = 916-(40*dlo)
25500 YHI% = 916-(40*dhi)
25600 VDU5
25700 MOVE XLO%,YLO%:PRINT "="
25800 MOVE XHI%,YHI%:PRINT "="
25900 VDU4
26000 ast%=ast%+1
26100 ENDPROC
26200 :
26300 DEF PROCnewast
26400 d=d+1
26500 X%=X%-1200
26600 ENDPROC
26700:
26800 DEF PROCformest
26900 INPUT"Set time on model formestimate when light comes on ";lion
27000 INPUT"Set time on model formestimate when lights go off ";liof
27100 FOR n = 1 TO p:T = T + dp(n) + lp(n):NEXT n
27200 INPUT"What day of entrainment do you want to produce form estimate ";day
27300 CLS:MODE128:PRINT TAB(2,1)"Model Form Estimate for day ";day;" T = ";T
27400 MOVE 100,100:DRAW 100,924:DRAW 1180,924:DRAW 1180,100:DRAW 100,100
27500 DRAW 100,60:DRAW 1180,60:DRAW 1180,100:MOVE 100,100
```

```
27600 MOVE 100+(lion*(1080/T)),60:DRAW 100+(lion*(1080/T)),924
27700 MOVE 100+(liof*(1080/T)),60:DRAW 100+(liof*(1080/T)),924
27800 ct%=(100+(lion*(1080/T)))+((6-CT(day,1))*(1080/T))
27900 IF ct%>1080 THEN ct%=ct%-1080
28000 CASE strain OF
28100    WHEN 1:ha%=(4.2*(1080/T))
28200    WHEN 2:ha%=(6.1*(1080/T))
28300    WHEN 3:ha%=(6.5*(1080/T))
28400 ENDCASE
28500 startact%=ct%-ha%
28600 IF startact%>1080 THEN startact%=startact%-1080
28700 IF startact%<100 THEN startact%=startact%+980
28800 endact%=ct%+ha%
28900 IF endact%>1080 THEN endact%=endact%-1080
29000 IF endact%<100 THEN endact%=endact%+980
29100 IF endact%<startact% THEN GOTO 29300
29200 MOVE 100,110:DRAW startact%,110:DRAW startact%,880:DRAW endact%,880:DRAW
endact%,110:DRAW 1180,110
29300 MOVE 100,880:DRAW endact%,880:DRAW endact%,110:DRAW startact%,110:DRAW
startact%,880:DRAW 1180,880
29400 ENDPROC
29500 :
29600 DEF PROCerror
29700 CLOSE#0
29800 REPORT:PRINT ERL:PRINT ERR
29900 END
30000 ENDPROC
30100:
30200 DEF PROCprint
30300 VDU2
30400 *HARDCOPYMX
30500 VDU3
30600 ENDPROC
```

Program Name : Pace-slave

Aim: To model the photoperiodic response of *Drosophila melanogaster* females using a two oscillator model based upon external coincidence (Gillanders & Saunders, 1992).

```
10 ON ERROR PROCerror
30 REM ENTRAINABLE SLAVE
40 REM PHOTOPERIODISM MODEL
50 REM WITH Q10 FOR DAYS SLOPE UB
55 REM OF BOTH OSCILLATORS
60 REM INDSUM VARIABLE THRESH FOR +S
70 REM PACEMAKER/SLAVE EXT COINCIDENCE
75 *FX 225,1
80 *KEY 1 CLS|M MODE 135|M LIST 1660,1810|M
90 *KEY 2 RUN|M
105 VDU 23,48,60,102,102,102,102,102,60,0
110 DIM D(2,43)
115 DIM LIGHT(2)
120 DIM SLOPE(2)
125 DIM LUX(2)
130 DIM UB(2)
140 DIM TD(2)
150 DIM CT(2)
160 DIM LOSS(2)
170 MODE 128
180 PROCreaddata
190 PROCtemp
195 PROCexpt
200 PROCprintvalues
210 PROCclear
220 PROCinit
230 PROCcalc
```

```
240 SOUND 1,-15,53,40
250 SOUND 2,-15,69,40
260 SOUND 3,-15,81,40
270 IF GET =32 THEN PROCprinter
280 END
290 :
300 DEF PROCreaddata
320 READ TEMP
330 READ CF
340 READ THRESH
350 READ DAYS
360 READ INDSYN
370 READ DS
380 READ HOUR
390 READ DUR1
400 READ GAP
410 READ DUR2
420 READ NUM
430 READ PERIOD
440 READ LUX(1)
445 READ LUX(2)
450 FOR OSC%=1 TO 2
460 READ SLOPE(OSC%)
470 READ UB(OSC%)
480 READ TD(OSC%)
490 READ LOSS(OSC%)
500 NEXT OSC%
510 ENDPROC
520 :
530 DEF PROCinit
540 LIGHT(1)=0:LIGHT(2)=0:PREVIOUS=100:INDSUM=0
550 MAOLD=0:MAGRAD=0:SAOLD=0:SAGRAD=0
560 CYCLE=0:STATE=0:PHASE=-4:CT(1)=10:CT(2)=10
570 START=(DS-1)*72+HOUR
580 FINISH=START+PERIOD*NUM
585 PRINT TAB(1);"0 1 2 3 4 5 6 7 8 9 10 11 12 1 2 3 4 5 6 7 8 9 10 11 "
590 PRINT
TAB(1);"________________________________________________________________"
600 HEIGHT= THRESH*5
610 MOVE 1,HEIGHT:DRAW 1289,HEIGHT
620 MOVE 0,35
630 FOR OSC%=1 TO 2
640 FOR J%=1 TO TD(OSC%)
650 D(OSC%,J%)=5
660 NEXT J%
670 NEXT OSC%
680 ENDPROC
690 :
700
710 DEF PROCprintvalues
720 @%=131594
730 PRINT:PRINT:PRINT
740 PRINT TAB(1);"OSC";TAB(8);"S R";TAB(15);"U B";TAB(22);"T D";TAB(28);"L R"
750 PRINT
TAB(0);"MASTER";TAB(8);SLOPE(1);TAB(15);UB(1);TAB(22);TD(1);TAB(28);LOSS(1)
760 PRINT
TAB(0);"SLAVE";TAB(8);SLOPE(2);TAB(15);UB(2);TAB(22);TD(2);TAB(28);LOSS(2);:PRINT
TAB(40);"COUPLING FACTOR ";CF
770 PRINT "INDUCTION SYN ";INDSYN;" DAYS ";DAYS;
780 PRINT TAB(40);"THRESHOLD ";THRESH;" TEMP ";TEMP
790 PRINT "LIGHT BEGINS ON DAY ";DS;
800 PRINT TAB(40);"TIME UNIT ";HOUR
810 PRINT "DURATION OF PULSE ONE ";DUR1;" TIME UNITS";
820 PRINT TAB(40);"DURATION OF GAP ";GAP
830 PRINT "DURATION OF PULSE TWO ";DUR2;" TIME UNITS";
840 PRINT TAB(40);"NO OF PULSES ";NUM
850 PRINT "PERIOD OF LIGHT CYCLE ";PERIOD;
```

```
860 PRINT TAB(40);"LIGHT INTENSITY ON P ";LUX(1)
865 PRINT "LIGHT INTENSITY 0N S ";LUX(2)
870 VDU3
880 ENDPROC
890 :
900 DEF PROCcalc
910 FOR D=1 TO DAYS
920 PRINT CHR$(124);
930 FOR I% = 1 TO 72
940 PROClight
950 FOR OSC%=1 TO 2
970 DIFF=30-D(OSC%,TD(OSC%))
980 SYN=DIFF*SLOPE(OSC%):IF SYN>UB(OSC%) THEN SYN=UB(OSC%)
983 SUBT=LIGHT(OSC%)
985 IF OSC%=2 THEN SUBT=LIGHT(2)-(CT(1)*CF*0.005)
990 CT(OSC%)=CT(OSC%)+SYN-SUBT-(CT(OSC%)*LOSS(OSC%))
1000 IF CT(OSC%)<1 CT(OSC%)=1
1010 PROCdelay
1020 NEXT OSC%
1040 PLOT 69,TU,CT(2)*5
1050 PLOT 69,TU,CT(1)*5+130
1060 CHR=32
1070 IF CT(2)>=THRESH ANDCT(2)<SAOLD AND SAGRAD<1.0 CHR=43:PHASE=TU
1075 SAGRAD=SAOLD/CT(2)
1080 SAOLD=CT(2)
1090 IF TU<PHASE+1 CHR=43
1105 FOR OSC%=1 TO 2
1110 IF LIGHT(OSC%)>0 AND CHR=43 CHR=42
1120 IF LIGHT(OSC%)>0 AND CHR<>42 THEN CHR=46
1125 NEXT OSC%
1130  IF CHR=43 AND D<.15*DAYS THEN INDSUM=INDSUM+(INDSYN-
(D*(INDSYN/(.15*DAYS)))) ELSE IF CHR=43 AND D>=.15*DAYS THEN
INDSUM=INDSUM+((INDSYN/7)-(D*((INDSYN/7)/DAYS)))
1140 IF CHR=42 THEN INDSUM=INDSUM-(3-(D*(3/DAYS)))
1150 IF CT(1)<MAOLD AND MAGRAD<1.0 CHR=94
1160 MAGRAD=MAOLD/CT(1)
1170 MAOLD=CT(1)
1180 PRINT CHR$(CHR);
1190 NEXT I%
1200 PRINT CHR$(124);(INT(INDSUM*100))/100
1210 NEXT D
1220
1230 @%=&131594
1240 PRINT:PRINT;"INDUCTION SUM ";INDSUM
1250 @%=&90A
1260 VDU3
1270 ENDPROC
1280 :
1290 DEF PROClight
1300 TU=(D-1)*72+I%
1310 DAWN=START+PERIOD*CYCLE
1320 FOR OSC%=1 TO 2
1325 LIGHT(OSC%)=0
1330 IF (TU>START
ANDTU<=FINISH)+(TU<=DAWN+DUR1+GAP+DUR2)+((TU<=DAWN+DUR1) OR
(TU>DAWN+DUR1+GAP))=-3 THEN LIGHT(OSC%)=LUX(OSC%)
1335 NEXT OSC%
1340 IF TU=START+(PERIOD*(CYCLE+1)) THEN CYCLE=CYCLE+1
1350 ENDPROC
1360 :
1370 DEF PROCdelay
1380 FOR T=TD(OSC%) TO 2 STEP-1
1390 D(OSC%,T)=D(OSC%,T-1)
1400 NEXT T
1410 D(OSC%,1)= CT(OSC%)
1420 ENDPROC
1430 :
```

```
1440 DEF PROCtemp
1450 IF TEMP=18 THEN ENDPROC
1460 SLOPE(1)=10^((LOG(SLOPE(1))*(10/(TEMP-18))+.07920)/(10/(TEMP-18)))
1470 SLOPE(2)=10^((LOG(SLOPE(2))*(10/(TEMP-18))+.07920)/(10/(TEMP-18)))
1480 UB(1)=10^((LOG(UB(1))*(10/(TEMP-18))+.0792)/(10/(TEMP-18)))
1490 UB(2)=10^((LOG(UB(2))*(10/(TEMP-18))+.0792)/(10/(TEMP-18)))
1500 DAYS=INT(10^((LOG(DAYS)*(10/(TEMP-18))-.415)/(10/(TEMP-18))))
1510 INDSYN=10^((LOG(INDSYN)*(10/(TEMP-18))-.301)/(10/(TEMP-18)))
1520 ENDPROC
1530 :
1540 DEF PROCclear
1550 PRINT:PRINT"PRESS SPACE T0 COPY INFO ANY OTHER KEY TO RUN"
1560 IF GET=32 THEN PROCprinter ELSE GOTO 1570
1570 CLS
1580 ENDPROC
1590 :
1600 DEF PROCerror
1610 REPORT: PRINT ERL
1620 VDU3
1630 END
1640 ENDPROC
1650 :
1660 DATA 18:REM TEMPERATURE
1670 DATA 2:REM C0UPLING (0-20)
1680 DATA 20:REM THRESH0LD
1690 DATA 14:REM N0 0F DAYS
1700 DATA 20:REM INDUCTION SYNTHESIS
1710 DATA 1: REM DAY 0F PULSE
1720 DATA 36:REM H0UR 0F PULSE
1730 DATA 3:REM DURATI0N 0F PULSE
1740 DATA 33:REM DURATI0N 0F GAP
1750 DATA 0: REM DURATI0N 0F PULSE 2
1760 DATA 100:REM NO OF PULSES
1770 DATA 72:REM PERIOD OF LD CYCLE
1780 DATA 2.2:REM LIGHT INTENSITY 0N P
1785 DATA 2.2:REM LIGHT INTENSITY 0N S
1790 REM SLOPE UPPER BOUND TD LOSS
1800 DATA .14,10,23,0.1:REM PACEMAKER
1810 DATA .1,10,29,0.1:REM SLAVE
1820 END
1830 :
1840 DEF PR0Cprinter
1850 VDU2
1860 *HARDC0PYMX
1870 VDU3
1880 ENDPR0C
1890 :
1900 DEF PROCexpt
1910 CLS:INPUT"D0 Y0U WISH T0 RUN A REPEATING L00P ";A$
1920 IF A$="Y" THEN GOTO 1930 ELSE ENDPROC
1930 INPUT"D0 Y0U WISH T0 ALTER THE PH0T0PERI0D WITHIN 24 HOURS ";B$
1940 IF B$="N" THEN GOTO 1960
1945 PROCphoto
1950 END
1960 PRINT"A NANDA-HAMNER THEN ?"
1970 PRINT"PRESS SPACE TO CONTINUE"
1980 REPEAT UNTIL GET=32
1990 PROCnanda
2000 ENDPROC
2010 :
2020 DEF PROCphoto
2030 INPUT"First light pulse ";a%
2040 INPUT"Last light pulse ";b%
2050 INPUT"In increments of ";c%
2055 DIM FININD(b%)
2057 YAXIS=0
2060 FOR DUR1=a% TO b% STEP c%
```

```
2070 CLS
2090 PROCinit
2100 PROCcalc
2105 FININD(DUR1)=INDSUM
2115 IF FININD(DUR1)>YAXIS THEN YAXIS=FININD(DUR1)
2130 NEXT DUR1
2135 PROCfinind
2140 ENDPROC
2150 :
2160 DEF PROCnanda
2170 INPUT"What is the photoperiod ";a%
2180 INPUT"What original period of darkness ";b%
2190 INPUT"What final period of darkness ";c%
2200 INPUT"Increments of ";d%
2210 DUR1=a%
2220 st% = a%+b%
2230 en% = a%+c%
2235 DIM FININS(en%)
2237 YAXIS=0
2240 FOR PERIOD = st% TO en% STEP d%
2250 CLS
2270 PROCinit
2280 PROCcalc
2290 FININS(PERIOD)=INDSUM
2295 IF FININS(PERIOD)>YAXIS THEN YAXIS=FININS(PERIOD)
2300 NEXT PERIOD
2310 PROCnfinind
2320 ENDPROC
2330 :
2340 DEF PROCfinind
2345 MODE 0
2350 LINE 39,150,1239,150
2360 LINE 39,150,39,850
2362 b=b%-a%
2370 x=1200/b
2380 y=700/(YAXIS+10)
2385 NO=1
2390 FOR DUR1=a% TO b% STEP c%
2400 Z=((x*DUR1)-(a%*x))+39
2410 MOVE Z,150:DRAW Z,143
2420 W=(y*(10*NO))+150
2425 TENS=10*NO
2430 NO$=STR$(TENS)
2440 MOVE 39,W:DRAW 32,W
2445 IF W>840 THEN GOTO 2490
2450 VDU5
2460 MOVE 1,W:PRINT NO$
2470 VDU4
2480 NO=NO+1
2490 NEXT DUR1
2590 FOR DUR1=a% TO b% STEP (5*c%)
2595 DUR=DUR1/3
2600 D$=STR$(DUR)
2610 VDU5
2620 MOVE ((x*DUR1)-(x*a%))+39,140:PRINT D$
2630 VDU4
2640 NEXT DUR1
2700 MOVE 39,150
2710 FOR DUR1=a% TO b% STEP c%
2720 X=((x*DUR1)-(x*a%))+39
2730 Y=(y*FININD(DUR1))+150:IF Y<150 THEN Y=150
2740 DRAW X,Y
2750 NEXT DUR1
2755 IF GET<>32 THEN GOTO 2765
2760 PROCprinter
2765 ENDPROC
2770 :
```

```
2780 DEF PROCnfinind
2790 MODE 0
2800 LINE 39,100,1239,100
2810 LINE 39,100,39,900
2820 x=1200/en%
2830 y=800/(YAXIS+10)
2835 NO=1
2840 FOR PERIOD = 0 TO en% STEP d%
2850 Z=(x*PERIOD)+39
2860 MOVE Z,100:DRAW Z,96
2870 W=(y*(10*NO))+100
2880 TENS=10*NO
2890 NO$=STR$(TENS)
2900 MOVE 39,W:DRAW 32,W
2910 IF W>900 THEN GOTO 2960
2920 VDU5
2930 MOVE 1,W:PRINT NO$
2940 VDU4
2950 NO=NO+1
2960 NEXT PERIOD
2961 FOR PERIOD=0 TO en% STEP 36
2962 PER=PERIOD/3:D$=STR$(PER)
2963 VDU5
2964 VDU5:MOVE (x*PERIOD)+39,90:PRINT D$:VDU4
2966 NEXT PERIOD
2970 A=(a%*x)+39
2980 LINE 39,125,A,125
3000 LINE A,125,A,100
3010 FOR PERIOD=st% TO en% STEP d%
3020 X=(x*PERIOD)+39
3030 Y=(y*FININS(PERIOD))+100:IF Y<100 THEN Y=100
3040 DRAW X,Y
3050 NEXT PERIOD
3060 IF GET<>32 THEN GOTO 3080
3070 PROCprinter
3080 END
3090 :
3100 DEF PROCprinter
3110 VDU2
3120 *HARDCOPYMX
3130 VDU3
3140 ENDPROC
```

Appendix iv

Biol. Cybern. 67, 451–459 (1992)

Biological Cybernetics

© Springer-Verlag 1992

A coupled pacemaker-slave model for the insect photoperiodic clock: interpretation of ovarian diapause data in *Drosophila melanogaster*

S. W. Gillanders and D. S. Saunders

Institute of Cell, Animal and Population Biology, Division of Biology, University of Edinburgh, West Mains Road, Edinburgh EH9 3JT, Scotland, U.K.

Received December 18, 1991/Accepted in revised form May 4, 1992

Abstract. A coupled circadian oscillator model for the insect photoperiodic clock is described which consists of a hierarchically arranged pacemaker and slave. The pacemaker is self-sustained, temperature compensated, and entrainable by the light cycle; the slave is a damping oscillation receiving entrainment from two sources, from the pacemaker via a coupling factor, and also directly from the light. The damping slave oscillation is seen as the "photoperiodic oscillator", equivalent to that proposed earlier by Lewis and Saunders (1987). The present simulations describe the effect of the strength of the coupling factor between hypothetical short- and long-period pacemaker oscillations (modelled on the "clock" mutants per^{S} and per^{L2} in *Drosophila melanogaster*) and a slave oscillation with a period of about 24 hours. The output is presented in terms of photoperiodic response curves and Nanda–Hamner, or resonance, plots. With a high coupling strength, the pacemakers strongly entrain the slave, but with a low coupling strength the slave's properties are more evident. The model is presented as a possible explanation for recent ovarian diapause data in *D. melanogaster* "clock" mutants (Saunders 1990), but also as a more general model for the role of the insect circadian system in seasonal time measurement.

1 Introduction

Temperate-zone insects, like many other organisms, develop and reproduce during the long days of summer, but enter a period of dormancy (diapause), in which endocrine regulation of development is temporarily suspended, in response to shorter days (or longer nights) of autumn. This photoperiodic response usually shows a sharp transition or "critical daylength" separating the diapause and non-diapause pathways, indicating some sort of time measurement within the system (Saunders 1981).

The fruit fly *Drosophila melanogaster* has recently been shown to possess a photoperiodic response in which newly eclosed females transferred to short days at a moderately low temperature ($< 14°C$) suspend ovarian maturation for up to 6 weeks, whereas those transferred to long days at the same temperature undergo slow but uninterupted vitellogenesis (Saunders et al. 1989; Saunders and Gilbert 1990). Diapausing (short day) flies were shown to synthesise yolk polypeptides (YPs) as readily as non-diapausing (long day) flies, but to have "immature" corpora allata, very low rates of juvenile hormone synthesis, and thus a failure to transport the YPs from the haemolymph to the ovaries (Saunders et al. 1990).

There is now ample evidence to suggest that photoperiodic time measurement is a function of the circadian system (Bünning 1936; Pittendrigh 1972; Saunders 1982) responsible for maintaining an organism's daily temporal organization, and of particular phase relationships established either between endogeneous circadian oscillation(s) and the daily light cycle, or between constituent oscillations within the circadian system, when the organism is subjected to photic entrainment. Some of the strongest evidence comes from so-called Nanda–Hamner or "resonance" experiments (Nanda and Hamner 1958; Saunders 1973, 1981) in which the organism is exposed, in different experimental sub-sets, to light cycles containing a constant photophase (say 12 h) and a range of dark periods (say 6 to 72 h) to give light cycles (T) of, in this case, 18 to 84 h, covering several multiples of the free-running period (τ) of the circadian system. These experiments normally show a high incidence of diapause when the "short day" ($L = 12$ h) occurs in cycles close to T 24, 48 or 72 h (modulo τ), but a *low* incidence of diapause in cycles close to T 36, 60 or 84 h (modulo $\tau + \frac{1}{2}\tau$) (Saunders 1973, 1981). Nanda–Hamner experiments with *D. melanogaster* have produced such results, indicating that the circadian system in this fly is somehow involved in photoperiodic time measurement (Saunders 1990).

Correspondence to: D. S. Saunders

The experimental advantage of *D. melanogaster* over other insect models for photoperiodism is, of course, its unrivalled background in classical and molecular genetics which should aid further analysis of the phenomenon. Of particular interest here are the circadian "clock" or *period* mutants first isolated by Konopka and Benzer (1971) following chemical mutagenesis. Mutation at the *per* locus, in the *zeste-white* region of the X-chromosome (Hall and Rosbash 1987; Hall and Kyriacou 1990), produced rhythms of pupal eclosion and adult locomotor activity with either shorter-than-normal circadian periods of about 19 h (per^S), longer-than-normal periods of about 29 h (per^L, per^{L2}), or apparently arrhythmic phenotypes (per^O). Behaviourally arrhythmic deletions of the *period* locus (per^-) are also available (e.g. Smith and Konopka 1981; Saunders 1990).

If the "same" circadian oscillation(s) are causally involved in both overt rhythmicity *and* photoperiodic time measurement, strong a priori predictions arise when these mutated flies are entrained to either T 24 h photocycles, or to the varied T cycles used in Nanda–Hamner experiments. Thus, long period flies (per^{L2}, τ 29 h) would be expected to show a critical daylength considerably shorter than wild type and Nanda–Hamner diapause peaks about 29 h apart, whereas short period flies (per^S, τ 19 h) would be expected to show a critical daylength longer than wild type, and Nanda–Hamner peaks about 19 h apart. Photoperiodic responses of arrhythmic per^O and per^- flies would be uncertain, but likely to be severely disrupted or absent.

Contrary to these expectations, studies of the photoperiodic responses of *per* mutant flies have shown that the critical daylength of per^S and per^{L2} are indistinguishable from that of the "parental' wild type, Canton-*S* (see Fig. 3), and that Nanda–Hamner profiles show peaks of high diapause at 24 h intervals in all three strains (Saunders 1990). Arrhythmic flies (per^{O1} and per^-) were also able to distinguish long days from short days, although the critical daylengths were shortened by 3 and 5 h respectively (Saunders 1990).

These unexpected results suggest that the circadian oscillations involved in photoperiodic time measurement are somehow separate ("different") from those involved in overt behavioural rhythmicity, and may be oscillations whose period is not altered by mutation at the *per* locus. This paper describes a theoretical model for photoperiodic induction in *D. melanogaster* (and other species) which might explain this anomaly. Following the lead of Pittendrich and Bruce (1957, 1959) and Pittendrigh (1981), it suggests that the circadian system is a two-tier or hierarchical structure consisting of self-sustaining, light entrainable "pacemaker" oscillation(s), whose free-running periods are encoded by *per*, coupled to a light sensitive but damping "slave" oscillation(s) which comprise the photoperiodic clock (*sensu stricto*), but whose periods are not encoded by *per*. The coupling between the pacemaker and the slave system is seen to be weak in *D. melanogaster* thus allowing virtual independence of photoperiodic time measurement from overt rhythmicity in certain circumstances. Theoretical considerations of such a model also suggests ways in which photoperiodic responses, although rooted in the circadian system, may have evolved differences to overt behavioural rhythmicity in, for example, geographical races of the same species.

2 The two oscillator model

The proposed model is an adaptation of the damped circadian oscillator model Lewis and Saunders (1987) designed to account for diapause induction in the fleshfly *Sarcophaga argyrostoma* and is based upon the Vaz Nunes et al. coupled oscillator model (1991a, b) but with the "circadian effect" removed and a simplified method for determining INDSUM used. In the latter model and the one presented here, two oscillations control the diapause response, one is termed the "pacemaker" whilst the other is the "slave". Both oscillations consist of a periodically varying amount of a "substance", c_{pace} and c_{slave}, the value of either of these depending upon its temperature-dependent synthesis interacting with a concentration-dependent loss. When a time-delay (TD) is incorporated into the system, oscillations in the level of c arise. The two oscillations are coupled together by a "coupling factor", CF, such that the "pacemaker" oscillation influences the "slave" but not vice versa. The systems diagram is given in Fig. 1.

The rate of synthesis of either c_{pace} or c_{slave} is determined by an error signal which, when positive, activates synthesis. The size of the error signal is determined by the difference between the value of c at this time and a reference value of c. The ratio of the synthesis rate (SR) to the error signal is linear up to an upper bound (UB) and is the main method of changing the amount of damping acting upon the oscillation, a low SR of about 0.09 producing a damped oscillation whilst an SR of 0.13 results in a self-sustained oscillation. SR is moderately temperature dependent ($Q_{10} = 1.2$). The values of the parameters for the pacemaker have been chosen to give a self-sustained oscillation whilst those for the slave produce an oscillation which damps below a constant threshold value for c after only two cycles. Both oscillations can be entrained by light-dark cycles because c is degraded by light by an amount proportional to the intensity of the light (or sensitivity of the oscillator to the light). The nature of the feedback system ensures that when the light signal stops, the value of c "bounced" back up to a high level (except if the time delay is 0 or the length of the light pulse is very long and in effect continuous).

The coupling between the pacemaker and the slave oscillation occurs because at a given time a fraction of c_{pace}, determined by the value of the coupling factor (CF) divided by 100, is added to the value of c_{slave}. The coupling factor varies between 0 (no coupling, in which the pacemaker and slave oscillate independently according to the light entraining each of them) and 20 (strong coupling in which the pacemaker imposes its characteristics upon the slave).

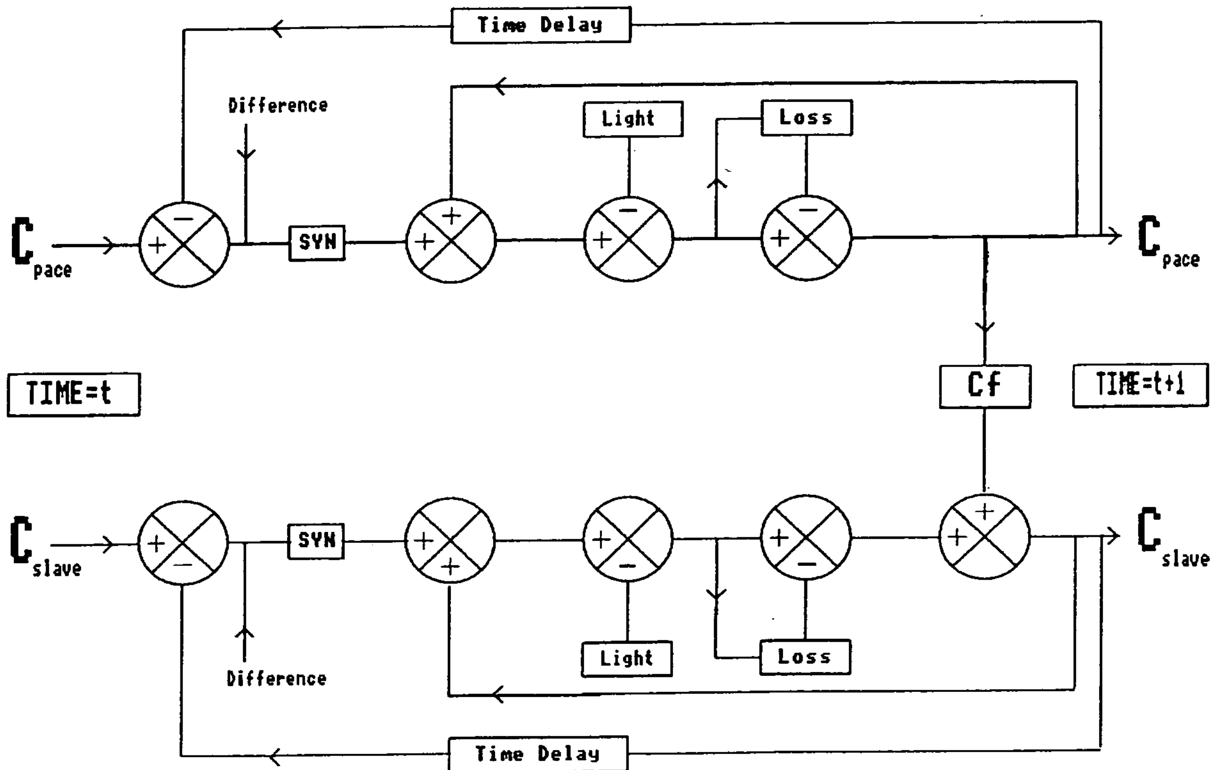


Fig. 1. The systems diagram of the coupled pacemaker-slave model. The levels of an oscillating chemical, *c*, for both the pacemaker and the slave are produced through a feedback loop each with a time delay (TD). Both oscillations are entrained by light because light degrades *c* proportional to the sensitivity of the oscillators to the light cycle. The pacemaker further entrains the slave through a fraction, determined by the coupling factor, of *c* for the pacemaker being added to the level of *c* for the slave

The model determines the diapause response by producing and recording an amount of an hypothetical diapause titre or "INDSUM" (Lewis and Saunders 1987). The higher the value of "INDSUM" the greater the likelihood of entering diapause and, given a large population of insects, the higher the percentage of adults seen to enter diapause. The generation of "INDSUM" is determined by the slave oscillation using the "external coincidence" principle (Pittendrigh 1966; Saunders 1981) whereby a given phase in the oscillation, the photoinducible phase (ϕ_i), causes the organism to develop along one of two pathways depending upon whether it is illuminated or not. This photoinducible phase is near the end of the organism's subjective night and, as the daylength increases, the entrainment of the rhythm to the light-dark cycle means that the dawn light signal is forced "back" into the end of the subjective night until, at the critical daylength, ϕ_i is illuminated and the non-diapause developmental pathway is taken. In this model, ϕ_i is the peak of the slave oscillation when this peak is greater than a critical threshold value. Light falling upon ϕ_i induces the degradation of "INDSUM" as though the organism were exposed to long days, whereas when ϕ_i is not illuminated "INDSUM" is produced and stored. The rate of production of "INDSUM" is temperature dependent ($Q_{10} = 1.2$) "INDSUM" being produced or degraded for a limited length of time (the sensitive period) which is again temperature dependent ($Q_{10} = 1.4$). In all previous models based upon this feedback system, and using this simple method of INDSUM production and degradation, a value for INDSUM of 40 was taken to be equivalent to 100% diapause within the population, and this convention will be used here.

3 Presentation of model predictions

Simulations of photoperiodic responses were carried out on a BBC Archimedes microcomputer programmed in BASIC. The results of photoperiodic response curves (PPRCs) and Nanda–Hamner, or resonance, experiments are plotted in terms of levels of "INDSUM" which are assumed to be proportional to diapause. The computer calculated values for INDSUM of consecutive photoperiods and *T*-cycles and plotted PPRCs and Nanda–Hamner experimental results.

4 Computer simulations of the coupled pacemaker-slave model

As the model developed from an attempt to replicate, in part, the photoperiodic responses of *Drosophila melanogaster*, the initial approach was to look at the

effect upon PPRC and Nanda–Hamner plots of the coupling between a slave oscillation, with a period of around 24 h (24.3 h, TD = 29), damping due to a synthesis rate (SR) equal to 0.1 and a light intensity (or sensitivity) of 2.2, and one of two abnormal pacemaker oscillations. The parameters for the slave were chosen so that the simulated PPRC resembled that experimentally produced for a wild-type Canton-*S* strain of *Drosophila melanogaster*, with a CDL of 14.5 h (Saunders et al. 1989; Saunders and Gilbert 1990). The pacemakers represented two period mutants per^S and per^{L2}, whose eclosion and locomotor rhythms show short and long periods respectively. Both abnormal pacemakers were self-sustained in constant darkness (SR = 0.14) and had a light sensitivity 1.96. The short period pacemaker, P_S, had a period of 19.8 hours (TD = 23), the long period pacemaker, P_L, had a period of 29.4 h (TD = 37). In order to determine PPRCs for these pacemaker oscillations, a ϕ_i was temporarily assumed and the program was run without reference to the slave oscillation, i.e. a circadian oscillator model was used to define the photoperiodic response of such mutant oscillators alone if such things existed. The coupling factor was then systematically varied from 0 to 20.

The lower panel of Fig. 2. shows the PPRC for the slave oscillator. The shape of the PPRC involves a drop in INDSUM in continuous darkness, a high level at short photoperiods, a sharp drop around the critical day length of 14.5 h and an increase in INDSUM with ultra-long photoperiods. This PPRC describes the photoperiodic response of the model system when the slave is not coupled to either pacemaker and closely resembles the PPRC of *Drosophila melanogaster* Canton-*S* (see Fig. 3).

The top left panel of the same figure shows the simulated PPRC for the short period mutant, P_S. The short period of the pacemaker results in a long CDL and a response which never drops to zero INDSUM. This can be compared to the PPRC produced by the long period mutant (top right) which has no steady high level of INDSUM, the CDL being very short and INDSUM values rapidly falling with increasing photoperiods.

When either mutant pacemaker is coupled to the slave (the slave now being the only oscillation with a photoinducible phase) the effects of increasing coupling strength upon CDL and PPRC shape can be seen. The left hand side of Fig. 2 shows the simulated PPRCs produced with a short period pacemaker and with coupling strengths of 2, 10 and 20. The low value has little effect upon the PPRC, and this is what we assume to happen in the case of *Drosophila melanogaster*, whilst increasing the coupling causes the pacemaker to impose itself upon the slave to a greater degree, lengthening CDL. However, even with a CF of 20, the model photoperiodic system is not entirely controlled by the pacemaker, the CDL being less than that of the pacemaker alone. This is because the pacemaker period is shorter than the slave and the latter always phase lags the former and so ϕ_i encounters light slightly before an equivalent, if imaginary, ϕ_i of the pacemaker.

A similar observation is seen with the long period pacemaker (right hand panels). When CF is 2 the PPRC is almost identical to that of the slave yet when CF increases to 10, 12 and 20 the pacemaker can be seen to impose itself upon the slave, the CDL shortening and collapsing after a CF of 12.

The results of the Nanda–Hamner simulations are shown in the same way in Fig. 4. The lower panel applies to the slave rhythm alone. The two peaks of high diapause incidence are 24 h apart; this is the inter-peak interval (IPI) and corresponds to the free-running period of the photoperiodic system (Saunders and Lewis 1987). The resonance plot tails off to a constant level of INDSUM production quite quickly, because the circadian component of the photoperiodic mechanism is quite weak due to the damping of the oscillation when SR = 0.1, and in very long *T*-cycles the slave rhythm is completely damped out. The effects, upon the resonance plots of the P_S system, of increasing coupling strength are shown on the left hand side of the figure, whilst the right hand side shows the P_L coupled pacemaker-slave system. For P_S, increasing the coupling results in an increasing circadian component to the diapause response. The mean IPI shortens to 20.1 h when CF = 20, and consequently more peaks of diapause are induced during the simulated resonance experiments. When CF = 1 the resonance plot resembles experimentally revealed resonance plots for per^S flies (Saunders 1990). Also, for the P_L system the resonance plot produced when CF = 1 is similar to that for P_S and an experimentally produced per^{L2} resonance plot (Saunders 1990). Increasing CF within the P_L system produced a lengthening of the mean IPI (at CF = 20 IPI = 29.0 h) and a consequential decrease in the number of diapause peaks.

5 Discussion

The pacemaker-slave model for photoperiodic induction (Vaz Nunes et al. 1991a, b) is an extension of the model proposed by Lewis and Saunders (1987), and this present version was designed to explain aspects of induction of ovarian diapause in *D. melanogaster*, particularly the responses of the *period* mutants originally isolated by Konopka and Benzer (1971).

In *D. melanogaster*, the critical daylengths for ovarian diapause induction and the responses to Nanda–Hamner experiments were shown to be almost identical for the short-period mutant (per^S), the long period mutant (per^{L2}) and the "parental" wildtype strain (Canton-*S*) (Saunders 1990) (Fig. 3). Critical daylengths for an "arrhythmic" strain (per^{O1}), and for flies with the *per* locus homozygously deleted (per^-) were also clearly defined, although shorter, indicating that photoperiodic time measurement does still occur in flies in which the "main" circadian oscillator (and the gene controlling it) is either seriously impaired, or totally absent. It was therefore concluded that the photoperiodic response is controlled by a gene (or genes) common

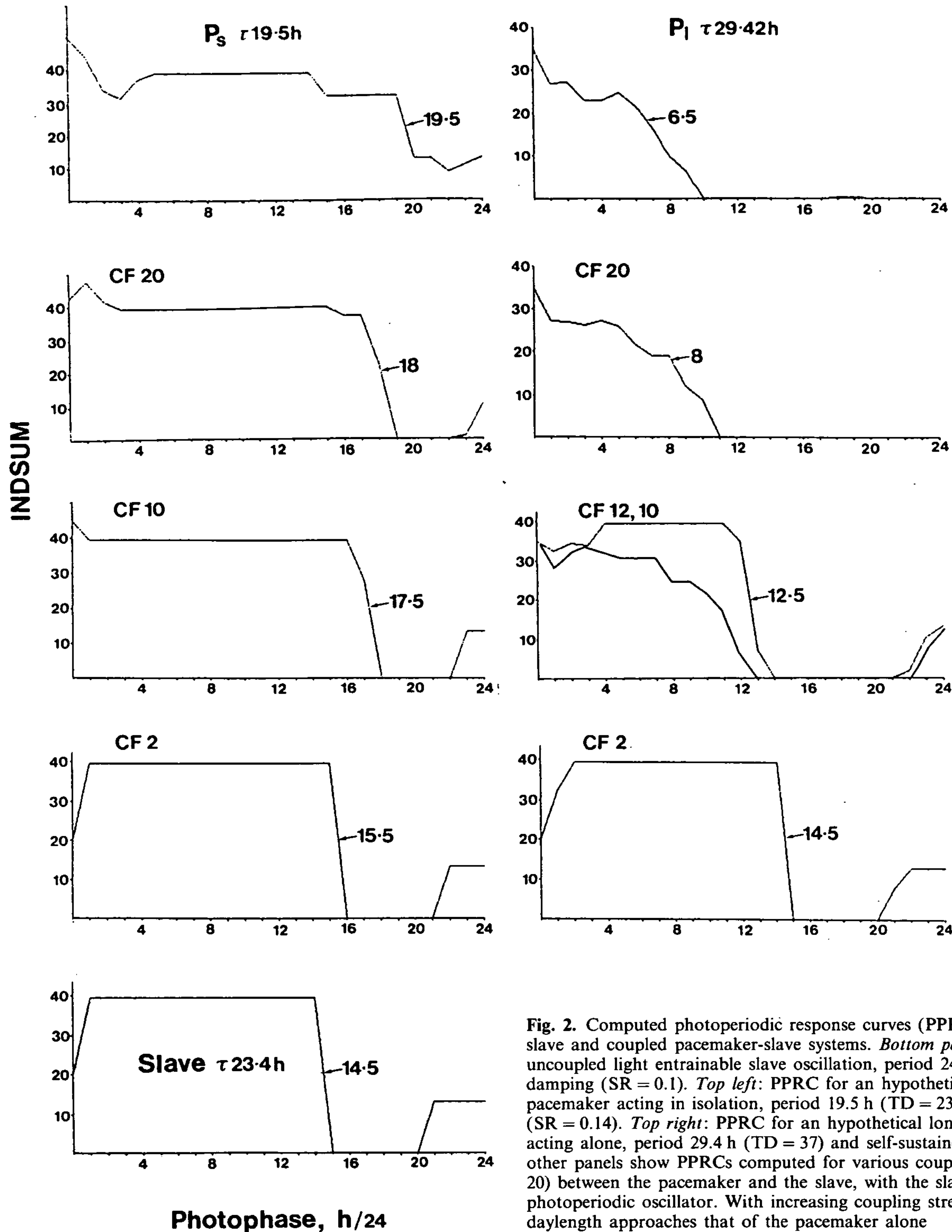


Fig. 2. Computed photoperiodic response curves (PPRCs) for pacemakers, slave and coupled pacemaker-slave systems. *Bottom panel*: PPRC for the uncoupled light entrainable slave oscillation, period 24.3 h (TD = 29) and damping (SR = 0.1). *Top left*: PPRC for an hypothetical short-period pacemaker acting in isolation, period 19.5 h (TD = 23) and self-sustained (SR = 0.14). *Top right*: PPRC for an hypothetical long-period pacemaker acting alone, period 29.4 h (TD = 37) and self-sustained (SR = 0.14). The other panels show PPRCs computed for various coupling strengths (CF 2 to 20) between the pacemaker and the slave, with the slave being the main photoperiodic oscillator. With increasing coupling strength the critical daylength approaches that of the pacemaker alone

to these mutants and wildtype that is separate from *period*. In other words, the *period* locus, and the oscillations it apparently regulates, are not "causally" involved in the photoperiodic time measurement (PPTM). However, in this and other organisms, Nanda–Hamner experiments clearly show that the circadian system is somehow involved in PPTM. The model we propose has then to allow a circadian input into the photoperiodic system whilst the *period* gene pacemaker does not control time measurement directly.

The coupled pacemaker-slave model described here resembles the model for the regulation of pupal eclosion proposed by Pittendrigh and Bruce (1957, 1959) and Pittendrigh (1981) but with one important differ-

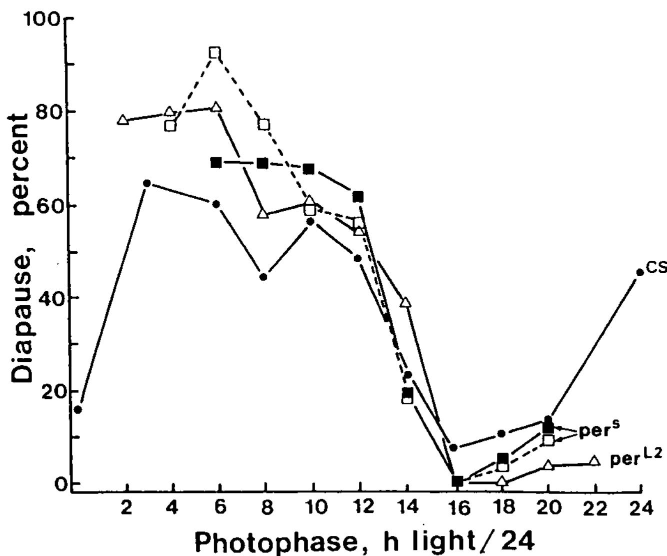


Fig. 3. Photoperiodic responses of *Drosophila melanogaster* at 12°C. CS – Canton-*S* wild type; per^S – short period mutant; per^{L2} – long period mutant (Taken from Saunders 1990)

ence, the slave oscillation is also entrained by the light-dark cycle. The pacemaker is self-sustained, temperature-compensated and entrainable to the environmental cycle of light and darkness; its free-running period is also regulated (encoded) by the *period* gene (Hall and Kyriacou 1990). The slave, on the other hand, is a damped oscillation responding to two sources of entrainment, the first from the driving pacemaker oscillation (as in the eclosion model), and the second from the light-dark cycle. The slave oscillation provides the photoperiodic mechanism itself by containing a photoinducible phase at a point in the oscillation and is equivalent to the damped circadian clock of Lewis and Saunders (1987). Coupling between pacemaker and slave is seen to be weak, or very weak in *D. melanogaster* leading to a virtual independence of the photoperiodic time measuring oscillation from the central pacemaker encoded by *period.* The photoperiodic mechanism proposed for *D. melanogaster* is shown diagramatically in Fig. 5.

The main difference between this model and Pittendrigh's (1981) eclosion model is that the slave is also light entrainable. This is a functional requirement for the operation of the model (Vaz Nunes et al. 1991b), but finds possible support in the vertebrate photoperiodic system, with its circadian pacemaker in the suprachiasmatic nuclei, coupled to the pineal which, at least in birds, is also light sensitive (Follett 1982). In *D. melanogaster*, the pacemaker is most probably in the brain (Handler an Konopka 1979; Hall and Kyriacou 1990) but the location of the putative photoperiodic slave is unknown. The entire mechanism could, for example, also be in the brain as indicated by in vitro and transplantation studies in *Manduca sexta* (Bowen et al. 1984), or the slave could be (for example) in a part of the retrocerebral complex.

The model demonstrates that if overt circadian rhythmicity (eclosion or locomotor activity) and photoperiodic induction were controlled by the "same" oscillation, or if coupling between the supposed pacemaker and slave were strong, any change in the period of the pacemaker would have profound effects upon photoperiodic time measurement. For example, an abnormally short period pacemaker, as in per^S, would lead to an abnormally long CDL and very short interpeak intervals in Nanda–Hamner experiments. Conversely, an abnormally long period pacemaker, as in per^{L2}, would lead to an abnormally short CDL and a very long interpeak interval. If such a pacemaker incorporated "arrhythmic" mutations such as per^{O1} and per^-, a complete disruption of nightlength measurement and Nanda–Hamner responses would undoubtedly ensue. The coupled pacemaker-slave system described here, however, allows for an apparently independent slave-based photoperiodic clock, or a variable influence of the pacemaker through a coupling of one to the other.

Insect circadian systems almost certainly involve more than two oscillators. Any central pacemaker will probably entrain a whole series of slave rhythms which depend, at least in part, on the pacemaker to synchronize them to external conditions. Circadian pacemakers are probably also "complex" in that they may comprise several components (Pittendrigh 1981; Konopka et al. 1989), and may also be constructed from shorter period or ultradian oscillators (Dowse et al. 1987; Dowse and Ringo 1987). Many overt rhythms maintain an appropriate phase relationship to the light cycle in order to function optimally during seasonal change. For example, the pupal eclosion rhythm in *D. pseudoobscura* displays a phase relationship close to the start of the photophase, irrespective of daylength, because the optimal time for eclosion appears to be close to dawn (Pittendrigh 1958). The importance of such a rhythm probably means that its phase relationhip must be maintained, even in the face of evolutionary changes in other, related, aspects of the species' temporal organization. If, for example, pupal eclosion and photoperiodic induction were governed by the "same" oscillator, appropriate changes in one (say latitudinal changes in CDL) might only be achieved at the expense of unwanted changes in, for example, the phase relation of the eclosion rhythm to the light cycle. This would suggest that evolutionary adaptations in part of the circadian system could be brought about by natural selection acting on processes downstream of a central pacemaker, on slave oscillations, or on their coupling to a pacemaker, because any change in the period of the pacemaker might impose temporal penalties on an individual's daily organization.

In the case of adaptations in photoperiodic responses where the circadian system is used to measure daylength and act as a switch, the CDL marks the transition from an active summer state to a dormant winter state. In the northern hemisphere, winter begins earlier in the year, the further north one looks. This means that populations of organisms that enter diapause but

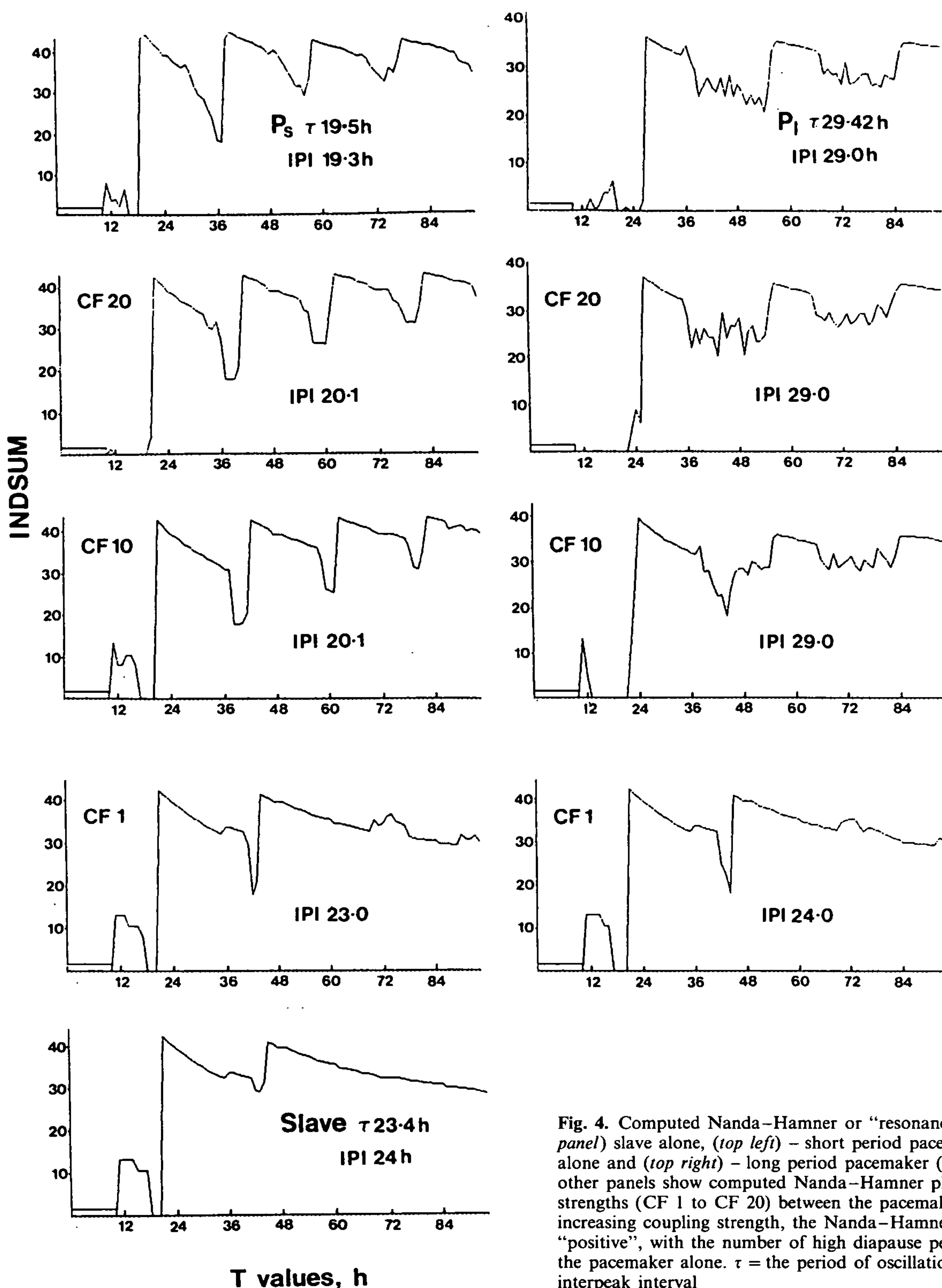


Fig. 4. Computed Nanda–Hamner or "resonance" plots for (*bottom panel*) slave alone, (*top left*) – short period pacemaker ($P_s\tau$ = 19.5 h) alone and (*top right*) – long period pacemaker ($P_l\tau$ = 29.4 h) alone. The other panels show computed Nanda–Hamner plots for various coupling strengths (CF 1 to CF 20) between the pacemaker and the slave. With increasing coupling strength, the Nanda–Hamner response becomes more "positive", with the number of high diapause peaks approaching that of the pacemaker alone. τ = the period of oscillation, in h. IPI = the interpeak interval

which have a large latitudinal spread need to have local adaptations of CDL in order to enter diapause at an appropriate time of year for the conditions at their latitude; namely CDL must be longer the further north a population is. If these adaptations were brought about solely by changes in pacemaker τ other important behaviours controlled by the circadian system would also be effected not necessarily for the better. There is experimental evidence to suggest that the lengthening of CDL in more northerly populations (of *D. littoralis*) may be associated with a decrease in the free-running period of the eclosion rhythm (Lankinen 1986). Closer

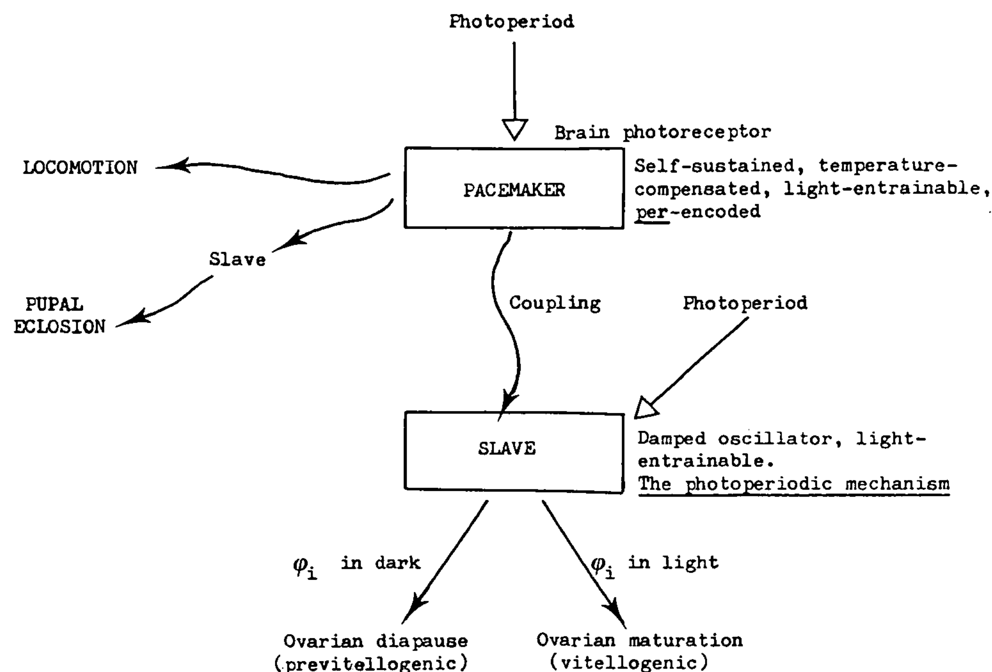


Fig. 5. Proposed coupled pacemaker-slave model for the photoperiodic clock in *Drosophila melanogaster*. The pacemaker is self-sustained, temperature compensated, light entrainable and *per*-encoded. The slave is a damping oscillator, also light entrainable and is the "main" photoperiodic mechanism, as in Lewis and Saunders (1987), controlling diapause induction (in long nights) or non-diapause development (in short nights). The slave oscillation receives entrainment from the pacemaker *via* a coupling factor, and also from the light cycle. The central pacemaker also regulates the overt daily rhythms of adult locomotor activity, and pupal eclosion, the latter probably *via* a slave system

examination of this association, however, reveals that the relationship is not causal; that is, the change in τ in the eclosion rhythm, and hence the central pacemaker, is not producing the expected changes in CDL showing that at least in *D. littoralis* local adaptations in CDL are not brought about by alterations in τ. The coupled pacemaker-slave model proposed in this paper, however, can explain such changing photoperiodic responses without necessarily demanding parallel, and possibly disadvantageous, changes in the pacemaker. With a pacemaker coupled to a light-entrainable photoperiodic slave, changes in the strength of the coupling between the two could produce subtle changes in the critical daylength without alterations in the period of a pacemaker that may have other timekeeping "responsibilities".

Abbreviations, symbols and terms

T	The period of the experimental light-dark cycle in hours.
τ	The free-running (unentrained) period of the circadian oscillation, in hours.
CNL	The critical night length, the amount of darkness in a 24 hour period which induces 50% of a population to enter diapause.
CDL	The critical day length, the reciprocal of CNL, i.e. if CNL = 10 h then CDL = (24 − 10) = 14 h.
PPRC	Photoperiodic response curve.
PPTM	Photoperiodic time measurement, the measurement of either day length or night length, the amount of light (or dark) being used as a signal which allows individuals to "tell" what time of year it is and to develop in a seasonally appropriate way.
IPI	Inter-peak interval, the length of time between peaks in a resonance plot, corresponding to the endogeneous period of the photoperiodic rhythm.
ϕ_i	The photoinducible phase, a component of the external coincidence model of the photoperiodic clock. When illuminated during entrainment ϕ_i induces development in one way (non-diapause) whereas a different developmental path is taken (diapause) if this phase point is not illuminated (Pittendrigh (1966).
P_S	The model photoperiodic system containing a short period central oscillator, or pacemaker.
P_L	The model photoperiodic system containing a long period central oscillator, or pacemaker.
c_{pace}	Hypothetical oscillating chemical of the pacemaker in the coupled model.
c_{slave}	Ditto for the slave.
INDSUM	Induction sum, the accumulated value of the diapause inducing titre produced when ϕ_i is not illuminated (Lewis and Saunders 1987).
CF	The coupling strength between the pacemaker and the slave.
SR	Synthesis rate of oscillating chemical c.
UB	Maximum rate of synthesis of c, the Upper Bound.
TD	The time delay incorporated into the negative feedback control of the level of c.

Acknowledgements. This work was supported by a grant from the Science and Engineering Research Council. Thanks also go to W. Engelmann, R. Lewis and M. Vaz Nunes for reading the manuscript.

References

Bowen MF, Saunders DS, Bollenbacher WE, Gilbert LI (1984) *In vitro* re-programming of the photoperiodic clock in an insect brain-retrocerebral complex. Proc Natl Acad Sci USA 81:5881–5884
Bünning E (1936) Die Endogene Tagesrhythmik als Grundlage der Photoperiodischen Reaktion. Ber dt Bot Ges 54:590–607
Dowse HB, Hall JC, Ringo JM (1987) Circadian and ultradian rhythms in *period* mutants of *Drosophila melanogaster*. Behav Genet 17:19–35
Dowse HB, Ringo JM (1987) Further evidence that the circadian clock in *Drosophila* is a population of coupled ultradian oscillators. J Biol Rhythms 2:65–76

Follett BK (1982) Photoperiodic physiology in animals. In: Brady J (ed) Biological timekeeping, Society of Experimental Biology Series 14:83–100, Cambridge University Press, Cambridge, England

Hall JC, Rosbash M (1987) Genetic and molecular analysis of biological rhythms. J Biol Rhythms 2:153–178

Hall JC, Kyriacou CD (1990) Genetics of biological rhythms in *Drosophila*. Adv Insect Physiol 22:221–298

Handler AM, Konopka RJ (1979) Transplantation of a circadian pacemaker in *Drosophila*. Nature 279:236–238

Konopka RJ, Benzer S (1971) Clock mutants of *Drosophila melanogaster*. Proc Natl Acad Sci USA 68:2112–2116

Konopka RJ, Pittendrigh CS, Orr D (1989) Reciprocal behaviour associated with the altered homeostasis and photosensitivity of *Drosophila* clock mutants, J. Neurogenetics 6:1–10

Lankinen P (1986) Geographical variation in circadian eclosion rhythm and photoperiodic adult diapause in *Drosophila littoralis*. J Comp Physiol 159:123–142

Lewis RD, Saunders DS (1987) A damped circadian oscillator model of an insect photoperiodic clock I. Description of the model based on a feedback control system. J Theoret Biol 128:47–59

Nanda KK, Hamner KC (1958) Studies on the nature of the endogeneous rhythm affecting photoperiodic response of biloxi soy bean. Bot Gaz 120:14–25

Pittendrigh CS (1958) Perspectives in the study of biological clocks. In: Buzzati–Traverso AA (ed) Perspectives in marine biology. University of California Press, USA pp 239–268

Pittendrigh CS (1966) The circadian oscillation in *Drosophila pseudoobscura* pupae: a model for the photoperiodic clock. Z Pflanzenphys 54:275–307

Pittendrigh CS (1972) Circadian surfaces and the diversity of possible roles of circadian organization in photoperiodic induction. Proc Natl Acad Sci USA 69:2734–2737

Pittendrigh CS (1981) Circadian organization and the photoperiodic phenomena. In: Follett BK, Follett DE (ed) Biological clocks in seasonal reproductive cycles. John Wright and Sons Ltd. Bristol, UK pp 1–35

Pittendrigh CS, Bruce VG (1957) An oscillator model for biological clocks. In: Rudnick D (ed) Rhythmic and synthetic processes in growth. Princeton pp 75–109

Pittendrigh CS, Bruce VG (1959) Daily rhythms as coupled oscillator systems and their relation to thermoperiodism and photoperiodism. In: Withrow RB (ed) Photoperiodism and related phenomena in plants and animals. Am Ass Adv Sci, Washington, USA pp 475–505

Saunders DS (1973) The photoperiodic clock in the flesh-fly, *Sarcophaga argyrostoma* J Insect Physiol 19:1941–54

Saunders DS (1981) Insect photoperiodism: the clock and the counter. Physiol Entomol 6:99–116

Saunders DS (1982) Photoperiodic induction of pupal diapause in *Sarcophaga argyrostoma*: Temperature effects on circadian resonance. J. Insect Physiol 28:305–310

Saunders DS (1990) The circadian basis of ovarian diapause regulation in *Drosophila melanogaster*: is the *period* gene causally involved in photoperiodic time measurement? J Biol Rhythms 5:315–331

Saunders DS, Henrich VC, Gilbert LI (1989) Induction of diapause in *Drosophila melanogaster*: photoperiodic regulation and the impact of arrhythmic clock mutations on time measurement. Proc Natl Acad Sci USA 86:3748–3752

Saunders DS, Gilbert LI (1990) Regulation of ovarian diapause in *Drosophila melanogaster* by photoperiod and moderately low temperature. J Insect Physiol 36:195–200

Saunders DS, Lewis RD (1987) A damped circadian oscillator model of an insect photoperiodic clock III. Circadian and "hourglass" responses. J Theoret Biol 128:73–85

Saunders DS, Richards DS, Applebaum SW, Ma M, Gilbert LI (1990) Photoperiodic diapause in *Drosophila melanogaster* involves a block to the juvenile hormone regulation of ovarian maturation. Gen Comp Endocrin 79:174–184

Smith RF, Konopka RJ (1981) Circadian clock phenotypes and chromosome aberrations with a breakpoint at the *per* locus. Mol Gen Genet 183: 243–251

Vaz Nunes M, Lewis RD, Saunders DS (1991a) A coupled oscillator feedback system as a model for the photoperiodic clock in insects and mites I. The basic control system as a model for circadian rhythms. J. Theoret Biol 152:287–298

Vaz Nunes M, Saunders DS, Lewis RD (1991b) A coupled oscillator feedback system as a model for the photoperiodic clock in insects and mites II. simulations of periodic responses. J Theoret Biol 152:299–317