Locomotor Activity Rhythms and Photoperiodic Time-measurement in the Blowfly Calliphora vicina R-D

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

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Abstract

It has been established that locomotor activity in *Calliphora vicina* is controlled by a circadian system comprising several oscillating elements which free-run with a combined period of 23.14 hours. During entrainment these oscillators are thought to be 'pulled' into two groups, one phase-set by the dawn and the other by the dusk. The patterns observed in constant dark after various light/dark cycles, so-called after-effects of entrainment, can be explained in terms of the splitting and remergence of some of these rhythmic elements. The crucial factors controlling these patterns are the periods and mutual coupling strengths of the oscillators, the latter only achieving its final form during larval development. Furthermore, some differences are thought to exist in both parameters between the two strains of *C. vicina* investigated.

The photoperiodic response is considered to be controlled in a similar manner, but utilizes a different set of oscillating components. The evidence for this came initially from the combined period for the photoperiodic system being close to 24 hours, and subsequently from the different phase positions which locomotion and photoperiodism are thought to adopt in light/dark cycles. It was also apparent that the Scottish and U.S. strains exhibited marked differences in their photoperiodic responses. These differences, and those found in locomotor activity, were attributed to differing selection pressures on the two blowfly populations.

In conclusion, although locomotor activity and photoperiodism are both controlled by oscillating circadian systems which may exhibit some functional similarities they cannot be considered to be different 'hands' of the same clock.

CONTENTS

Glossary of Symbols and Terms

Activity time (α). This is the part of a sleep-wake cycle when an animal 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, that 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 zero point is defined arbitrarily, but the whole cycle always encompasses a change from Ct 0 to Ct 24.

Critical daylength (or nightlength) (CDL and CNL respectively). The length of the light (or dark) fraction of the light/dark cycle which separates long day from short day effects. By convention this is taken to be the photoperiod which elicits a 50% response.

Diapause. A period of arrested growth and development enabling a particular species to overwinter, aestivate or synchronize its developmental cycle to the seasons. Most diapause involves the cessation of neuroendocrine functions, and is usually induced by photoperiod.

Entrainment. The coupling of a self-sustained oscillation to a Zeitgeber (or forcing oscillation) so that both adopt the same frequency (τ=T) or that the frequencies are integral multiples (frequency demultiplication).

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

Free-running rhythm. A biological rhythm or oscillation which continues with its own 'natural' period (τ) in the absence of entrainment (i.e. in constant temperature, DD or LL).

Hourglass. A non-repetitive (i.e. non-oscillatory) timer which is set in motion at dawn or dusk and runs its allotted time course. Such systems do not persist in constant conditions.

Internal Coincidence Model (ICM). A model for photoperiodic clocks in which two or more oscillators are independently phase-set by dawn and dusk. Photoperiodic induction is a function of the phase relationship of these constituent oscillators (Pittendrigh, 1972).

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

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

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

Photoinducible phase (ϕ_i). A hypothetical phase point in an oscillator (or a driven system) which is light-sensitive, and an integral part of the External Coincidence Model (Pittendrigh, 1966).

Photoperiod. The length of the light in the daily light/dark cycle, measured in hours.

Photoperiodic response curve (PPRC). The response of a population of insects to a range of stationary photoperiods, usually including the critical daylength.

Rest time (ρ). The time in a sleep-wake cycle in which the animal is inactive, measured in hours.

Subjective day. The first half of the circadian cycle (Ct 0-Ct 12), and the part in which the 'day' normally occurs.

Subjective night. The second half of the circadian cycle (Ct 12-Ct 24), and the part in which 'night' normally occurs.

Zeitgeber. That forcing oscillation which entrains a biological oscillation, e.g. the environmental cycle of light and dark.

Zeitgeber time (Zt). Comparable to circadian time but defining one environmental cycle.

LD: Light/dark cycle. LD 8:16 represents 8 hours of light and 16 hours of dark in each 24 hour cycle.

LL: Continuous light.

DD: Continuous dark.

τ: Natural period of an endogenous oscillator as revealed in free-run.

T: Period of the Zeitgeber.

ϕ_i: Photoinducible phase.

α: Activity time.

ρ: Rest time.

PRC: Phase response curve.

PPRC: Photoperiodic response curve.

CDL: Critical daylength.

CNL: Critical nightlength.

Ct: Circadian time.

Zt: Zeitgeber time.

CHAPTER 1

Introduction, Materials and Methods

General Introduction

All organisms have had to adapt to a constantly fluctuating daily and seasonal environment. The most successful adaptations to this have involved the accurate timing of various elements of the organism's life-cycle, such as reproduction, feeding and seasonal dormancy. For the majority of organisms the most influential physical cycle which allows the synchronization of these life history events to the environment is photoperiod: the daily cycle of light and dark.

Since the daylength, and the concomitant nightlength, change in a very precise and predictable manner throughout the seasons of the year, any specific photoperiod is a reliable indicator of the time of year in any one part of the world. This being so, any favourable or unfavourable changes throughout the year can be anticipated to the maximum benefit of the organism. In the specific case of insects this is most frequently expressed as annual cycles of development and dormancy. The matter is, however, complicated by the same photoperiod occurring twice in each annual cycle, once when the daylength is increasing and once when it is decreasing. One might therefore expect to find insects which can respond to the direction of change of daylength. In fact for many insect species this is not a problem because the developmental and environmental conditions necessary for them to be able to respond to photoperiod prevent them from seeing most photoperiods more than once. Some species do, however, respond to the direction of change of daylength. The red locust *Nomadacris septemfasciata* (Norris, 1965), the lacewing *Chrysopa carnea* (Tauber and Tauber, 1970) and several species of the univoltine carabid beetle *Pterostichus* (Thiele, 1967, 1971) all display intense diapause when the insects are moved from long days to short days during their development.

In order for photoperiod to provide a suitable basis for synchronization to the environment, insects must possess a physiological mechanism for day or nightlength measurement. Such a system does indeed exist and appears to be astonishingly accurate: changes of as little as 15 minutes in day or night

duration can result in very large changes in the photoperiodic response of insects. The molecular basis of this photoperiodic time-measurement is, as yet, unknown but many genetic mutations affecting various 'timed' events have been identified, and to date several models have been proposed which involve membrane structure and function (review in Hall and Rosbash, 1987). Although these studies are increasingly providing a real basis for theoretical models, the experimental analysis of the system has had, on the whole, to rely on indirect means. This has generally involved exposing the insect to certain photoperiodic inputs and analysing the resultant rhythmic output, a so-called 'black-box' experimental technique. Over the past 20 years this approach has provided a great deal of knowledge of the formal properties of the system.

Long before this however, persistent rhythms had been observed in the absence of any temporal cues (i.e. in total darkness and constant temperature). Characteristically these rhythms had a period which was close to, but significantly, rarely equal to the environmental cycle, i.e. 24 hours. Subsequent observations showed that the periods of these rhythms were highly homoeostatic in a large number of species, many exhibiting a large degree of temperature-compensation (Caldarola and Pittendrigh, 1974; Pittendrigh, 1960; Pittendrigh and Caldarola, 1973; Pittendrigh et al., 1973). Such homoeostasis, in conjunction with a relatively stable period for the rhythm, is an essential feature of any device designed to measure time.

Apart from rhythms evolving to match a 24 hour environmental periodicity (circadian rhythms), endogenous oscillations matching other physical cycles have been found: annual (period c. 1 year), lunar (c. 29.4 days), semilunar (c. 14.7 days) and tidal (c. 12.4 days). The endogenous nature of these oscillations is illustrated by their ability to persist in the absence of any environmental cues.

It therefore appears that in almost every species investigated there is a strong endogenous oscillation which exhibits the features one would expect to find in a system designed to measure the passage of time. Many aspects of insect physiology and behaviour appear to be controlled by such a clock, e.g. daily locomotion, feeding, mating, pupal eclosion and oviposition. However, these are usually restricted to particular times of day. Although photoperiodism also involves such a clock for the production of a dormant stage in the life-cycle, this phenomenon usually only occurs once in the life time of insects,

and there is in most cases a critical time during the lifespan when there is a maximum sensitivity to environmental photoperiod. As this usually occurs well before the expression (output) of the photoperiodic response, the experimental regimes used must rely on the 'black-box' principle. In terms of the output (usually expressed as the percentage of the population entering diapause) it is useful to determine responses not only to 24 hour photoperiods, but also to cycle lengths which are either longer or shorter than 24 hours (termed diel and non-diel photoperiods respectively). Such experimental protocols yield a great deal of information about the mechanism underlying photoperiodic effects. This is especially true of non-diel cycles which, although never experienced by insects in natural conditions, have been found to give highly reproducible results and have therefore been interpreted as useful indicators of the formal structure of the 'clock' (for review see Saunders, 1982a).

The most influential of these protocols, and the most convincing with regard to the involvement of the circadian system in insect photoperiodism, was developed by Nanda and Hamner in 1958 while investigating induction of flowering in the soy bean *Glycine max*. These experiments combined a photophase (the light portion of a photoperiod) of constant duration with a variable scotophase (the dark portion of a photoperiod). This resulted in light/dark cycles (LD cycles) of up to 72 hours or more. The response produced by these cycles had alternating high and low incidences of photoperiodic induction. A variety of theoretical models have been developed to explain the results obtained from this, and many other protocols involving diel and non-diel photoperiods.

Historically, the accuracy of the timing system of photoperiodic events in natural 24 hour cycles led early workers to observe that a small range of day to nightlength ratios could produce a switching over of the physiological response. The photoperiod at which this change was expressed by 50% of the population became known as the 'critical photoperiod'. This photoperiod marks the boundary between long days (=short nights), which will usually allow insects to break or avoid their dormancy and to become reproductively mature, and short days (=long nights) which will induce dormancy to allow survival over winter.

Initially the mechanism behind this, although generally unstated, was assumed to be an 'hourglass'. In this system dawn or dusk acted as a switch to

begin some physiological change which was terminated at the next LD transition. If this terminating transition occurred before the process had reached a certain stage, the photoperiod was interpreted as a short day; if on the other hand the process reached completion before the transition the photoperiod was interpreted as a long day. In such a scheme a Nanda-Hamner protocol would be expected to show no successive peaks and troughs once the nightlength had exceeded the critical value.

Although this was thought to be the controlling feature in the majority of insect species it now appears that it only applies in a few cases, most notably the green vetch aphid *Megoura viciae* (Lees, 1973) and some Lepidoptera (Takeda and Masaki, 1976). An alternative mechanism was originally proposed in 1936 by Erwin Bunning, whose insight was that the system of distinguishing different photoperiods was based on an endogenous oscillation. His model divided each 24 hour period into two parts: the first 12 hours being the photophil (or 'light requiring') and the second 12 hours being the scotophil (or 'dark requiring'). Short day effects were produced when the daylight illuminated only the photophil; long day effects were brought about when the light extended into the scotophil.

A more explicit version of his model was devised by Pittendrigh and Minis (1964), and Pittendrigh (1966), in which the function of the light was redefined as 1. Entrainment with phase readjustment and 2. Photoinduction.

Entrainment is the process whereby the endogenous oscillation attains the period of the physical cycle and establishes a unique phase relationship to it. The agent which brings this about (the Zeitgeber), light in most cases, therefore allows the insect to lock on to and thereby measure local sidereal time. Bünning's original model did not consider the importance of entrainment because the endogenous oscillation was assumed to have a period (termed 'τ') equal to the environmental period (termed 'T'). It is now known that endogenous circadian rhythms rarely have periods equal to the physical environmental cycle and therefore the importance of entrainment is evident.

Circadian oscillations are able to entrain because they have a periodically changing sensitivity to light. This can be shown experimentally by applying single short light pulses to the rhythm in a free-running state, i.e. where it is not experiencing the action of any Zeitgeber. Such light pulses cause phase

shifts of differing magnitude and direction depending on which phase of the oscillation is illuminated. The result is a phase response curve (PRC) which illustrates the variability in light-sensitivity in one cycle of the oscillation. This PRC not only enables us to understand entrainment but also allows us to predict the response of the system to novel photoperiods.

In the entrained state τ becomes equal to T; light must therefore cause a phase change in each cycle equal to the difference between τ and T. This is achieved in the steady state by light falling on the part of the circadian oscillation that produces a phase change of exactly the required direction and magnitude. Although the system as a whole takes several cycles to reach a steady state phase relationship, the oscillator may be reset instantly (Pittendrigh, 1981), implying that the system may involve several interacting levels.

The adoption of a mutual phase relationship between the endogenous rhythm and the environment also means that every phase point in the rhythm corresponds to a particular time of day. Thus, by coupling any cellular event to a certain phase of the circadian oscillation, selection will ensure its occurrence at an appropriate time of day. This is important for it allows events to begin in anticipation of changes in the environment as well as allowing temporal organization of internal and external biological processes.

Entrainment alone is not responsible for the initiation of the photoperiodic process; this is only made possible by the second function of the light, photoinduction. In Pittendrigh's revised model this operates in much the same manner as Bunning had proposed, except that the inductive phase of the oscillation is reduced to a specific part of the subjective night (the new term for the scotophil). Critical photoperiods could thus be explained in terms of the illumination or non-illumination of this inductive phase, ϕ_i.

This revised model, the 'External Coincidence Model' (ECM) as it became known (Pittendrigh, 1972), assumed for simplicity that there was only one oscillator driving the system. It has become apparent however, that many of the properties of circadian rhythms can only be explained in terms of more than one oscillator. Experimental evidence comes from many sources: the 'splitting' of activity periods into two or more components; spontaneous changes in the free-running period; 'after-effects' of entrainment on the

free-running period; and evidence that the structural organization of circadian systems is complex (Page, 1981a, 1984; Engelmann and Mack, 1978). This consideration has given rise to another model based on the precept that the mutual phase relationship of the constituent oscillators could be altered by a change in the photoperiod (Pittendrigh, 1972, 1981; Tyshchenko, 1966). This is related to the ECM because induction occurs in some entrained steady state(s). As in the ECM, light serves as the entraining agent, however it does not undertake photoinduction. This is achieved only when the oscillators attain a certain phase relationship to one another and is not dependent on an external light source. For this reason the model has come to be termed the 'Internal Coincidence Model' (or ICM) (Pittendrigh, 1972).

Some models have been advanced to incorporate such a multioscillator system. One of the most important of these (Pittendrigh, 1967; Pittendrigh and Bruce, 1957, 1959; Pittendrigh et al., 1958) hypothesized the existence of a hierarchy of oscillators. At the top of this hierarchy is a main driving oscillation (the pacemaker, or 'A' oscillator) which is entrained and phase set by the external light cycle, and has a temperature-compensated frequency. This drives, by a temperature-dependent coupling, a second peripheral oscillation (the slave, or 'B' oscillator) which in its turn is responsible for the observed output. The phase relationship of 'B' to both 'A' and the external light cycle is therefore also temperature-dependent. The driving rhythm ('A') is considered to be self-sustaining and acts to reinforce the 'slave' oscillation ('B') which would rapidly damp out in its absence. Phase changes imposed by light on the circadian system are assumed to produce instantaneous effects on the 'A' oscillator, thus the transient cycles that are observed in the production of phase response curves (PRCs) are interpreted as a feature of the peripheral 'slave' oscillator system.

A test of the instantaneous resetting of the pacemaker was carried out by Chandrashekaran (1967a, b) based on phase resetting experiments on the pupal eclosion rhythm of *Drosophila pseudoobscura*. The flies were first exposed to one light pulse at a known circadian time, and again, shortly afterwards, another of equal duration and intensity. The first pulse produced a certain phase shift as determined by the PRC, while the shift produced by the second pulse indicated the effect the first pulse had had in the short intervening time. Thus the first pulse was found to have produced the full phase shift expected, i.e. the pacemaker had been reset instantly. Pittendrigh (1974) subsequently

provided further support for this instantaneous resetting using the same two-pulse experimental regime.

Further evidence of a hierarchical system comes from experiments in which two strains of *D. pseudoobscura*, which differed in their eclosion peaks by about 4 hours, were selected for very 'early' and very 'late' eclosers (Pittendrigh, 1967, 1974). The PRCs of the two strains were found to be identical. Therefore, as the PRC is taken to be an indication of the phase relationship of the pacemaker to the light cycle, it must be concluded that selection had altered the phase relationship between the slave and the pacemaker, rather than that between the pacemaker and the environment.

A similar model has been proposed for the control of locomotor activity in birds (Menaker and Zimmerman, 1976; Zimmerman and Menaker, 1979) whereby the pineal was shown to be a self-sustained oscillator. Removal of the pineal prevented the expression of a free-running rhythm, but entrainment to light/dark cycles was still possible perhaps indicating a peripheral oscillating system.

It has been assumed so far that induction of the photoperiodic response is entirely brought about by the 'clock'. However this does not adequately explain why experimentally several cycles of any given photoperiod are required to complete induction. The answer to this may lie in the proposal by Saunders (1971, 1981b) that a 'counter' device may exist which quantitatively accumulates successive long or short nights until a threshold is reached, at which point induction is complete. The counting mechanism is temperature-compensated and this, when viewed in conjunction with temperature-dependent development, may account for many of the effects of temperature, diet, etc. on the photoperiodic response.

The photoperiodic process therefore could be considered to involve two inter-related processes: time-measurement and the summation of photoperiodic information.

Vaz Nunes and Veerman (1982b) have proposed a scheme which involves such a photoperiodic 'counter'. This came about because of the inadequacy of existing models to explain the photoperiodic responses seen in the spider mite *Tetranychus urticae* (Vaz Nunes and Veerman, 1979a, b, 1982a; for review see Veerman and Vaz Nunes, 1984). The basis for their model derives, firstly, from

an idea suggested by Pittendrigh (1972) that the success with which time is measured may depend upon the proximity of the system to 'resonance' with the entraining LD cycle, i.e. how near the period of the oscillator is to the period of the driving cycle. Therefore, irrespective of the role of the circadian system in time-measurment, it may also have further effects on other parts of the pathway from photoreception to photoperiodic response. Veerman and Vaz Nunes viewed this involvement as affecting the summation of the photoperiodic information. The second aspect of their model was based on the hourglass clock developed by Lees (1973, 1981) for the aphid *Megoura viciae*. Their proposition thus involved an hourglass clock distinguishing between long and short nights and a counter mechanism modulated by a circadian clock. It was therefore termed the 'Hourglass timer: oscillator counter' model (Vaz Nunes and Veerman, 1982b).

Apart from the hourglass, the models so far proposed to account for a circadian element in photoperiodism could perhaps be summarized as in Pittendrigh et al. (1984):

1. A non-clock role for the circadian system. This refers to models in which the circadian system is not responsible for time-measurement. The best example are the 'resonance' theories, e.g. the Vaz Nunes and Veerman model (1982b).
2. A clock role for the circadian system. The ECM and ICM are placed in this category. Although theoretically these two models are quite distinct, in practice all the observations on photoperiodic induction are consistent with both of them. Therefore it may be more useful to consider the two systems as complimenting each other because, while the ECM may prove to be the way in which the system operates, most insects appear to have more than one oscillator.

Recent reappraisals of the kinetics of hourglass and circadian oscillators have come to the conclusion that the differences in their formal properties may simply be a matter of semantics. The primary reasons for this come from Nanda-Hamner experiments in which a single insect species could be made to display either an hourglass or a circadian type of response simply by altering the temperature or comparing strains from different latitudes. The fleshfly, *Sarcophaga argyrostoma*, displays an hourglass response at 16°C which can be transformed to a circadian response when the temperature is raised (Saunders, 1973a, 1982b). The same is also true for the ovarian diapause of *Drosophila auraria* (Pittendrigh et al., 1984) and the determination of the seasonal morphs in the aphid *Aphis fabae* (Hardie, 1987). This same type of

change was also observed in the carabid *Pterostichus nigrita*. The northerly strain of this species (64-66°N) exhibited an hourglass while the southern strain (51°N) displayed peaks of induction 24 hours apart (Thiele, 1977a, b). Takeda and Skopik (1985) showed that southern and northern strains of *Ostrinia nubilalis* altered their response with a change in temperature. The classic hourglass insect, *Megoura viciae*, does however retain its response when the temperature is raised (Lees, 1986). Furthermore, in a recent study (Claret, 1985) *Pieris brassicae* was shown to use a circadian system for diapause induction and an hourglass for its termination. Thus, the presence of two systems controlling the same response must imply some similarity in their basic structure.

Since it is unlikely that these organisms can switch from one clock to another it must be assumed that the same system is operating to produce both types of response. Therefore one might conclude that much of the photoperiodic research could be unified if hourglass clocks are regarded as being very strongly damped oscillators which can only achieve one cycle before damping out. This concept of damping is consistent with both the ECM and ICM because it could be interpreted as the action of one oscillator or as an increase in asynchrony between constituent oscillators.

A recent model which incorporates this principle has been devised (Lewis and Saunders, 1987; Saunders and Lewis, 1987a, b). It is based on the ECM as it appears to operate in *S. argyrostoma* (Saunders, 1976, 1978, 1979, 1981a) and on the feedback model for the activity rhythm of *Hemideina thoracica* (Gander and Lewis, 1979; Christensen et al., 1984). To date, this model seems to be one of the most useful in terms of predicting the response of the system to various experimental regimes and in explaining photoperiodic results in a large number of species.

It was in the context of these models and concepts that the present investigation of some aspects of the biological clock in the blowfly *Calliphora vicina* (or *C. erythrocephala*) was undertaken. The physical manifestations of the clock are expressed in many forms, but of special interest in this study were the photoperiodic responses and the locomotor rhythms of the fly.

The photoperiodic response in *C. vicina* is expressed as a diapause in the post-fed larval stage, just before pupariation (Vinogradova and Zinovjeva, 1972;

Saunders et al., 1986). The photoperiodically sensitive period is in the adult stage preceding this, in other words the photoperiodic response is maternally controlled. *Calliphora vicina* is a suitable species for study because it displays clear and sharp responses to the natural and unnatural photoperiods that are essential for investigating photoperiodism.

Although larval diapause is seen in many Diptera, a study of *C. vicina* was considered to be useful, initially from a purely comparative viewpoint and subsequently, via chemical perturbation, as a means to a greater understanding of the photoperiodic system. The existing oscillator models provided the best basis for explaining the results that were obtained.

An equally strong locomotor rhythm was found in *C. vicina*. Although most Diptera must show such activity rhythms comparatively little research has been carried out in this Order, except perhaps in the Culicidae (Taylor, 1969; Taylor and Jones, 1969; Nayer and Sauerman, 1971). One of the interesting features of the activity rhythm of *C. vicina* is that it displays a diurnal activity pattern, which in itself makes it unusual, as the majority of insect locomotor studies have concentrated on nocturnal species. As it was also possible to easily and accurately record the activity patterns of individual flies, using computer facilities, many features were observed in greater detail than ever before. As in the case of the photoperiodic system, chemicals were used to disrupt the activity patterns. The results obtained from all of these experiments thus provided a particularly detailed picture of locomotion in a higher Dipteran.

The majority of the work was carried out on a British strain of *C. vicina* (Musselburgh, 55°N), but a strain from the United States was also made available (Chapel Hill, N.C., 36°N) and was used to investigate some of the latitudinal effects on the locomotor and photoperiodic circadian systems.

The extensive nature of the literature in both photoperiodism and locomotion has made it necessary to discuss them in separate experimental sections. This was also considered in some ways appropriate because, although superficially they appear to be completely different areas of research, they are in fact simply two sides of the same coin. For both locomotion and photoperiodism can be thought of as the 'hands' of a circadian clock. Thus, by drawing together the evidence which is made available through their separate study, a more complete picture of the true nature of circadian systems may

emerge. Therefore, although each of the experimental chapters will make references to the existing circadian models, the true relationship between these two outputs will be considered more fully in the final discussion.

The strains of the blowfly, *Calliphora vicina* Robineau-Desvoidy (Diptera: Calliphoridae), used in this study were collected in Musselburgh, Scotland (55°N), in 1984 and Chapel Hill, N.C., U.S.A. (36°N), in 1987.

Stock Cultures

Both strains were maintained as stock cultures in constant temperature rooms at 25±1°C, which were illuminated continuously by large fluorescent strip lights (700 lux). The adult flies were kept in breeding cages (26x21x19 cm) consisting of a rectangular metal frame placed in a metal dish. This frame supported a fine white muslin stocking which was knotted at both ends. The base of each cage was covered with a sheet of white paper to facilitate cleaning. The flies were provided with sugar and water *ad libitum*, the latter from a 150 ml jar inverted onto a piece of cotton wool. Meat, in the form of beef muscle, was provided as a protein source to allow full ovarian development and a site for oviposition.

The eggs collected were used to set up larval cultures maintained under the same conditions as the adult cages. The pieces of meat with newly deposited eggs were covered by a clean perspex box for about 24 hours, thereby providing a humid environment for a sufficient length of time to allow most of the eggs to hatch. Subsequently the hatched larvae were transferred to fresh meat on a supplementary dried milk-yeast-agar medium in plastic dishes (8.5x9x25 cm). The details of this medium are given below. The number of larvae were limited to between 300-400 in each culture; any greater number can impair the growth and survival of the larvae due to over-crowding (Shahein, 1986). Each plastic dish was then placed in a larger tray (16x12x33 cm) which contained fine sawdust to a depth of 2 cm. The larval cultures were protected from contamination by other flies by placing a lid on the large tray with a gauze-covered ventilation hole. After 5 or 6 days the mature 3rd instar larvae left the food and wandered into the sawdust where, after a further 2-3 days, they began to pupate. The pupae were then collected by sieving the sawdust, and transferred to Kilner jars (c. 200/jar) covered with a muslin gauze. The pupae were then kept in constant light at 25°C until the first adults emerged.

Experimental Cultures

All of the experiments were carried out in walk-in constant temperature incubators which could be maintained at the desired temperature to an accuracy of half a degree Centigrade. The experimental adult cages (which were identical to the stock cages) were placed in light-tight wooden boxes in these incubators. Each box was illuminated with a 4 Watt fluorescent strip light (300 lux) surrounded by a water jacket and controlled by an individual Venner time switch. The temperature, measured using a Rustrak temperature probe, did not increase significantly during light phases in the boxes. Larval cultures on the other hand, tended to be 2-3°C higher than the ambient temperature, particularly towards the end of the larval feeding phase (Richard et al., 1986).

When experimental cultures were being set up the pieces of meat, which were covered with eggs, were removed from the adult cages daily and left in the boxes beside the cages for about 24 hours rather than being covered by a perspex dish. These cultures were then placed in an incubator set at 11°C. This greatly protracted the period of larval development and allowed full expression of diapause in each culture, thus enabling diapausing and non-diapausing larvae to be easily distinguished. Although the exact details of each experimental protocol will be discussed in the relevant sections, it should be noted that the flies used for every experiment were all members of a cohort, i.e. they came from the same larval culture and eclosed at the same time, and thus were all of the same age.

N.B. The units for illumination (lux) can also be stated in Watts/m^2, such that 1 lux equals 0.00146 W/m^2, therefore 700 lux becomes 1.022 W/m^2 while 300 lux is equivalent to 0.438 W/m^2.

Supplementary Larval Medium

The recipe for this was originally devised by Peterson (1953, cited in Haupt and Busvine, 1968). For the purposes of this study the following quantities were used for every 10 cultures required:

250g	Dried milk powder (Millac; instant, spray-dried, separated milk, with added vegetable fat and vitamins A, C and D).
30g	Dried yeast (Distillers Co.; autolysed yeast granules).
20g	Davies agar (Mackay and Lynn Ltd.).
4 litres	Water

Method

The dried milk and yeast were mixed in a plastic basin and 1.5 litres of warm water was gradually added until a smooth paste was formed. The remaining water was heated and while warming, the agar was slowly added until it was fully dissolved. The milk/yeast paste was added to this and the mixture was brought to the boil. The heat was then removed and the medium was poured into the larval plastic dishes. Once the medium had set the dishes were stored at 4°C until they were used.

CHAPTER 2

Circadian Rhythms of Activity in *Calliphora vicina*

1. Introduction to Locomotion

Introduction

The first experimental sections of this thesis are concerned with the locomotor rhythms of insects. Although attention is focused on the Diptera, the whole spectrum of insect locomotor rhythms must be considered as relevant to the system operating in the blowfly *Calliphora vicina*. In order to discuss such an extensive field of research it is convenient to divide the subject into three sections. These are, respectively: the activity patterns insects adopt in natural or experimental 24 hour light/dark cycles; the evidence for the endogeneity of such patterns; and the insights which such work gives us as to the structure of circadian systems.

Many insects restrict their activity to particular times in the natural diel cycle. Such activity can be expressed in a variety of forms, for example general locomotion, flight, feeding or oviposition. These activities appear as nocturnal (night-active), diurnal (day-active) or crepuscular (twilight-active) rhythms, depending on the particular activity involved. It is now generally accepted that the mechanism controlling this system is probably some form of endogenous oscillation which is continually modulated by environmental cycles of light and temperature, together with a variety of direct, exogenous, effects of light. Much recent work has been focused in this area because, as with photoperiodism, such an oscillation will impart a degree of temporal control to the physiological functions that result in activity. Therefore studies in this area can provide a means of directly comparing the different outputs of the circadian system. The only technical difference between the two processes is that the 'hands of the clock' (as both activity rhythms and photoperiodic expression are taken to be) are observed at the individual level in behavioural studies, and for the population as a whole in photoperiodic investigations.

Activity Patterns in Natural Light Cycles

The earliest work in this field was generally of a purely descriptive nature and involved the collection of insects in the wild. A great many studies of this type were carried out, most notably that of Lewis and Taylor (1965). Unfortunately these investigations can only be given superficial consideration because of the lack of evidence for the endogenous nature of the rhythmic patterns observed. Such evidence can only be obtained in the controlled environment of the laboratory, and until recently such work was limited to insects of medical or economic importance, or simply those which were the easiest to study.

One of the first insect groups to attract attention in this respect was the Dictyoptera, the cockroaches. Their large size (allowing various devices to be effectively used to record their activity) and their role as pests made them obvious candidates for such intensive study. The locomotor activity of these insects was found to be almost entirely nocturnal in light/dark (LD) cycles, activity beginning at, or soon after, the onset of darkness and continuing for most or all of the dark phase (Harker, 1954; Roberts, 1960). However, it became apparent that considerable individual variation existed in terms of age, sex, and physiological state. For example, females displayed a somewhat erratic rhythm as their physiological reproductive state tended to interfere with the expression of their locomotor activity (Roberts, 1960; Leuthold, 1966; Lipton and Sutherland, 1970). The crickets (Orthoptera), although not exclusively nocturnal, proved to have features of their locomotor rhythm in common with cockroaches, displaying a marked degree of individual variation in the expression of their activity (Lutz, 1932; Cymborowski, 1973).

Until recently higher insects have only been represented by a small number of examples, namely mosquitoes, fruitflies and tsetse flies. Interestingly the smaller of these flies have tended to show crepuscular locomotor activity patterns in LD cycles, e.g. mosquitoes displayed a bimodal pattern of flight in accordance with their crepuscular habit. *Anopheles gambiae*, a malarial mosquito, is nocturnal and displayed two flight/activity peaks. The larger occurred after lights off and a smaller, more diffuse, secondary peak was seen later in the night (Jones et al., 1967, 1972b). *Aedes taeniorhynchus* had a similar nocturnal bimodal pattern (Nayer and Sauerman, 1971), as did *Culiseta incidens*, the Cool-weather mosquito (Clopton, 1984a) and *Culex pipiens*

quinquefasciatus (=*C. p. fatigans*, Jones, 1976). However, the Yellow-Fever mosquito (*Aedes aegypti*) was diurnally bimodal, having a main peak of activity just before lights off and a smaller peak at dawn (Taylor and Jones, 1969).

The locomotor pattern of individual tsetse flies, on the other hand, was made up of short intense periods of activity followed by long periods of inactivity. Calculating the mean hourly activity level for several individuals did, however, show very clear diurnal rhythms with almost no activity in the dark. Adopting this technique for the tsetse fly *Glossina morsitans* revealed a bimodal pattern biased towards the morning peak (Brady, 1972). *Glossina austeni* also displayed bimodal behaviour, but with a bias to the evening peak; *G. palpalis*, on the other hand, appeared to have a single peak associated with dusk (Crump and Brady, 1979).

Calliphora vicina (=*C. erythrocephala*) belongs to the Dipteran sub-order Cyclorrhapha, which includes some of the most advanced of all the flying insects. Although a few members of this sub-order have been investigated (all displaying a diurnal activity pattern), most of the data are concentrated on the fruitflies (*Drosophila* spp.), which appear to have a bimodal flight pattern with peaks at dawn and dusk. A number of species have been shown to exhibit similar patterns in the field (Lewis and Taylor, 1965) and in the laboratory, e.g. *D. pseudoobscura* (Engelmann and Mack, 1978) and *D. melanogaster* (Helfrich and Engelmann, 1983). Such bimodal behaviour does not, however, appear to be a universal feature of locomotor activity in higher flies, as the remaining examples from this sub-order have all shown a unimodal diurnal pattern, with activity uniformly distributed throughout the light portion of the day. For example: the house fly *Musca domestica* (Parker, 1962; Helfrich et al., 1985); the greenbottle *Lucilia cuprina* (Smith, 1983); *Phormia regina* (Green, 1964a) and the blowflies, *Calliphora stygia* (Waddell, 1984) and *Calliphora vicina* (Grosse, 1985a). Interestingly, some Diptera also showed marked bimodal patterns of activity, especially in extended light cycles. The significance of this will be discussed in the next section.

The Endogenous Nature of Activity Patterns

Locomotor activity is one of many behavioural patterns which insects display. Such activity exists, presumably, to aid each insect to survive and reproduce maximally in a constantly changing environment. Therefore, in order to achieve this insects must be capable of responding to a variety of environmental factors. If, as is believed, the control of locomotor rhythms is largely endogenous, we must have some means of distinguishing between this endogeneity and any exogenous influences. Observing locomotor patterns in LD cycles alone cannot provide sufficient information to allow us to do this. However, the degree of endogeneity of the rhythm can be established if the insect is transferred from a LD cycle to continuous light (LL) or to continuous darkness (DD), provided that all other possible Zeitgebers are held constant (e.g. temperature). In this 'free-running' state the natural period of the endogenous oscillator, τ, will be revealed. The fact that the periods of the rhythms that are obtained in this way are rarely equal to 24 hours is in itself powerful evidence for the existence of an endogenous oscillator, which has been released from the control of the Zeitgebers associated with the solar cycle.

The cockroach again provided many of the early examples which illustrate the endogenous nature of locomotor rhythms. Roberts (1960) was able to show that three species of cockroach (*Leucophaea maderae, Byrsotria fumigata* and *Periplaneta americana*) could free-run in DD. The rhythms proved to be very persistent, lasting as long as several months. This work also demonstrated the variability of the free-running period, both between individuals and within the record of each individual (period values varying between 23 and 25 hours). The changes in period within an individual's free-run occurred spontaneously and were frequently very abrupt. Similar features have been seen in another Dictyopteran, *Nauphoeta cinerea*, in its free-run in DD (Saunders and Thomson, 1977). As will be discussed later, such alterations in period are thought to provide important information as to the structure of the circadian system controlling locomotor rhythms.

Further interesting features were revealed when cockroaches were allowed to free-run in LL. Firstly the period was lengthened, and secondly a bimodal pattern was seen, the extra peak occurring approximately 10 hours after the onset of the main activity band (Roberts, 1960; Wiedenmann, 1977a). These

factors suggest that more than one oscillator may be involved in the control of activity rhythms.

Lutz (1932) reported that *Acheta* (=*Gryllus*) *domesticus* (Orthoptera) had a persistent free-running rhythm in DD with a period of less than 24 hours. This period was subsequently found to lengthen to more than 24 hours when released into LL (Nowosielski and Patton, 1963). Another Orthopteran, the New Zealand weta *Hemideina thoracica*, provided a particularly good example of a free-running rhythm. Christensen and Lewis (1982) showed that the period of the rhythm was between 23.3 hours and 28 hours in DD, and increased to 31.8 hours in LL. The rhythm persisted for several months in DD but tended to damp out in LL (Lewis, 1976). The free-running rhythm in this species proved to be very complex with enormous spontaneous changes of period in DD.

In an attempt to clarify the variability in the period of free-running rhythms in LL, a series of 'rules' were devised, the so-called 'Aschoff's Rules' (Pittendrigh, 1960). These stated that 1. the free-running period increased with an increase in light intensity or on transfer from DD to LL, for nocturnal animals, and 2. under the same conditions the free-running period would decrease for diurnal animals. The locomotor activities of birds and mammals have tended to agree with these rules, but their application to insect rhythms seems to be of limited use (for review see Saunders, 1982a). Equally the 'Circadian Rule', developed as an addition to these earlier rules (Aschoff, 1960), proved to be unsatisfactory in predicting the behaviour of many insects. This new rule extended the criteria for the study of activity patterns to include the ratio of the active-time (α) to the rest-time (ρ) and the total amount of activity per circadian cycle. Aschoff predicted that both of these parameters would increase with increasing light intensity in diurnal animals but decrease for nocturnal animals. Again these new rules could be applied to mammals and birds with reasonable success (Hoffmann, 1965), but insects were not found to conform. For example, *Tenebrio molitor* (Lohmann, 1964), a nocturnal beetle, showed an increasing activity/rest-time ratio (α:ρ) as the light intensity increased.

The Diptera provide especially interesting examples in the investigation of the endogenous nature of rhythms, because of the occurrence of bimodal patterns of locomotion. The bimodal pattern seen in the mosquito *Anopheles gambiae* continues in DD with a period of about 23 hours, but is inhibited in LL

(Jones et al., 1967). The importance of being able to distinguish between the true endogenous components of the system and extraneous exogenous effects, usually brought about by the light, is clearly illustrated in a series of experiments by Jones et al. (1972a, b). In experimental LD cycles a sharp peak of flight activity was seen just after lights on. When the abrupt light/dark transitions associated with such experimental regimes were replaced with more gradual changes, which more accurately mimicked the natural light cycles, this 'extra' peak vanished. The other, endogenous, peak continued as before with no visible alteration. Apparent 'after-effects' were also observed in this mosquito, where the first cycle in DD was slightly longer than the period displayed in the rest of the free-run (Jones, 1973). This first period appeared to be a function of the length of the last light phase. *Aedes taeniorhynchus* and *A. aegypti* also showed a persistent bimodal rhythm in DD, free-running with periods of 23.5 hours (Nayar and Sauerman, 1971) and 22.5 hours (Taylor and Jones, 1969) respectively. The rhythm of *A. aegypti* also continued in LL with a period of 26 hours. In extended light cycles (e.g. 20 hours light: 4 hours dark, 20:4) the situation becomes more complex with the occurrence of extra peaks, frequently associated with the lights-on. A similar pattern was seen in *Anopheles farauti* when the light phase exceeded 12 hours (Taylor, 1969). Interestingly the two peaks in *A. aegypti* also appeared to be separately phase set by LD transitions, suggesting perhaps the existence of two oscillators, one associated with dawn and the other with dusk. *Culex pipiens quinquefasciatus* also displayed a persistent bimodal rhythm in DD (period about 24 hours; Jones, 1976), which became unimodal in LL, with a period of about 25 hours (Jones, 1982).

A more recent study of the Cool-weather mosquito *Culiseta incidens* (Clopton, 1984a, b, 1985) is of particular interest because it concentrated on extended locomotor records in DD. The results revealed that the activity patterns seen in this mosquito could be very similar to those seen in the higher insects (see below), where an initially short free-running period of less than 24 hours increased after a few days to greater than 24 hours. As well as there being obvious lability in the period of the rhythm, there was also evidence of infradian rhythmicity, where the period of the rhythm was far greater than 24 hours. In this case the period of the rhythm appeared to double its frequency from about 24 hours to about 48 hours. Although Clopton termed this 'circa-bi-dian' rhythmicity, it has all the appearance of a phenomenon more frequently described as 'day-skipping'.

Although tsetse flies usually displayed bimodal rhythms in LD, in DD these became unimodal. The bout of activity which persisted in DD appeared to be the evening peak in mature flies, while immature flies ('tenerals') retained the morning peak (Brady, 1988). This bears comparison to a mosquito example, *Culex pipiens quinquefasciatus*, which will be discussed later (Jones and Gubbins, 1979; Jones, 1982). Unfortunately, low activity levels and very short lifespans made it impossible to produce unequivocal evidence that the bimodal patterns did not persist, or to accurately measure the period of the rhythm (Brady, 1972, 1988; Crump and Brady, 1979).

The Drosophilidae display similar bimodal rhythms in LD cycles. For example, *Drosophila melanogaster* were shown to free-run in constant infra-red light with a mean period of 24.5 hours (Konopka and Benzer, 1971) and 23.6 to 24.0 hours (Helfrich and Engelmann, 1983). Although *Drosophila pseudoobscura* displayed a bimodal activity pattern in LD cycles, once the flies were transferred to DD only the dusk component persisted (with a period between 21.3 and 23.9 hours). The dawn peak must therefore have been an exogenous effect associated with the lights-on (Engelmann and Mack, 1978). An identical result was obtained for *D. robusta* when the rhythm was released into dim LL, i.e. the dusk activity peak alone continued, in this case with a mean period of about 24 hours (Roberts, 1956).

As has already been indicated, the species of Cyclorrhapha which are most closely related to *C. vicina* all showed a unimodal diurnal activity pattern in LD cycles. This unimodal pattern was seen to persist in constant conditions. *Musca domestica* free-ran in constant red light (RR) with an initial period of about 23 hours, which lengthened to about 25 hours after several days. The activity in LL was characterized by arrhythmicity and hyperactivity (Helfrich et al., 1985). Smith (1983) showed that locomotion in *Lucilia cuprina* became increasingly arrhythmic in LL with higher light intensities; above 5.0 lux the pattern was entirely arrhythmic. In DD a clear rhythm was observed. For individual flies the period of this rhythm ranged between 21.75 and 22.75 hours; for cages of 10 flies the mean period was 21.19 hours. The rhythm appeared to be phase set by the last light to dark transition, and once the light phase immediately preceding DD became longer than 12 hours the activity always began at the same phase.

Green's studies on *Phormia regina* (1964a, b) were more difficult to

interpret because the insects were systematically starved while they were free-running. Generally though a rhythm was seen in DD which had similarities to those displayed by related flies. The period of these rhythms depended on the LD regime from which the flies were released: 12:12 gave periods of less than 24 hours, and DD resulted in mean periods of 24.3 hours. The pattern in LL was arrhythmic. *Phormia terraenovae*, on the other hand, showed a clear free-running rhythm in LL, the period of which did not vary in any significant direction over a very wide range of light intensities from 2 to 2000 lux (Aschoff and von Saint Paul, 1982). This rhythm shared features with *Musca domestica*, in that the period was initially shorter than 24 hours but subsequently increased to a value greater than 24 hours. The lengthening of the period was considered to be independent of the age of the fly and of the prior LD cycle, therefore Aschoff did not believe it to be an after-effect. It was not possible for the rhythm to be recorded in DD as the fly became totally inactive at light intensities of below 1.0 lux.

The two *Calliphora* species for which data are available both showed an initial free-running period in DD of less than 24 hours: *C. stygia* had periods of 22 to 23.5 hours (Waddell, 1984); *C. vicina* had a mean period of 22.5 hours (Grosse, 1985c). *Calliphora stygia* also displayed a lengthening in its period, after several days, to more than 24 hours. Unfortunately the recordings of the rhythm for *C. vicina* were too brief to indicate whether a similar lengthening occurred. The pattern in LL was arrhythmic for both species, although below 25 lux a small amount of rhythmicity was seen in *C. stygia*.

As has been mentioned, some Diptera exhibit bimodal patterns of activity in extended light cycles. Usually however, the peak associated with dawn was found to disappear once the fly was removed from its entraining LD cycle. For example, *M. domestica* could manifest such a bimodal diurnal pattern, with a small amount of activity just after lights on and a more pronounced peak before the lights went off (Helfrich et al., 1985; Parker, 1962). The dusk component alone persisted in continuous red light (RR). This implies that the first peak was brought about as a direct effect of the lights-on. A comparable result was obtained with *C. stygia*, where the second peak disappeared on transfer to DD (Waddell, 1984). Although, as will be seen later, these flies appear to have a circadian mechanism made up of several components, these results serve to remind one of how important it is to be able to distinguish exogenous effects, so that in turn the endogenous components of the system

can be exposed.

The Structure of The Circadian System

It is clear that almost all insects display rhythmic activity in LD cycles. Within the Diptera all of the usual types of locomotor pattern can be clearly seen, i.e. nocturnal, diurnal and crepuscular.

The persistence of many of these rhythms in the absence of any apparent entraining agents (and with periods which do not equal 24 hours) leads one to conclude that these rhythmic patterns are the result of endogenous oscillations. However, they have other features which can also provide a more complete picture of the underlying circadian system, particularly in terms of its multioscillator nature, i.e. the 'splitting' of the activity rhythm into several components; spontaneous changes in the free-running period; and 'after-effects', where the free-running period can be altered following a variety of treatments, including entrainment.

Rhythm splitting was first recorded as a response of the Arctic ground squirrel *Spermophilus undulatus* when free-running in LL (Pittendrigh, 1960). Under these conditions a component of the normal free-running rhythm detached itself from the main activity band and, having a period longer than the main rhythmic element, was able to track across a full cycle and rejoin the main rhythm again. This process was very gradual, taking place over 100 cycles. The same phenomenon has been observed in a variety of rodents (Pittendrigh, 1974; Pittendrigh and Daan, 1976c; Pohl, 1972), the tree shrew *Tupaia belangeri* (Hoffmann, 1971), the rabbit (Jilge, Friess and Staehle, 1986), the starling *Sturnus vulgaris* (Gwinner, 1974) and a lizard *Sceloporus olivaceus* (Underwood, 1977). In most of these cases the splitting occurred after some form of treatment rather than being purely spontaneous.

The examples of splitting in insects are less clear. *Leucophaea maderae* spontaneously produced a split rhythm in RR, or after treatment with light or temperature pulses (Wiedenmann, 1977a). *Nauphoeta cinerea*, on the other hand, produced such a response in LL free-run (Thomson, 1976, cited in Saunders, 1982a). These responses differed from the vertebrate examples in two important respects. Firstly, the splitting occurred instantaneously with few transients (vertebrate examples tending to show a very gradual splitting of the rhythm), and secondly, the two components produced from the splitting usually

had the same free-running period. However, one example of insect rhythm-splitting which does bear comparison to the vertebrates was that seen in the New Zealand weta *Hemideina thoracica* (Christensen and Lewis, 1982), where a single light pulse was sufficient to bring about splitting when the rhythm was free-running in DD. At times the two components clearly displayed different periods and eventually merged after 150 days into a single discrete band of activity.

The degree of the observed splitting can cover a wide range from the simple bi-phasic split to rhythms which disintegrate into high frequency components. Although the existence of the latter short-term (or ultradian) periodicities has been known for some time, only recently have they come to be thought of as important elements in the temporal organization of behavioural rhythms. This was due, firstly, to the remarkable precision of the ultradian periodicities and secondly to the fact that such periods did not correspond to the periods seen in any known environmental cycle. The first examples of ultradian rhythms came exclusively from rodents, e.g. rats and mice (Szymanski, 1920). Subsequently, however, they have been found in a wide variety of other mammals including the common vole *Microtus arvalis* (Daan and Slopsema, 1978) and the rabbit (Jilge and Staehle, 1984). Also humans, primates, birds and one lizard species (*Gallotia galloti*, Molina-Borja et al., 1986) have shown evidence of similar types of rhythmicity (review in Daan and Aschoff, 1981). So far the only insect examples have been some period mutants of *Drosophila melanogaster* (Dowse et al., 1987).

The apparently ubiquitous nature of this phenomenon led some researchers to propose that many of the observed circadian rhythms may represent the coupled output of a number of high frequency pacemakers (Dowse et al., 1987). The fact that many intra-cellular molecular mechanisms operate rhythmically with periods close to the ultradian values obtained from activity records has given weight to this argument (Jerebzoff, 1987).

Although rhythm-splitting is considered to be one of the more important pieces of evidence supporting the multioscillator hypothesis, the other elements of free-running rhythmic patterns also support this. Vertebrate rhythms, although remarkably stable, have been known to show spontaneous alterations in their free-running periods (Pittendrigh and Daan, 1976a). Two of the insect examples cited above, *N. cinerea* and *H. thoracica*, have also

displayed such period changes (Saunders and Thomson, 1977; Christensen and Lewis, 1982), and Roberts (1960) observed similar spontaneous changes of period in DD free-runs in the cockroaches *L. maderae* and *Byrsotria fumigata*.

After-effects (as first described by Pittendrigh, 1960) have been shown to be present in several rodent species following a variety of pre-treatments: entrainment to exotic LD cycle lengths (i.e. < or >24 hours); entrainment to 24 hour cycles with differing photoperiods; entrainment to skeleton photoperiods, where the light phase in any one cycle is reduced to short pulses at its beginning and end; phase shifts by single light pulses; and exposure to LL (Pittendrigh and Daan, 1976a). Similar phenomena have been described for insects, again most notably the weta *H. thoracica* (Christensen and Lewis, 1982). Immediately after entrainment to natural cycles (T=24 hours) the free-running rhythm in DD was initially longer than 24 hours but subsequently decreased to about 24 hours. The free-running period following entrainment to non-24 hour cycle lengths was always increased, but the degree to which this occurred showed a number of predictable trends. Firstly, the change in period was dependent upon the difference between the entraining cycle length and the rhythm's period prior to entrainment, the direction of this change being related to the increase or decrease of the period of the rhythm that was being forced to entrain (i.e. if the length of the entraining cycle was systematically increased then the period after entrainment was found to increase). Secondly, the difference in the free-running period before and after long-period entrainment, and the duration of the after-effects were dependent upon the number of entraining cycles. Thirdly, these entraining cycles, to be effective in producing after-effects, had to be continuous. Exposing the rhythm to short light pulses, or allowing it to free-run in LL also produced a rhythmic period which was longer than that seen in DD. However, if the rhythm was re-released into DD from LL, further after-effects were seen. As was the case with non-24 hour cycles, the magnitude of these changes was dependent upon the difference between the period in LL and the prior free-running period in DD. In all of these examples the period in DD eventually shortened to a value close to that seen before the treatment, although in some cases it took nearly 40 days to do so. The cockroach *L. maderae* (Page and Block, 1980) has also been described as having after-effects to non-24 hour cycles.

In those cases where it was possible to observe it, Dipterans have shown similar spontaneous changes in the period of their rhythms in constant

conditions, e.g. the mosquito *Culiseta incidens* (Clopton, 1984a); *Drosophila melanogaster* (Helfrich and Engelmann, 1983; Helfrich, 1986); the house fly *Musca domestica* (Helfrich et al., 1985); *Phormia terraenovae* (Aschoff and von Saint Paul, 1982); and *Calliphora stygia* (Waddell, 1984). After-effects, on the other hand, have been less frequently recorded, but certainly appeared in *C. stygia* (Waddell, 1984) and, to a lesser extent, in *Anopheles gambiae* (Jones, 1973). In this latter example the first cycle in DD was slightly longer than the period of the remaining free-run, and this initial period appeared to be functionally related to the length of the last light phase.

Spontaneous rhythm-splitting has been recorded in very few Dipteran species, e.g. *C. stygia* (Waddell, 1984), *M. domestica* (Helfrich et al., 1985), *D. melanogaster* (Helfrich, 1986) and *C. vicina* (Helfrich, unpublished, cited in Helfrich, 1986). It was also shown to occur, with a very high frequency, in several mutant forms of *D. melanogaster* (Helfrich and Engelmann, 1983; Helfrich, 1986).

As has been indicated, the Diptera display a great variety of activity types. The bimodal patterns seen in the crepuscular species are especially interesting when one considers the structure of the oscillating system, because they suggest the existence of either more than one oscillator or a compound pacemaker. Possible confirmation of this comes when insects can be shown to establish a steady phase relationship between the two activity bands in a wide range of LD cycles, and can maintain that relationship in the free-running state. Such requirements are fulfilled by some of the bimodal patterns seen in mosquitoes, e.g. *Aedes aegypti* (Taylor and Jones, 1969) and *Culex pipiens quinquefasciatus* (Jones, 1982). Unfortunately many of the mosquito examples have not, as yet, been investigated sufficiently thoroughly to allow all of these criteria to be satisfied.

Clopton (1984a, b, 1985) proposed a model to explain the bimodal activity seen in the mosquito *Culiseta incidens*. This was based on an earlier model conceived by Pittendrigh and Daan (1976c) for the locomotor patterns seen in nocturnal rodents. This model stated that the pacemakers controlling locomotion consisted of two mutually coupled oscillators, 'M' and 'E', which exerted control over the morning and evening activity peaks respectively. These two components were viewed as having reciprocal responses to light intensity, with the period of 'E' lengthening with increasing light intensity and

that of 'M' decreasing. The effects of rhythm-splitting, spontaneous period changes and after-effects could thus be explained by varying degrees of desynchronization of the constituent oscillators. Another mosquito, *Aedes aegypti*, has provided further support for a system comprising two pacemakers, separately phase set by dawn and dusk (Taylor and Jones, 1969).

Jones (1982) put forward another model for mosquito activity patterns (based on *Culex p. quinquefasciatus*) which involved three components, representing evening ('E'), night ('N') and morning ('M') peaks of activity. 'E' and 'M' only occurred in virgin females; inseminated females tending to produce a third peak, 'N', at the expense of 'M'. This rhythmic change was associated with a switch from mating to host-seeking behaviour, which under natural conditions occurs in the middle of the night. Such behavioural alterations are thought to be initiated by a pheromone in the male's accessory fluid (Jones and Gubbins, 1979). The activity pattern in *A. gambiae* also appeared to be modified by this same means (Jones and Gubbins, 1978). This suggests many comparisons to the situation found in starlings, where testosterone was found to induce rhythm-splitting (Gwinner, 1974). Virgin female mosquitoes appeared to show differential responses to light in their two activity bouts, 'M' and 'E'. The 'E' component, in inseminated females, appeared to function in much the same way as it did in virgins. 'N' and 'M' also behaved in the same way. It is possible, therefore, that the 'M' and 'N' peaks may simply represent differing 'slave' outputs of the same oscillator; the phase relationship to the light cycle being readjusted by the pheromone transferred during insemination.

It is interesting to note that both Jones (1982) and Clopton (1984b) concluded that the 'E' component was far more labile than 'M' (or 'N'). The situation may therefore be described as one in which there are two mutually coupled oscillators with greatly differing strengths. The 'M' (or 'N') oscillator is the more stable element in the free-running rhythmic patterns, and would under normal circumstances be coupled to the less stable 'E'. Under certain conditions an uncoupling of the system may occur, e.g. by altering the light intensity, at which times the two elements of the clock may free-run with different periods.

The Cyclorrhaphan bimodal patterns observed in entrained states appear to be largely the result of exogenous effects, the patterns frequently becoming unimodal in DD and LL. In all cases it was the dusk component which appeared

to be the truly endogenous part of the system. However, if one assumes that locomotor rhythms are controlled by more than one oscillator, perhaps on the hierarchical basis proposed by Pittendrigh (1974), it could be argued that the system as it operates in the mosquito may also apply here. For this to be the case, the difference in strength between the two oscillators may have to be even greater than that seen in mosquitoes, with the result that in free-run they are very rarely separated. Furthermore, if the oscillators were able to be strongly phase set by light/dark transitions, they could be pulled apart in long light periods. This might explain why, in such extended photoperiods, bimodal patterns are frequently observed in the Cyclorrhapha.

In conclusion, it can be seen that many features of free-running rhythms, in both vertebrates and insects, cannot be accounted for by circadian models comprising a simple single oscillatory pacemaker; a more complex multioscillator mechanism is considered to be the only solution. Although this introduction has been limited to those direct features of activity rhythms which allow such a conclusion, further evidence exists in other areas of insect circadian research, for example, histology (Page, 1981a, 1984; Helfrich et al., 1985), genetics (Konopka and Benzer, 1971; Helfrich and Engelmann, 1983; Helfrich, 1986) and physiology (Brady, 1974; Carpenter and Grossberg, 1985; Hall and Rosbash, 1987).

The experimental protocols which follow are an attempt to establish whether the activity patterns shown by *Calliphora vicina* conform to the same principles which appear to apply in the insect examples outlined above.

The Analysis of Locomotor Rhythms

The aims of this section are to briefly summarize some of the statistical procedures which are available for analysing locomotor rhythms, and to provide some justification for the form of analysis which was adopted.

Time-series observations have, for many years, been one of the more important techniques in scientific research, with the result that many tenets of modern science have developed from their interpretation. In order to utilize these observations to the full, care must be taken in the selection of the most appropriate form of analysis. This is largely because the information which most ordinary statistical techniques require on the nature of the time dependence of data is simply not available for biological rhythms. The situation is further complicated by many of the characteristic features of circadian oscillations, e.g. a period which changes with time and a 'waveform' which is rarely a simple single harmonic oscillation or sine wave. As these observed rhythms are probably the combined output of several coupled pacemakers, such features may be important clues as to the structure and function of the circadian system. Therefore, whatever final form the analysis takes, it cannot completely ignore such 'extraneous' data.

While many of the analytical techniques used in simple physical oscillating systems treat time-serialized data as the sum of a series of additive sine and cosine components, it is probably inappropriate to make the same assumptions for biological rhythms. Their characteristic rhythmic features would, if anything, be obscured by such analysis. Despite this, some of the analytical protocols which have been applied to circadian rhythms have employed hypotheses of this nature. Although these have proved to be helpful in the initial analysis of rhythms, the results of this type of statistic should be interpreted with care.

Before any analysis of data can be undertaken it must be ensured that the observations have been collected at regular intervals, and in a wholly objective manner: in the present thesis this was guaranteed by an automated collecting system outlined in the next section. Once this has been established, any number of features of the rhythm are open to investigation, e.g. period, amplitude and waveform. Activity patterns are, in general, rather easier to

observe and analyse with respect to these variables than most oscillations. Fortunately those exhibited by *C. vicina* were found to be particularly good examples, most of the records displaying a waveform of large amplitude coupled with relatively low 'noise' level.

Although for the purposes of this study several aspects of circadian locomotor rhythms were considered, it was thought that period was the most worthwhile parameter of the rhythm to resolve. In order to achieve an estimate of this period from one cycle to the next it is necessary to have, within each cycle, a phase point which can be directly compared with a similar point in the preceding or succeeding cycles. It is necessary to assume that such phase-reference points (PRPs) are equivalent in different cycles. Although it is a moot point as to whether this is truly the case for rhythms which do not have a constant period or waveform, judicious use of analytical protocols should overcome most of the potential problems with this. While the choice of potential PRPs is endless, and will obviously depend on the rhythm involved, the selection of any reference point in the cycle should result in the same period estimate, as long as the waveform of the rhythm is stable.

Despite these difficulties, the activity on-sets in locomotor patterns are considered to provide a very reliable PRP for the estimation of period. Indeed the oldest, and most widely used, method of estimating locomotor activity period relies solely on this reference point. The statistic operates by calculating a least-squares regression line for a sequence of PRPs, with the time of day at which the PRP occurs in each cycle being the dependent variable and the day in the sequence the independent variable. Thus, when circadian rhythms display their endogenous nature by free-running in constant conditions, the period of this rhythm is shown as the slope of the regression line added to 24 hours. As the free-running period can be greater or less than 24 hours, the direction of the slope (either +ve or -ve) is also important. As the activity on-sets for locomotor rhythms are relatively clear and reliable, this statistic is usually quite sufficient for determining average periods.

The evaluation of such average periods is more difficult when a reliable PRP cannot be recognized. Enright (1981) summarized the statistics which attempted to deal with this as firstly multiplicative, in which one portion of the record is cross-multiplied with another or secondly, those which depend on an arithmetic averaging of sequential segments of the data. Autocorrelation

functions and the power spectra come into the first group, while periodograms, and their derivatives, make up the second group.

The autocorrelation function is the most elementary of the multiplicative procedures, and operates by determining the ordinary statistical correlation coefficient between the original data series, and that same series 'lagged' on itself by some fixed number of time units (one time unit equalling the time interval separating the observations in the original data series). A sequence of these coefficients can then be plotted against the number of lags. For persistent rhythms with only one dominant component this plot has the form of an oscillation, with the peaks, troughs and zero-crossing points all occurring at intervals corresponding to the period of the main rhythm. As the oscillations in the autocorrelation functions are usually smoother than the original data, direct estimations of the period can be attempted by averaging the intervals between successive PRPs. The major drawback of this is the very large variances associated with the period estimates; only very large numbers of observations can reduce this to any significant degree.

Power spectra rely on the mathematical principle that any sequence of data recorded at regular intervals can be fully characterized by discrete values, taken at the same intervals, from the sum of a series of sine and cosine waves. These values are known as the Fourier components of the data. The calculations are very long and complex, but basically involve the determination of certain coefficients that correspond to the amplitudes of the Fourier components. These amplitudes are then used to assign a degree of 'importance' to each of the possible periods into which the data can be divided. The period with the largest amplitude can thus be taken to be the best estimate of the major rhythmic element in the data. While this analytical method has been useful in the physical sciences, it has not proved to be wholly satisfactory for the study of biological rhythms. The reasons for this are twofold. Firstly, the period estimates have a low degree of resolution. For example, in a data series made up of T observations, the interval between each possible period estimate is 1/T. Therefore, if data are collected hourly for 10 days T will be 240, and the full range of estimates will be from 240/240 to 240/1. For biological rhythms the most meaningful period range is probably between 21 and 27 hours. If one looks at the power spectrum estimates available in this area, there are only three: 240/11, 240/10 and 240/9. These are 21.82 hours, 24.0 hours and 26.67 hours respectively. This is in no way accurate

enough for any investigation of biological pacemakers. The second problem is that the division of the data into its Fourier components makes certain assumptions about the waveform of any rhythmic elements present. Although these assumptions may describe the data adequately, theoretically any number of other models (based on quite different hypotheses) could produce equally valid period estimates.

This brings us to the second category of possible analytical procedures, periodograms. These have the advantage over the preceding methods in that no assumptions are made concerning the waveform of the recorded observations: the data set itself is used to produce any estimate of the waveform.

Schuster first described the principles behind periodograms in 1898, but their application only became practicable with the development of modern computers. Periodograms are based on a generalization of the 'Buys-Ballot' waveform estimation technique, and follow the initial assumption that a regular, phase-conservative rhythm is present in the data, with a period value, P_i. The data are divided into sequential segments of length P_i, and all the segments are then superimposed. An average value is then calculated for the level of activity in each recording interval over a cycle of length, P_i. If a single, dominant and stable rhythm of period P_i is present in the record, this average set of values will represent an estimate of its waveform. As it is unlikely that P_i will be the true period, the entire procedure is repeated for a range of possible period values. The periodogram is subsequently made up from a function of these waveform estimates (or simply form-estimates) plotted for each recording interval. High values of the statistic plotted produce peaks on the periodogram which can be read off on the abscissa as period values. From the above argument it follows that the form-estimate which produces the largest peak must be an indicator of the underlying waveform of the rhythm.

A variety of statistics can be used on the ordinate to characterize the form-estimates. Enright (1965a, b), suggested the use of either the range or the root mean square amplitude of the mean activity levels (which is derived from the average activity for all the intervals at each period tested). Williams and Naylor (1967), on the other hand, used the coefficient of variability in order to permit better comparisons between the results of different experiments. Enright (1965a) also produced simple procedures for obtaining form-estimates for

period values that were non-integer multiples of the basic time unit of the observations. Many subsequent extensions to the periodogram have been proposed, especially with regard to the assessment of the degree of significance of the period estimates (Williams and Naylor, 1978; Harris and Morgan, 1983). Although periodograms can have problems associated with their use and interpretation (Enright, 1965a), they appear to provide a very reliable and accurate method of estimating period.

This discussion of some of the statistical techniques which are available for analysing biological rhythms is obviously not exhaustive, but does include the most important procedures. Unfortunately, it is not possible, nor worthwhile, for a non-mathematician to consider the relative merits of these various period estimating statistics, particularly when very few papers have been produced which deal with this. However, Enright (1981) does discuss some of the relevant studies. These generally conclude that while each procedure can be useful, the periodogram is perhaps the most feasible, having relatively simple calculations which are economical in computer time without being any less accurate in the estimation of the period values. While combining accuracy and reliability with a simple mathematical basis, it also makes no assumption about the nature of the waveform of the rhythm. This is especially important when variable waveforms are so prevalent in biological oscillations. Furthermore, Enright (1965a, b) has been able to show how the simplicity of the periodogram can enable one to understand the ways in which it may be misleading as well as instructive, the relative complexity of the other statistical methods tending to preclude the assessment of these factors.

For these reasons, together with the fact that activity in *C. vicina* is usually clearly defined, the periodogram was chosen as the most suitable analytical procedure. However, one potential problem which emerged in the analysis of blowfly activity was the way in which period was seen to alter throughout the records. Periodograms cannot separate out the components in such cases of non-stationarity, as Enright (1981) termed these period alterations. However, as will be shown in the following section, this can be overcome by very carefully selecting the data blocks which were to be analysed.

The version of the periodogram used in the present research was originally devised by S.E.R. Bailey at Manchester University in 1982, and subsequently modified for the BBC range of microcomputers with the assistance of

R.D. Lewis of Auckland University. Bailey's program was based on Enright (1965a), and Williams and Naylor (1967). Significance levels were calculated from a 95% confidence limit placed on the slope of a randomization of the data (Lee and Lee, 1982). In the present work, the 'variance' of the hourly means within a form-estimate was taken as the estimate of amplitude, rather than the 'standard deviation' used by Enright (1965a). This type of periodogram has proved useful in elucidating the circadian components of the activity of the mollusc *Helix lucorum* (Bailey and Lazaridou-Dimitriadou, 1986). More recently, however, a 'chi-squared' periodogram (as first proposed by Sokolove and Bushell, 1978) has been adopted by many workers as an alternative to the other statistics. Unfortunately it was not possible to fully assess this periodogram with respect to that used here, but the results obtained from both appeared to be very similar.

The following section will describe in detail the data collecting system, while the subsequent chapters will concentrate on the results obtained from its use.

Methods of Recording Activity

Throughout the history of the study of circadian rhythms locomotor activity has been one of the most popular areas for investigation. The reasons for this are twofold; firstly, it has proved to be very simple to measure activity accurately over long periods of time, e.g. Harker (1956) found that an acceptable recording could be produced by simply tying the insect to a pen that marked a smoked drum. Secondly, since locomotion is performed as part of a number of behavioural patterns, it could, if necessary, be taken as the integrated response to several different endogenous stimuli. These factors have enabled activity studies to become integrated into a wide variety of quite different research fields.

Over this time a large array of more sophisticated techniques than that used by Harker have been developed to record insect movement, e.g. sound recording (Jones, 1964), running-wheels (Roberts, 1960), rocking actographs (Syzmanski, 1914 cited in Brady, 1974), light-beams (Brown and Unwin, 1961), electrostatic fields (Smith, 1983) and many modifications of all of these. Some of these limited the recording to only certain aspects of the activity. For example, sound and electrostatic fields were used to measure flight, while

running-wheels only recorded walking movements. While these two methods have the advantage of reducing the general 'noise' level in the observations, they can also result in the loss of some important components of the rhythmic behaviour. On the other hand, the rocking actograph and the light-beam recorders measured whole body movement and thereby simplified the recording to a question of motion or non-motion. However, all of these devices shared one common factor, namely the assumption that the daily pattern of locomotor activity was the result of an internal clock. Although this is generally accepted to be true it must also be considered that as locomotion is a form of behaviour it could be influenced by a variety of other non-rhythmic elements in the environment. One such source could be the way in which the insect responds to the actograph which is recording its behaviour. Thus, the cockroach *Periplaneta americana* when placed in a rocking actograph or a Brown and Unwin infra-red device has been shown to display a gradually declining amplitude in its activity peaks. Over a period of several days the rhythm could completely disappear, even in LD. However, if the cockroach was subsequently transferred to a running-wheel the rhythm re-emerged (Brady, 1967). This declining amplitude of activity was not, however, seen in the cricket *Acheta domesticus* even when it is kept in a rocking actograph for several weeks (Nowosielski and Patton, 1963). Mammals have also been shown to give differing experimental results in different recording environments, e.g. Aschoff et al., 1973 (cited in Aschoff and von Saint Paul, 1982) found that hamsters kept in spring-suspended cages had slightly shorter free-running periods than those kept in running-wheels. It is clear, therefore, that in making any inter-species comparisons the recording mechanism must be recognized as source of potential variability.

It is also obvious that, irrespective of the device used, the recording environment will always be very artificial. Different mechanisms will necessarily provide the insect with differing sensory information. For example, in a running-wheel the first steps taken by the insect will result in further stimuli from the movement of the wheel, and these may initiate a chain of running sequences that are artificially enhanced by positive feed-back. The light-beam recorders, on the other hand, will provide no such stimuli, so that no further behaviour is initiated. In this sense the non-invasive infra-red detectors should give a more realistic record of the activity behaviour of insects. This does not, however, explain why the amplitude of the activity should decline in the

cockroach example above, when the same devices (infra-red beams and rocking actographs) have proved capable of giving very long records in many insects. The only way to resolve such a problem is to measure activity in each insect species with more than one type of recorder. Unfortunately this has only been done in a very small number of cases.

When deciding the kind of device most suitable for *C. vicina* it was not feasible to design and use more than one recorder. Therefore, for comparative reasons, it seemed more appropriate to use the most common method which had been applied to species closely related to the blowfly. This was, in almost all cases, an infra-red recorder. This device, as well as being less likely to influence the data in unpredictable ways, produced results which seemed to be clear and persistent, with none of the undesirable features seen in the cockroach.

The original Brown and Unwin recorder was defective in that it tended to produce a very 'noisy' record. This was because the infra-red beam was too large relative to the size of the insect (being 2.5cm wide), and, in the confined space of the cage, the insect's other activities (i.e. feeding and preening) were constantly altering the light intensity striking the photocell. Thus, the locomotor rhythm had other elements superimposed on its pattern. Subsequent advances in miniaturization have enabled investigators to employ very much smaller infra-red beams, thereby reducing this hazard and allowing very small insects to be observed, e.g. *Drosophila* spp. (Hamblen et al., 1986).

In the present study the flies were placed in 9cm petri dishes bisected by an infra-red beam of 5mm diameter. The 'extra' activity associated with feeding and drinking was kept to a minimum by placing the sugar and water at opposite sides of the dish, well away from the beam. The bottom of the petri dish was covered with a double layer of filter paper to facilitate cleaning. The dishes, each containing one fly, were then mounted in pairs on a wooden platform. Two raised arms on either side of the platform were pierced with holes to accommodate the infra-red emitters and detectors (see Plate 1). The dishes were also separated by a thick piece of black card to prevent the flies from seeing each other, and thus influencing each other's locomotion. Each platform was housed in a separate light-tight box (as described in the General Methods) in a walk-in constant temperature room at 20±0.5°C, unless otherwise stated. Whenever possible the flies were only disturbed at the very

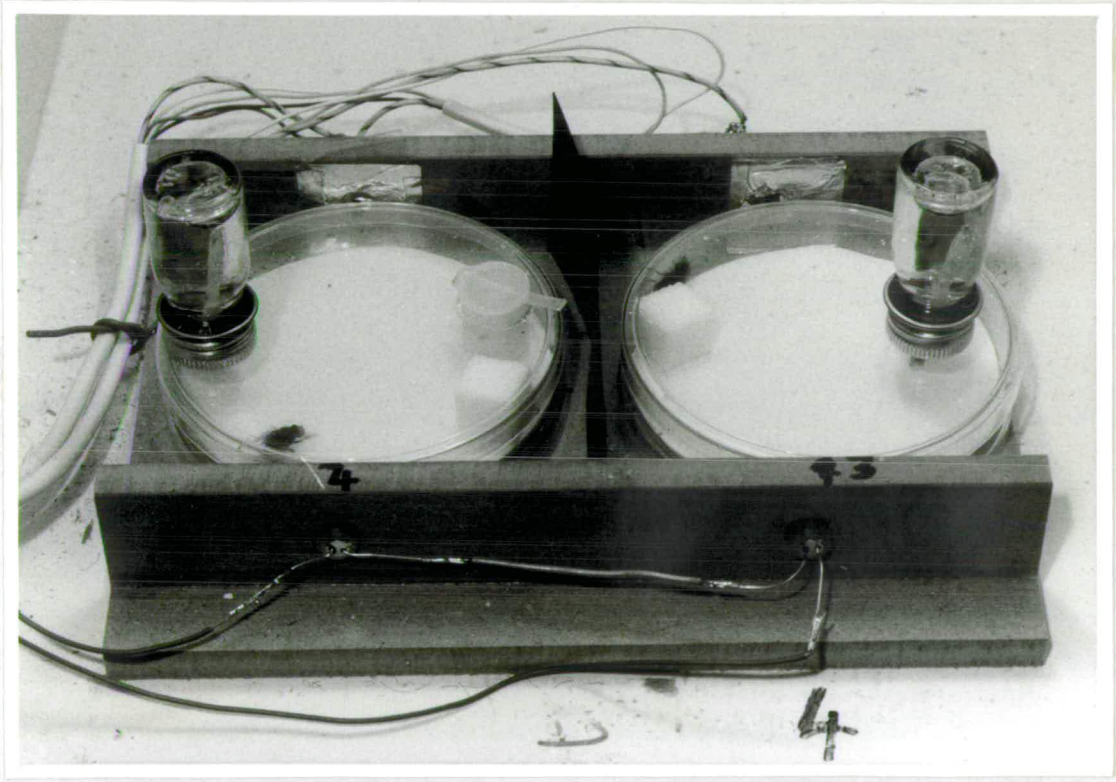

Plate 1. Photograph of the activity recording device. Note that there were two separate recordings occurring simultaneously within each device. Each petri dish was provided with fresh water and sugar at the beginning of every experiment. The petri dish on the left also contains a small plastic well (on the right) which if necessary could be used to supply meat to the flies. The infra-red emitters and detectors were located on opposite sides of the petri dish embedded in wooden blocks. A black card was also placed between each recorder to limit the visual stimulation between the flies.

beginning of each experimental run. Although this was usually carried out during the time when the lights were on it was occasionally necessary to handle the flies during the dark. At such times a red light source was used to provide the illumination, the reason being that light of this wavelength was thought to be invisible to the flies. Furthermore, throughout all of the experiments described here there was never any suggestion that this was not the case. Both sexes were used for all of the studies, as preliminary experiments had found no differences in their behaviour.

All activity experiments were automatically recorded, printed and analysed on a BBC microcomputer (the programs necessary for this are given in Appendices I to VI). The collection of the data was only made possible by a 32 channel digital interface, which was obtained from Mike Cook of Musbury Consultants, 8 Fairhill, Helmshore, Rossendale, Lancs. The most convenient time units for observing the activity were 10 minute intervals, with each breakage of the beam counting as a single datum. All of the 32 channels were continuously monitored on a VDU, and were subsequently divided into the individual record files using the programs in the Appendices. Each file was then printed out in the standard double-plotted format, whereby two copies of the entire record are displayed side by side, the right hand copy placed one day higher on the page. Figure 2.1 is an example of just such an arrangement, showing a free-running blowfly rhythm. This allows a clearer display of the locomotor pattern, as consecutive days are seen as a contiguous record.

As was indicated in the previous section the periodogram was chosen as the most appropriate statistical procedure for analysing the locomotor pattern of the blowfly. In order to understand the system of analysis it is convenient to consider each fly's record as a single data string. If the record lasted 14 days the number of units in the string would be 2016, this being the number of 10 minute intervals in that time. The versatility of the periodogram program is illustrated by its ability to analyse between any two units in this string, and within any range of periods, for the main rhythmic components of the locomotory pattern. While all of the locomotion observations described in this thesis were dealt with in this manner, the magnitude of the data prevented the inclusion of too much analytical detail in the results sections. Thus, much of the data will be presented in a tabular format. It was also considered useful, on some occasions, to split the observed patterns into those flies which showed a) clear circadian rhythms with a constant free-running period throughout the

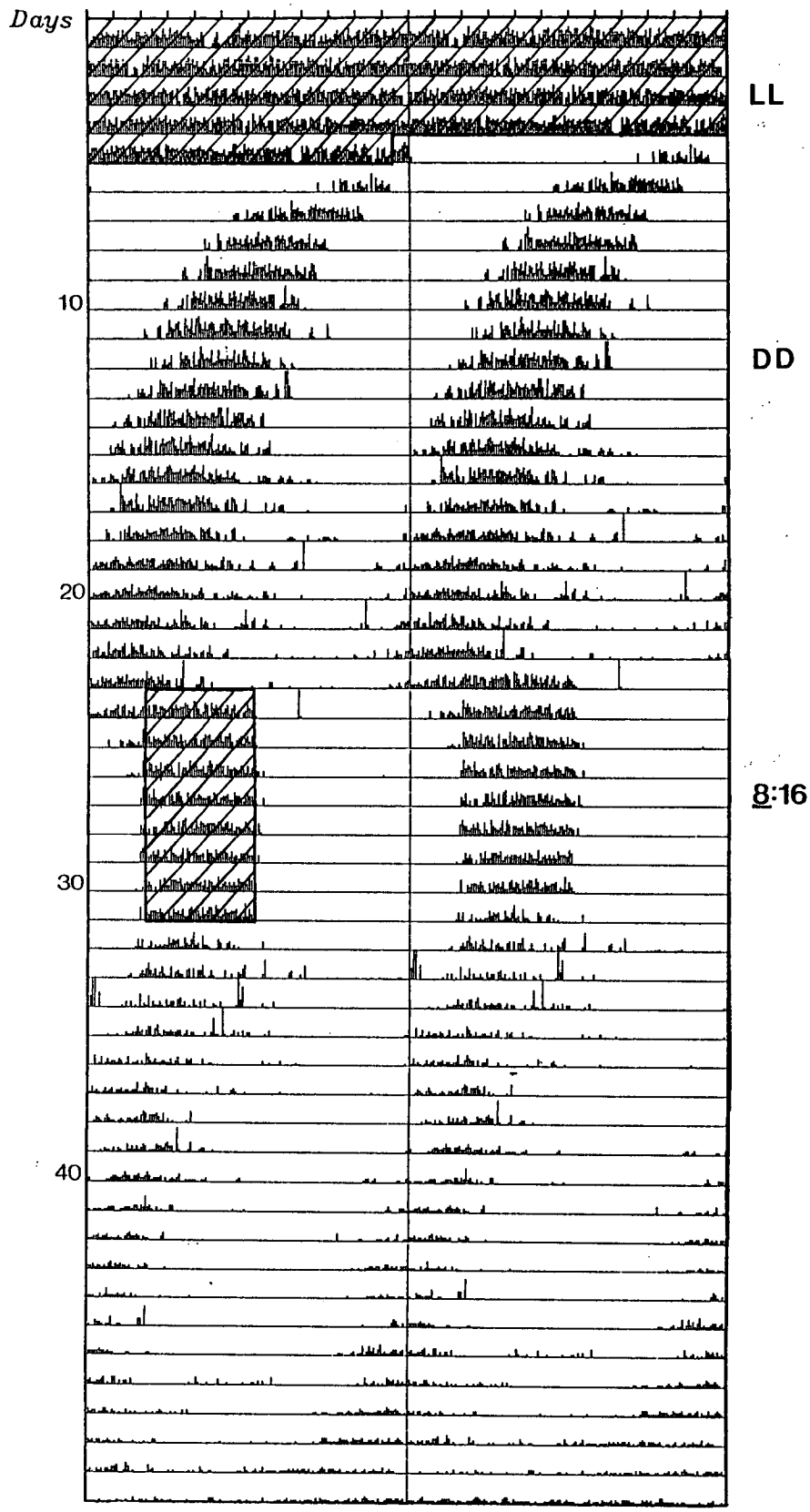

Figure 2.1. The activity pattern observed in adult blowflies when free-running in constant darkness (DD), after several days in constant light (LL). A brief entrainment to 8:16 is also shown.

recording time, b) clear rhythms with a single, or few, changes in period and, c) complex rhythmic patterns. This last group included patterns which were arrhythmic and those which appeared to comprise several discrete components. Those results which did not lend themselves to this form of categorization were presented simply in terms of the free-running period of their rhythm, the overall amplitude in each cycle and the duration of the active phase (α). More precise definitions of these components of the activity will be presented in the experimental sections. Some cases of particular interest were dealt with separately.

For comparisons between most experimental groups a two-tailed *t*-test was employed. This was necessary as many of the groups had differing sample sizes, and it could not be assumed that the variances within each sample were the same. The 5% probability level ($p=0.05$) was chosen as the cut-off point for all of these *t*-tests. One-way analysis of variance was also used, again with a 5% significance level.

CHAPTER 3

Circadian Rhythms of Activity in *Calliphora vicina*

2. Results and Conclusions

Activity Patterns in Light/Dark Cycles

The first experimental parts of this study follow on directly from the topics which were discussed in the introduction to locomotion. As the results from all of these experiments were highly interrelated it was decided to group the observations into one large experimental chapter. Thus an attempt will be made to describe, in detail, the patterns of locomotion as they appeared in steady-state entrainment to T24 and non-24 hour cycles, all of the flies involved in these experiments coming from the Scottish stock of *Calliphora vicina* (55°North). Observations will also be made on the form of the activity when the flies were released from the effects of the entraining Zeitgeber and allowed to free-run in constant darkness. The results will then be discussed with reference to the current locomotor models in terms of the structure of the circadian system governing activity behaviour.

Entrainment to 24 Hour Cycles

In any investigation of activity rhythms it is important to begin by observing the locomotion patterns which are present in those photoperiods which most closely mimic the natural light/dark (LD) cycles. For this reason the first experiments on the blowfly's locomotor rhythms were concerned with the patterns of activity present in rhythms entrained to 24 hour LD cycles. Thus the flies were exposed to a variety of cycles of T=24 hours for most, or all, of their lifetime. In all cases most of the activity was restricted to the light portion of the day. In long light periods (12 hours or greater) almost no activity was seen in the dark (see Figs. 3.1 to 3.3), but decreasing the length of light to 8 hours and finally 4 hours resulted in a pattern where activity began well before the 'dawn' (Figs. 3.4 and 3.5). In 4:20 (4 hours light: 20 hours dark) activity also extended beyond the lights-off (see Fig. 3.5). There were, however, certain features which seemed to be shared by all of the recordings. The most specific of these was the manner by which the activity reached a maximum

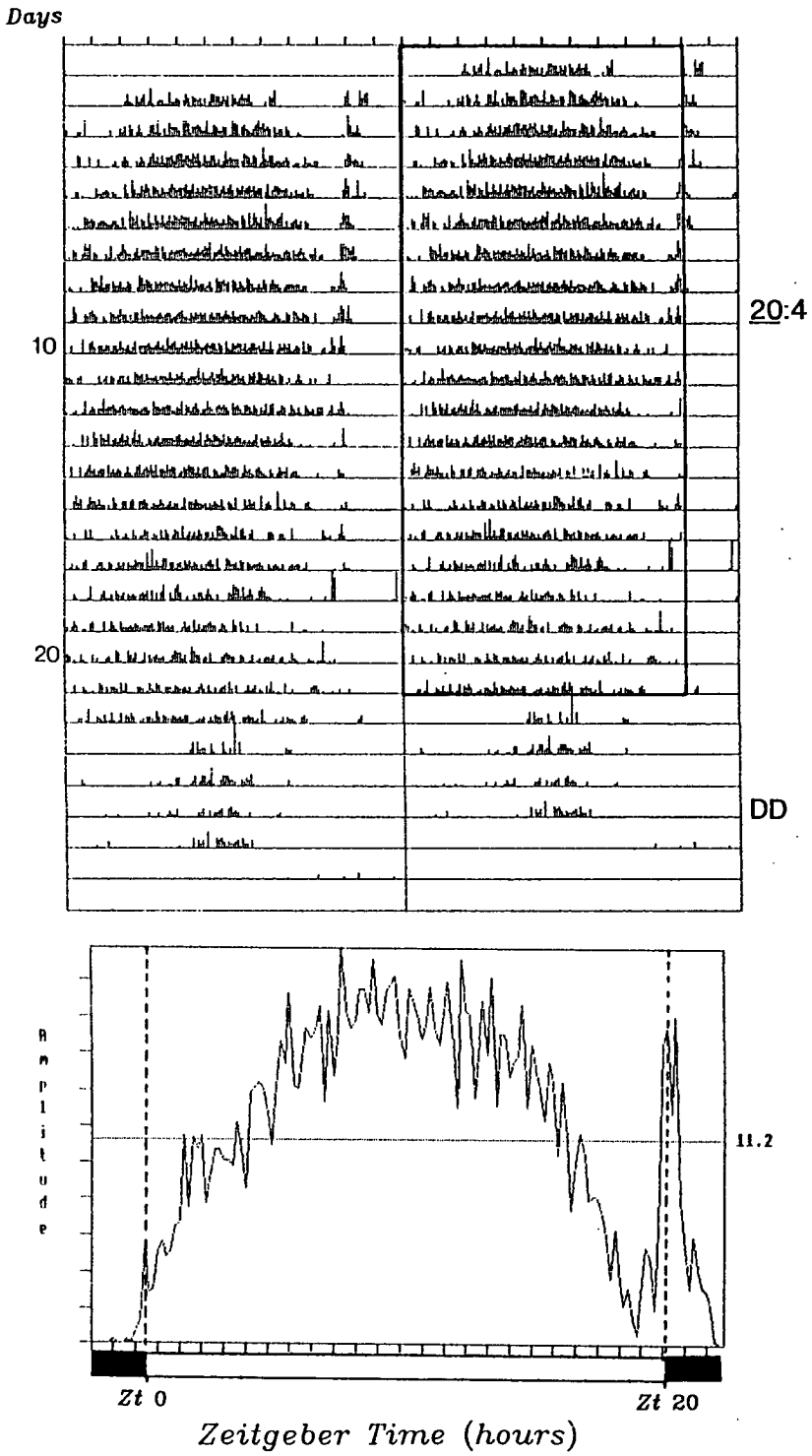

Figure 3.1. The activity patterns observed in a cycle comprising 20 hours light and 4 hours dark (usually termed 20:4).

Top. Daily activity pattern in standard double-plotted format, with the extent of the light cycle for one side of the plot being defined by the enclosed box.

Bottom. Average form of the activity pattern relative to the prevailing light cycle. By convention the beginning of the light phase is defined as Zeitgeber time (Zt) 0 hours. Detailed descriptions of this, and all of the subsequent figures, are given in the text.

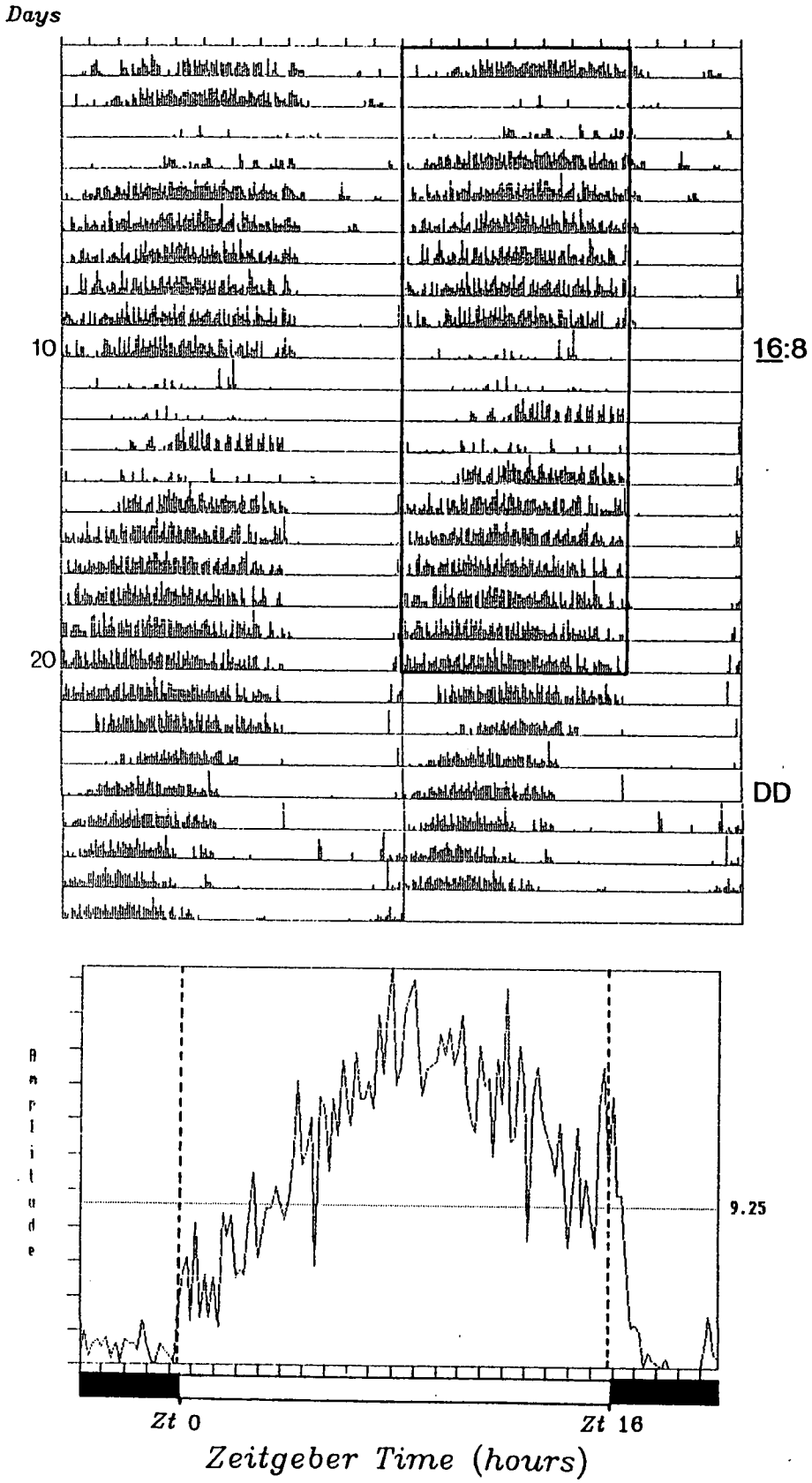

Figure 3.2. The entrained activity pattern in 16:8.

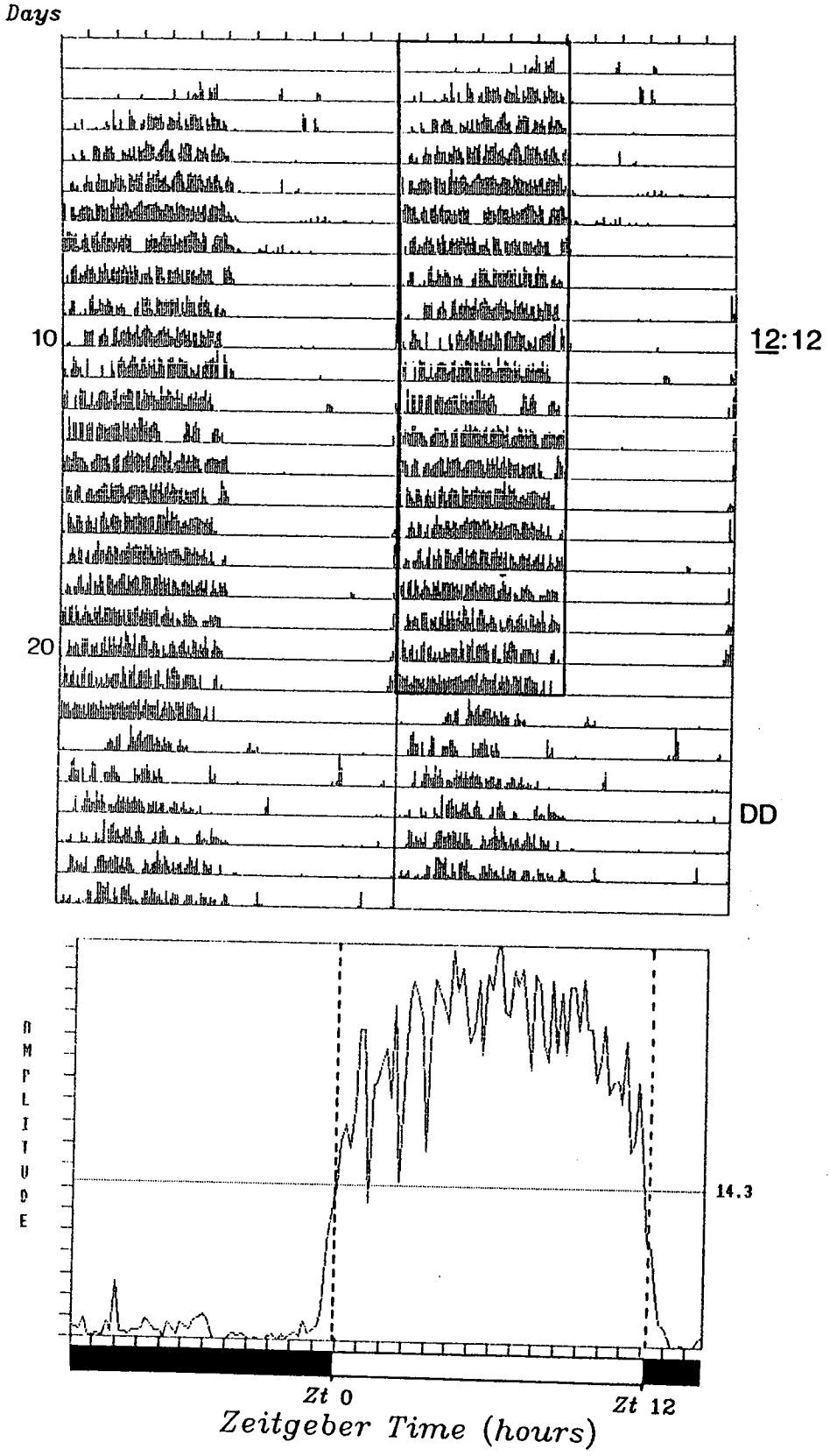

Figure 3.3. The entrained activity pattern in 12:12.

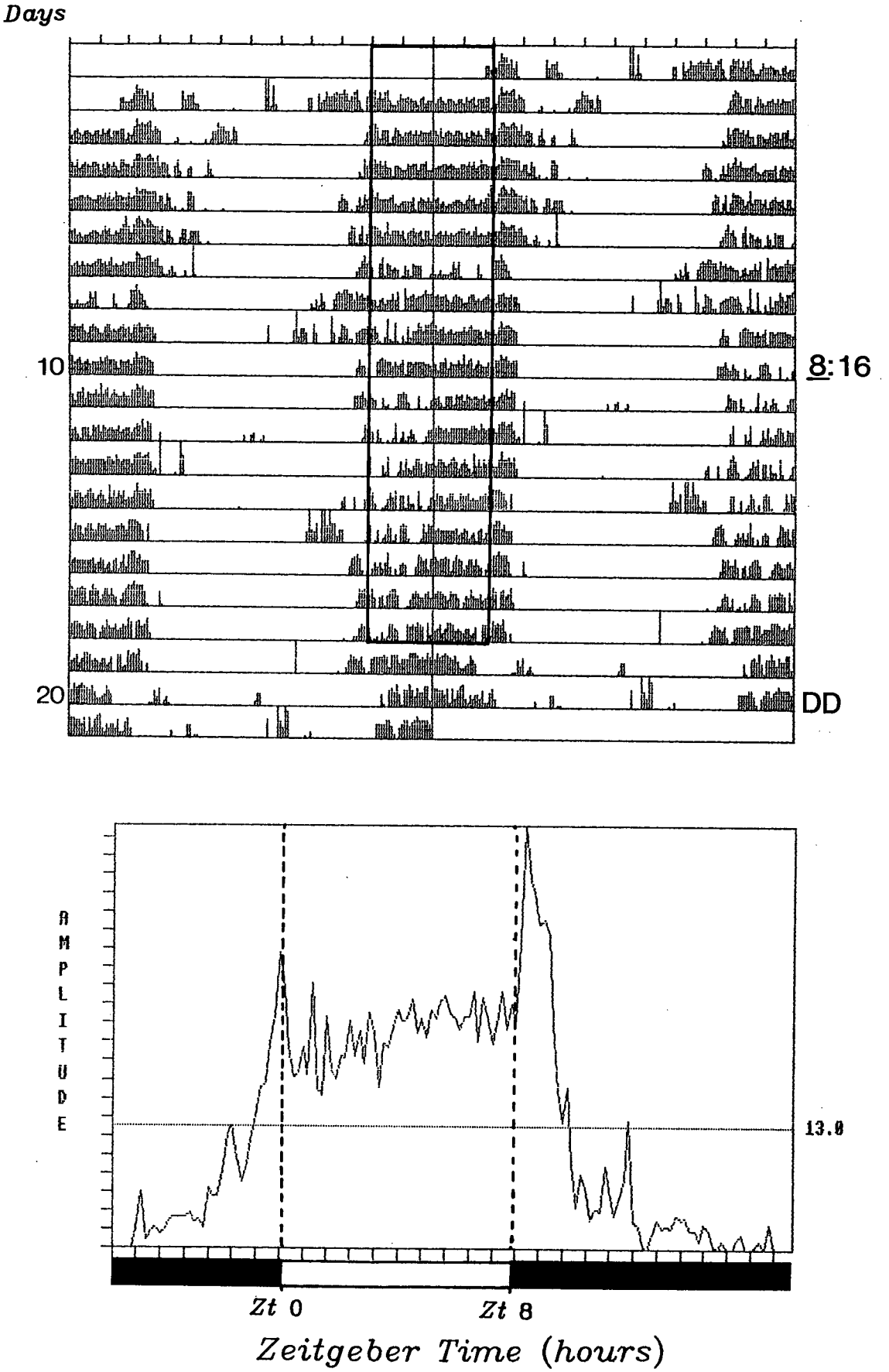

Figure 3.4. The entrained activity pattern in 8:16.

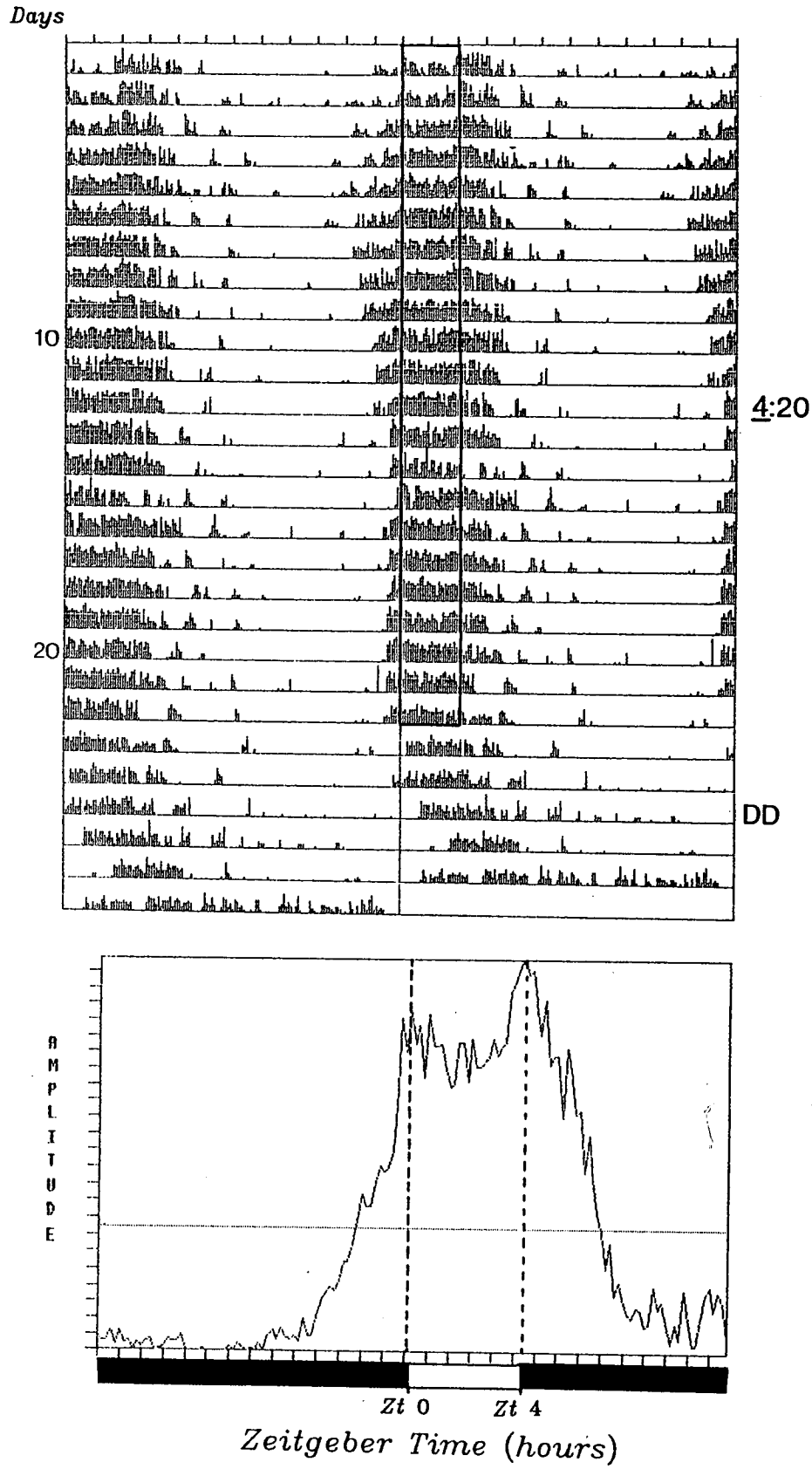

Figure 3.5. The entrained activity pattern in 4:20.

value. This was usually characterized by a gradual increase in the level of activity to the maxima, generally situated at the centre of the light phase, and a subsequent decline at roughly the same rate to the low levels seen in the dark. In long photoperiods this gradual increase occurred over a large part of the illuminated phase, although frequently beginning just before lights-on (Fig. 3.1). When the photoperiods were short (Fig. 3.5) most of the increase in activity occurred in the dark, thus maintaining the position of the maxima in the centre of the light. The anticipation of the 'dawn' (as indicated by the pre-lights-on commencement of activity in most photoperiods) was interpreted as evidence for an endogenous basis to this behavioural rhythm. However, imposed on this pattern were other, very short (1-2 hours) and very intense, activity peaks which appeared to be closely associated with the light to dark transitions. This association tended to suggest that these peaks were the result of a direct effect of the light on the rhythm, the 'dusk' transition being especially effective in this respect. As will be seen in subsequent sections the exogenous nature of these extra activity peaks was confirmed by their inability to continue in the absence of the entraining cycle, i.e. when the rhythm was released into constant dark, DD.

The remarkable degree of similarity in the entrained patterns produced by the various photoperiods prompted a fuller analysis of the features which comprised these locomotor rhythms. There are in general three fundamental characteristics of entrained rhythms which can be investigated. These are the relative phase relationship of the activity to the light cycle, the duration of the active phase (termed α, and *vice versa* the duration of inactivity, designated by ρ) and the amplitude of the active phase. Although theoretically the period of the rhythm could be considered as a feature of its entrainment it was evident that in all cases the rhythm of activity was able to adopt the period of the entraining cycle, i.e. 24 hours. As will be seen, when the cycle length was extended far beyond the natural period this ability became less certain.

When considering phase relationships at any level one is continually faced with the dilemma of the true temporal relationship between the activity rhythm which is observed and the circadian oscillation which underlies it. If one follows Pittendrigh's hypothesis (Pittendrigh, 1967; Pittendrigh and Bruce, 1957, 1959; Pittendrigh et al., 1958) of a circadian system comprising several hierarchical levels, then the observed activity patterns are probably the output of 'slave' oscillations rather than the overall 'pacemaker' which may govern

several slaves. As was discussed in the General Introduction it is the pacemaker which is entrained by the light pulses, altering its natural period (τ) to that of the entraining cycle (T) in order to do so. This pacemaker in its turn entrains the slave which controls the observed rhythms, in this case locomotion. By the time this process is complete the pacemaker will have adopted a specific phase relationship to the LD cycle and the slave will have attained a certain phase relationship to the pacemaker. In the present study it was difficult to know the true position of either of these, as no markers other than the position of the maxima of activity were available. Since, as has been indicated, such maxima coincided with the centre of the light cycle in all of the photoperiods, it has been construed that the phase relationship of the slave to the output (locomotion) and thence the slave to the pacemaking oscillator may be conserved over the differing entraining photoperiods.

The next consideration was the length and intensity of the activity bout in the entrained state. The measurement of these parameters was both easy and accurate as the computerized activity recorders guaranteed the availability of 'real' unaltered data. In both cases it was possible to produce, over a specified portion of the recording, an average profile of a single cycle of the rhythm (e.g. Figs. 3.1–3.5). The duration of the activity (in hours) was then directly measured from the points making up these profiles, whilst the average amplitude over one cycle was calculated automatically. Strictly speaking this average activity level is not the amplitude of the active phase but rather an average over one whole cycle. However, the clarity of the blowfly's locomotor rhythm (with very little activity in the dark) meant that these amplitude estimates were probably good estimates of the average activity levels of the active phase. It must also be stressed that this 'amplitude' referred solely to the observed activity patterns, and therefore does not imply anything about the nature of the amplitude of the underlying oscillator. These definitions must be borne in mind when the endogenous nature of the activity rhythms are considered in the following sections.

The resulting average activity lengths are shown in Table 3.1. As one might expect there was an overall increase in the duration of the activity between the 4:20 entraining cycle and 20:4. Although an analysis of variance test confirmed this ($F=7.806$ with 4 and 22 d.f.) the relationship did not appear to be linear, as most of the increase occurred after the light exceeded 12 hours in length. Indeed, up to that point there appeared to be a gradual decrease in activity

Hours of light per 24 hours	*Mean duration of activity in each cycle (hours±s.d.)*	*Number of observations*
4	15.78±1.86	6
8	15.07±1.69	5
12	14.57±1.33	5
16	17.75±0.72	6
20	19.30±2.14	5

Table 3.1. The data for the average duration of the active phase (α) in steady-state T24 LD cycles.

duration with 12:12 having the narrowest active phase. It was also of interest to note that the range of the overall increase was only in the region of 5 hours, compared with 16 hours for the duration of the light. It would seem, therefore, that the length of the active phase is only a partial reflection of the length of the light, and that its minimum duration (14.5 to 15 hours) is mainly physiologically controlled. The increases seen in longer photoperiods may then be the result of the light exogenously widening the activity band. The respective amplitudes associated with these active phases are given in Table 3.2. An analysis of variance test for these indicated no significant difference in the amplitude of the activity between the entraining LD cycles ($F=2.089$ with 5 and 28 d.f.). As can be seen, Table 3.2 also contains data for rhythms in constant light. Although the patterns produced by this regime (Fig. 3.6) always appeared to be arrhythmic when analysed using the periodogram, more recent work has indicated that very short ultradian rhythms may be present in such data. This will be discussed in a later section.

It appeared, therefore, that the pattern of rhythms entrained to 24 hour cycles could be described as gradually increasing and decreasing peaks of activity centred around the middle of the light period. In other words *C. vicina* had a clearly diurnal locomotor behaviour pattern. Changing the length of the light phase of the entraining cycle did produce small increments in the duration of the active phase but such changes as did occur were probably the result of the direct effect of light. In conjunction with this the amplitude of the activity showed no pattern associated with the increasing length of light, being at a relatively consistent level throughout. Therefore, all of the major descriptive parameters of the entrained locomotor rhythms (i.e. phase relation to light cycle, duration and amplitude) seemed to display some degree of homoeostasis in the differing photoperiods. It is tempting to assume that, as homoeostasis is a general feature of biological clocks, the reluctance of the locomotor rhythms to alter some of their basic features may be some manifestation of an endogenous oscillator.

Entrainment to Non-24 Hour Cycles

In addition to 'natural' cycle lengths the blowflies were also exposed to a variety of exotic cycles, i.e. T20, T30, T36, T48, T60, and T72. These comprised a 12 hour light pulse followed by a variable amount of darkness up to the required cycle length. These cycles were chosen in order to aid the later

Hours of light per 24 hours	*Mean amplitude of activity in one cycle (units±s.d.)*	*Number of observations*
4	12.49±3.39	6
8	14.49±1.96	6
12	14.03±1.28	5
16	14.08±5.30	7
20	8.48±2.16	5
24	17.31±8.57	5

Table 3.2. The data for the average levels of activity in one Zeitgeber cycle in entrained T24 LD cycles.

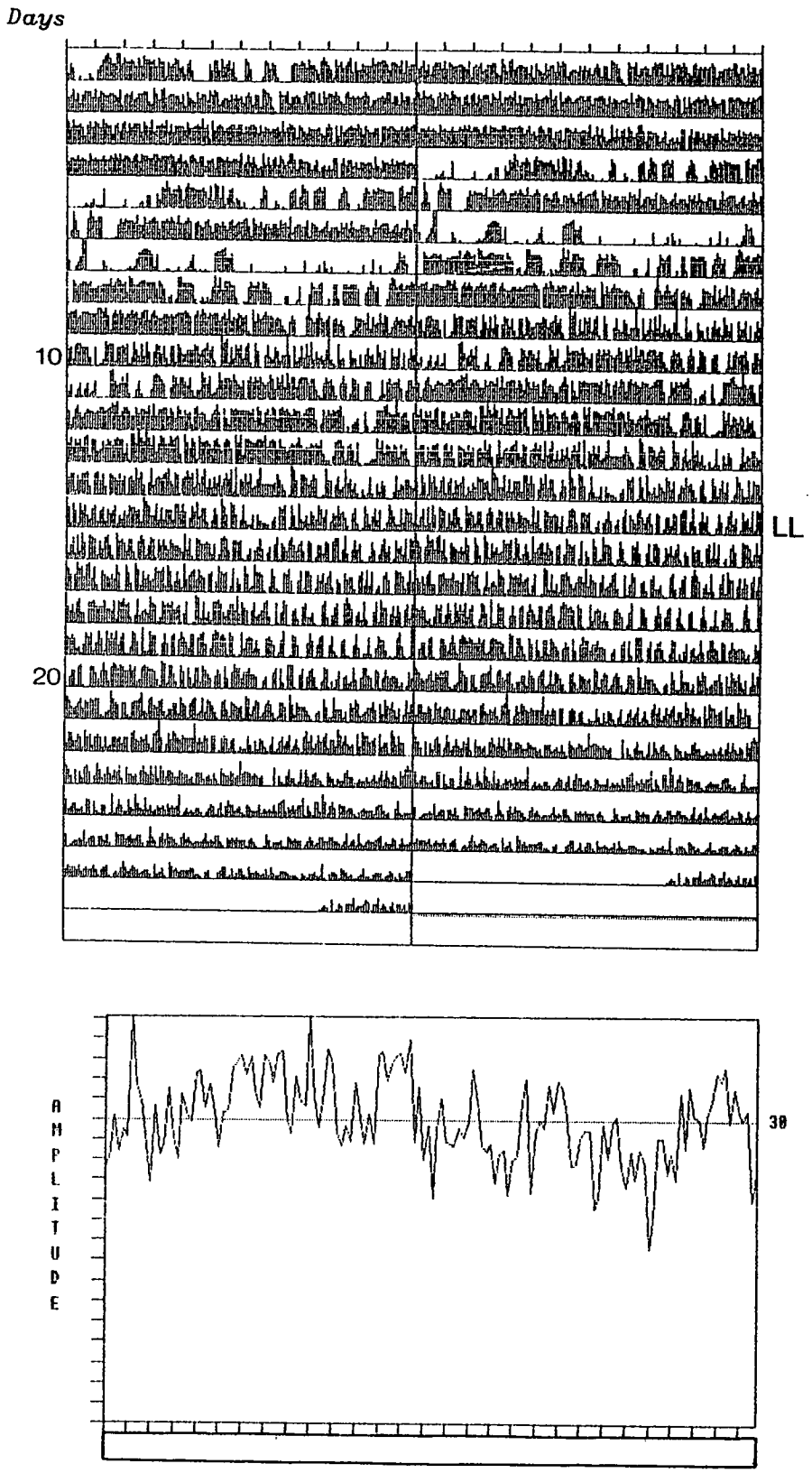

Figure 3.6. The activity pattern in LL. Note the absence of any oscillation in the activity level.

comparisons between locomotor rhythms and the photoperiodic clock which controls the induction of diapause. As will be seen, one of the most powerful experimental protocols available to investigators of photoperiodic induction is the Nanda-Hamner technique. This involves exposing flies to a range of T-cycles (comparable with those used here) and observing the percentage diapause induced at each T-value. Those species which are thought to have a circadian basis to the induction process produce a sequence of peaks and troughs in the diapause percentage. The inter-peak interval in these results is considered to be an indicator of the period of the photoperiodic oscillator and is frequently around 24 hours. Thus using this technique in an analysis of the locomotor behaviour over the same photoperiods may help in elucidating the mechanism behind diapause induction.

In the shortest cycle, T20, one obvious feature was always evident in the records. This was the large burst of activity which occurred immediately after lights-out (Fig. 3.7). This behaviour was of a consistently higher amplitude than that seen during the preceding light phase, and while it superficially resembled the 'exogenous' peaks observed in T24 cycles, it could be distinguished from them because of its relatively longer duration (2-4 hours). In the former T24 cycles such extraneous behaviour persisted for no more than 1-2 hours after each LD transition. No activity peaks were found at the lights-on as had been seen occasionally in T24, rather activity on-set seemed somewhat delayed, frequently beginning some time after the light phase had started (see Fig. 3.7). This pattern of extra activity was also present in T30 cycles, with the exception that the high amplitude activity bout preceded the lights-on (Fig. 3.8). Another short but intense peak of activity was also observed at the transition which terminated the light cycle, as had been seen in T24 cycles.

Extending the cycle length up to T36 resulted in a radical alteration in the relationship of the locomotion rhythm to its entraining cycle. The main active peak was now seen to occur entirely outside the light portion of the cycle (Fig. 3.9). In all cases there was some locomotion within the light, but it seemed as if this was largely exogenous in nature. This view was enhanced by the variety of amplitudes that were present during the illuminated phase, some flies displaying quite high activity levels while others had little or no activity (Fig. 3.9). In any event in all of the T36 examples the activity seen in the light was of a consistently lower amplitude than that in the dark. Extra activity peaks were again obvious around the LD transitions in many of these recordings.

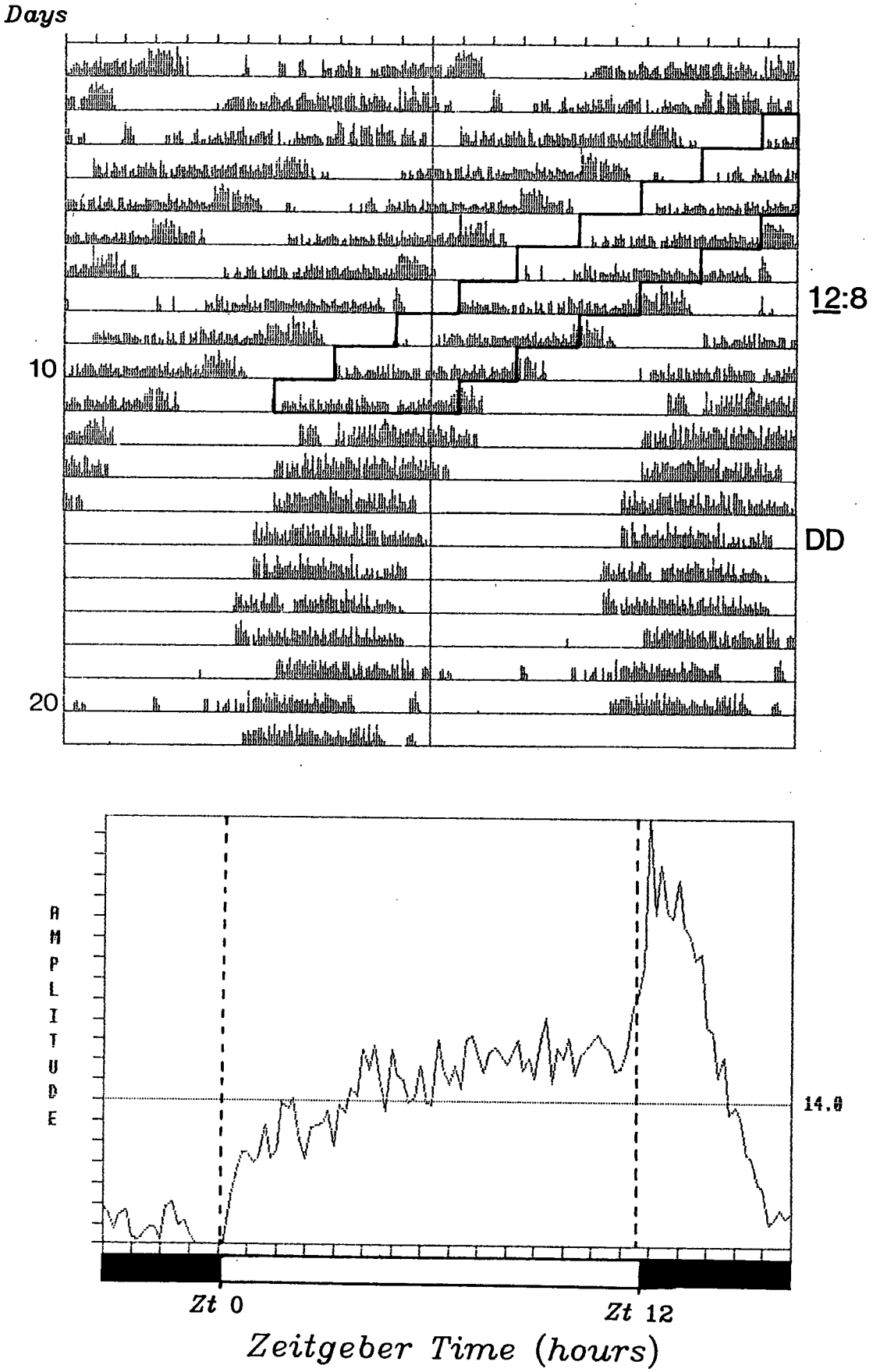

Figure 3.7. The entrained activity pattern in T20 (12:8). Note the excess activity after lights-off.

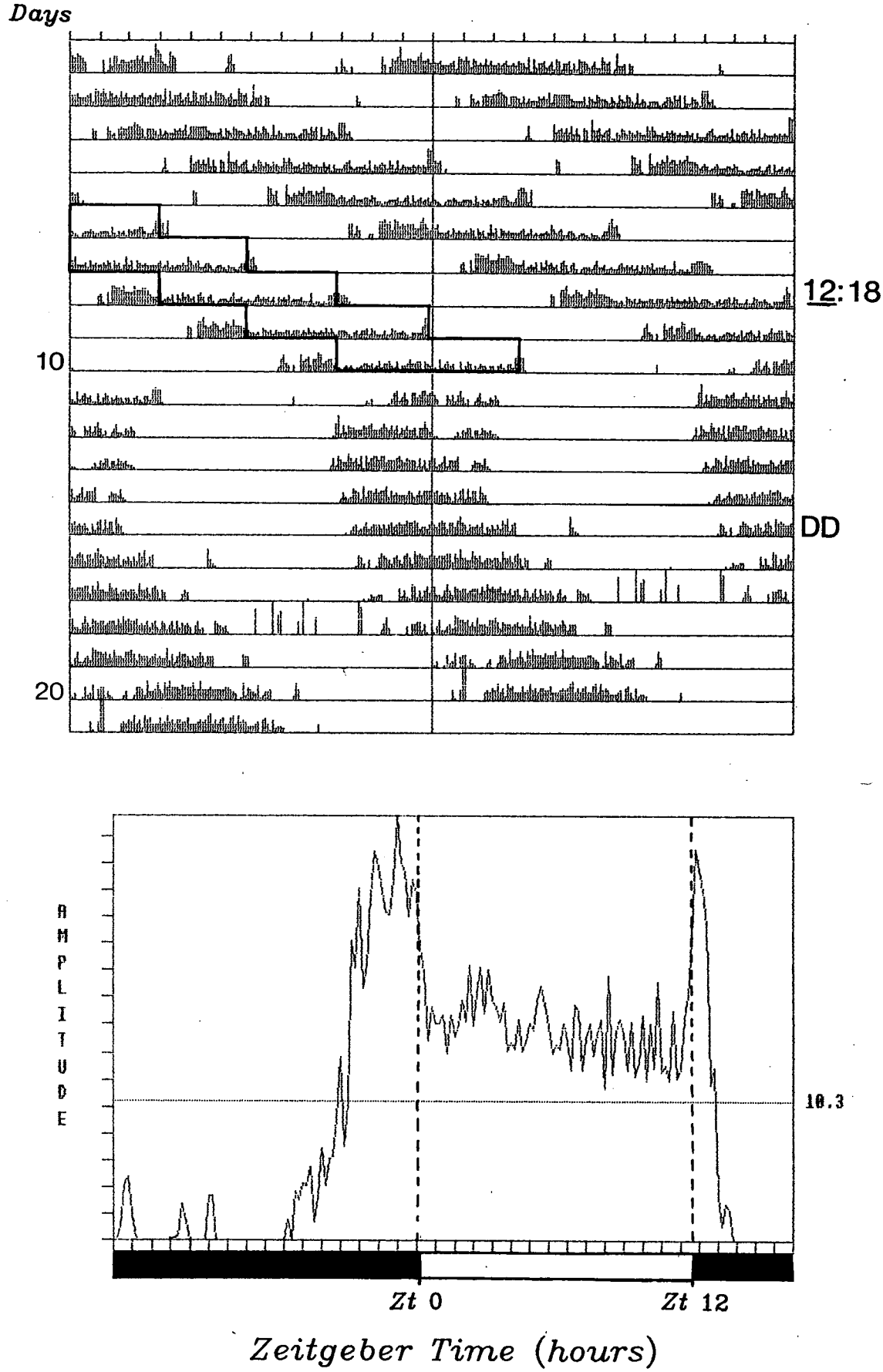

Figure 3.8. The entrained activity pattern in T30 (12:18). Note the extra activity just before lights-on.

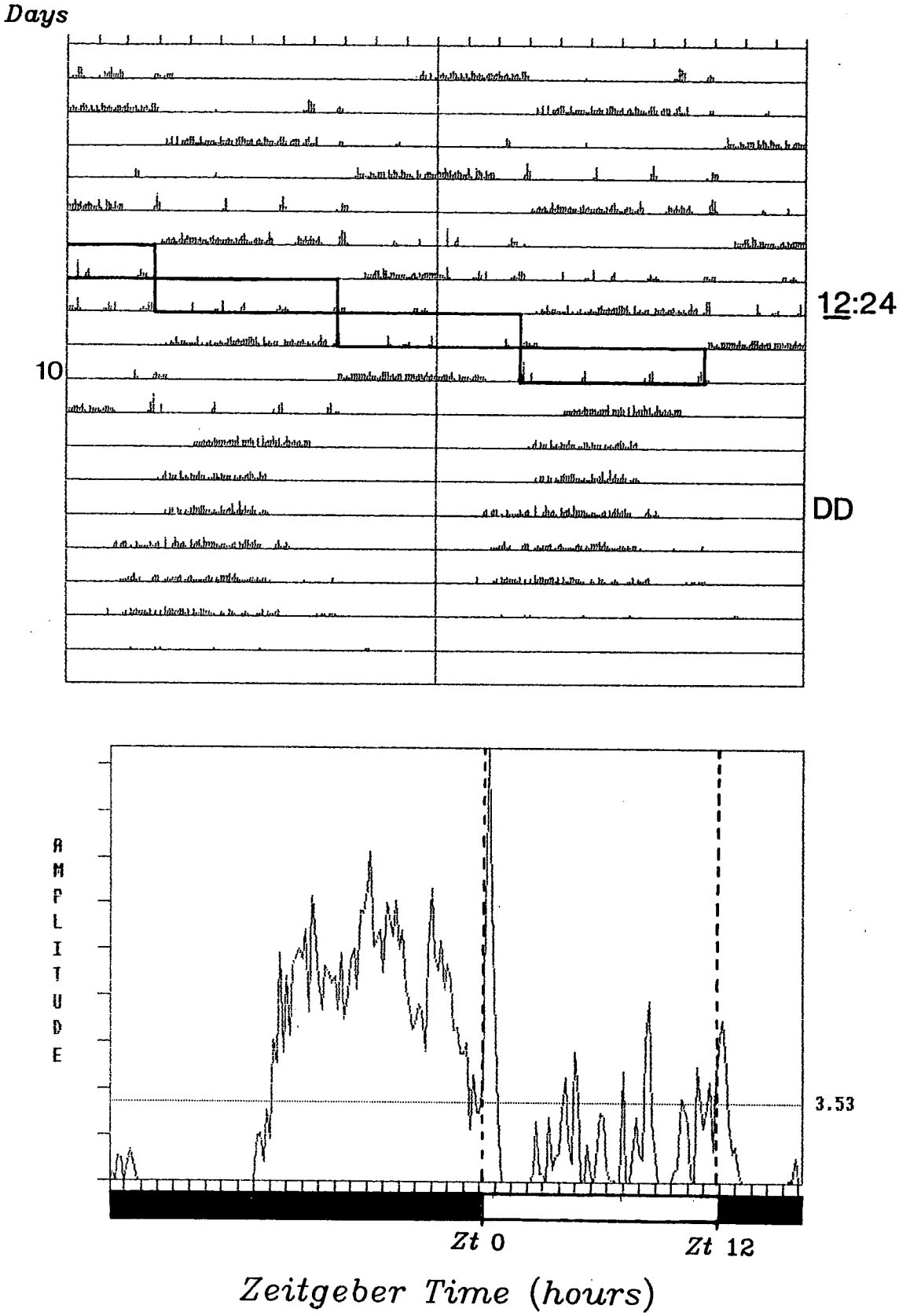

Figure 3.9. The entrained pattern in T36 (12:24). Most of the activity occurred outwith the light phase in these T-cycles.

The results observed in the T36 examples were at first sight rather odd, but could be explained by comparisons with the results of three longer cycles, T48, T60 and T72. Thus, it became evident that once the cycle length was substantially longer than 24 hours the activity rhythm began to free-run between the light pulses. T30 was just close enough to 24 hours to allow entrainment without any large phase changes by the rhythm. However, when T was increased to 36 hours the locomotor oscillator was able to free-run for one cycle before experiencing the light once more. Each time the light pulse hit the oscillator the rhythm underwent an instantaneous 180° phase change and altered the position of the next free-running peak, thus giving the appearance of a rhythm entrained to T36. A comparison with the entrainment patterns produced by T48, T60 and T72 (Figs. 3.10-3.12) supported this viewpoint. In all of these examples the rhythm clearly free-ran for 1 or more cycles before coming into the light again. Once the light was encountered it resulted in a 'resetting' of the free-running rhythm. In cycles which were modulo 24 hours, i.e. T48 and T72, this produced an entrainment pattern which was strikingly similar to that seen in T24; for those cycles which were far removed from T24, i.e. T36 and T60, the pattern was very complex, involving large phase jumps each time the light came on. Indeed the resemblance of the T48 and T72 records to T24 was generally greater than it may appear in Figs. 3.10 and 3.12, as these examples were chosen for their clarity in illustrating the free-running pattern between the light pulses. As a result the periodogram analyses of these cycles invariably indicated the presence of clear 24 hour periodicities.

Thus, one could argue that it is possible for very long (modulo 24 hour) cycles to result in an illusory 'entrainment' not dissimilar to T24, being limited only by the degree of damping which may be present and the period of the free-running rhythm. A heavily damped rhythm would disappear if the time between light pulses became too long, forcing the clock to start up again each time the light came on, thus losing the 'appearance' of entrainment. This was not considered to be a significant factor for the blowfly's rhythms, as all of the evidence for *C. vicina* (see the next sections) indicated that damping was either not present, or was so slight that the fly died before it had occurred to any significant degree. More important, however, was the period of the rhythm. In the blowfly this tended to be close to 24 hours (see subsequent sections) and therefore one might suppose that the cycles which could produce a T24-like pattern would be limited to those which were multiples of this number. This

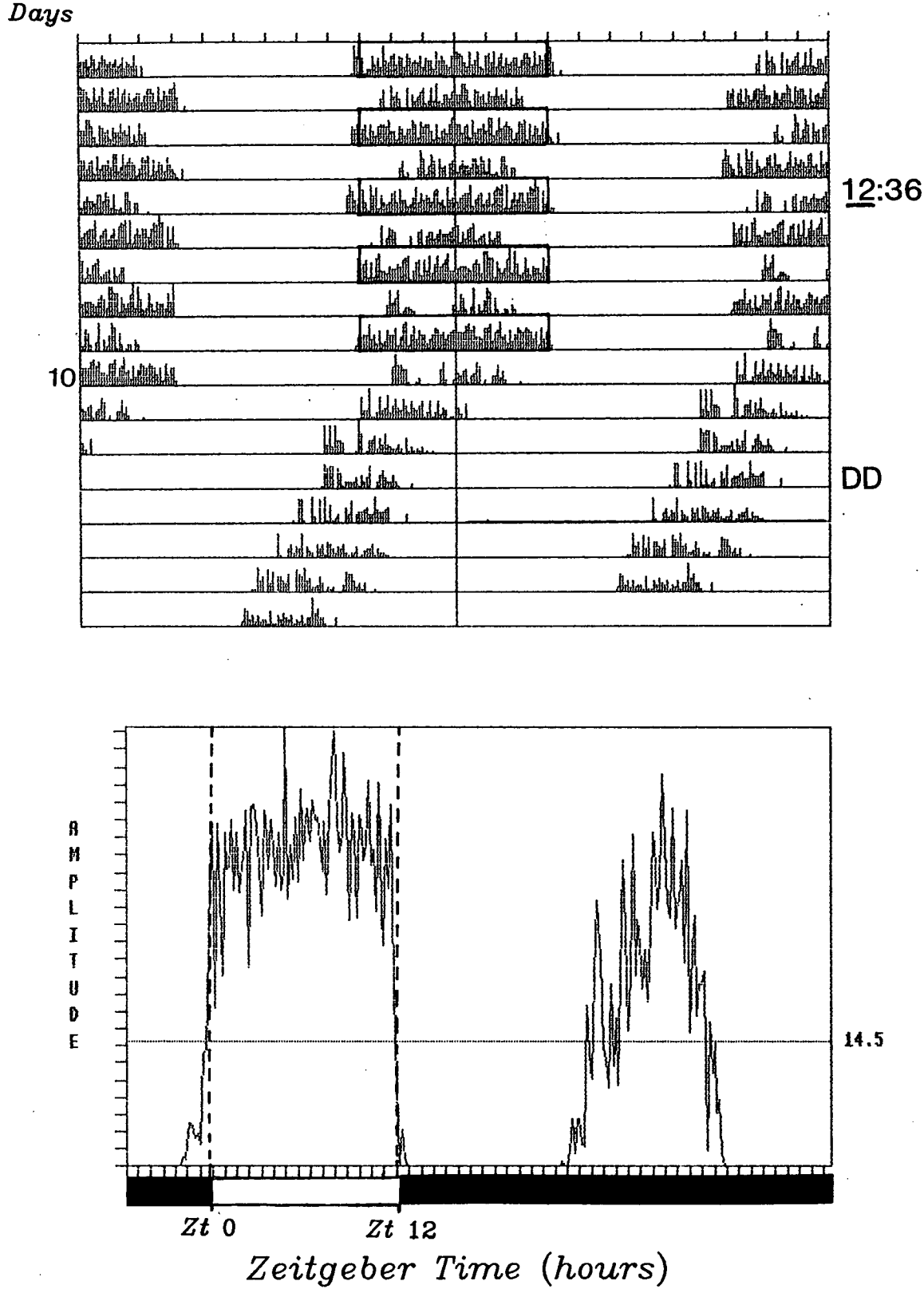

Figure 3.10. The entrained activity pattern in T48 (12:36). As well as large levels of activity during the light phase, one free-running peak was also seen.

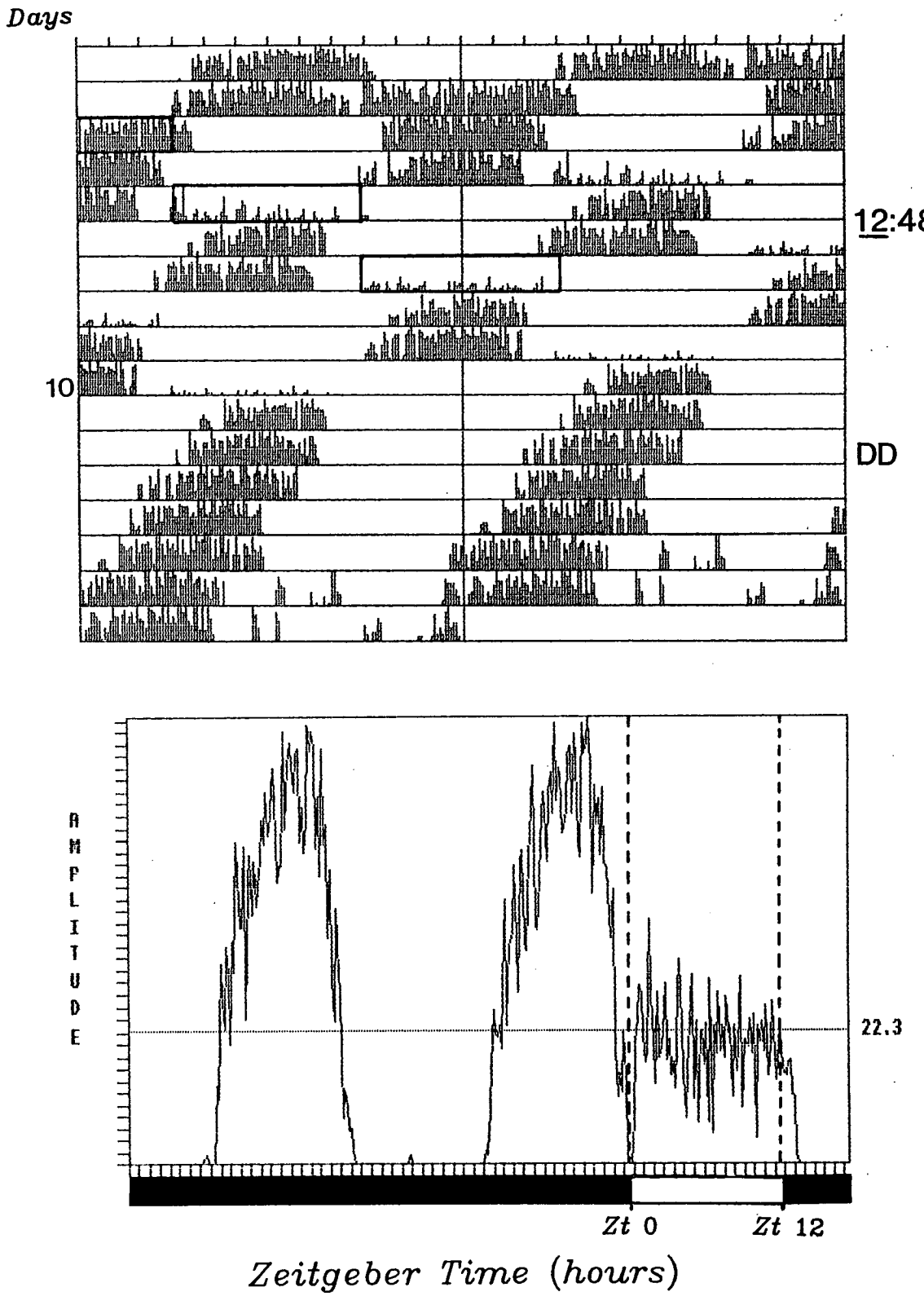

Figure 3.11. The entrained activity pattern in T60 (12:48). In contrast to T48, two free-running peaks of activity were seen in the dark. It is evident that the light cycle is out of phase with the free-running cycle at this T-value. Note also that little activity occurred in the light.

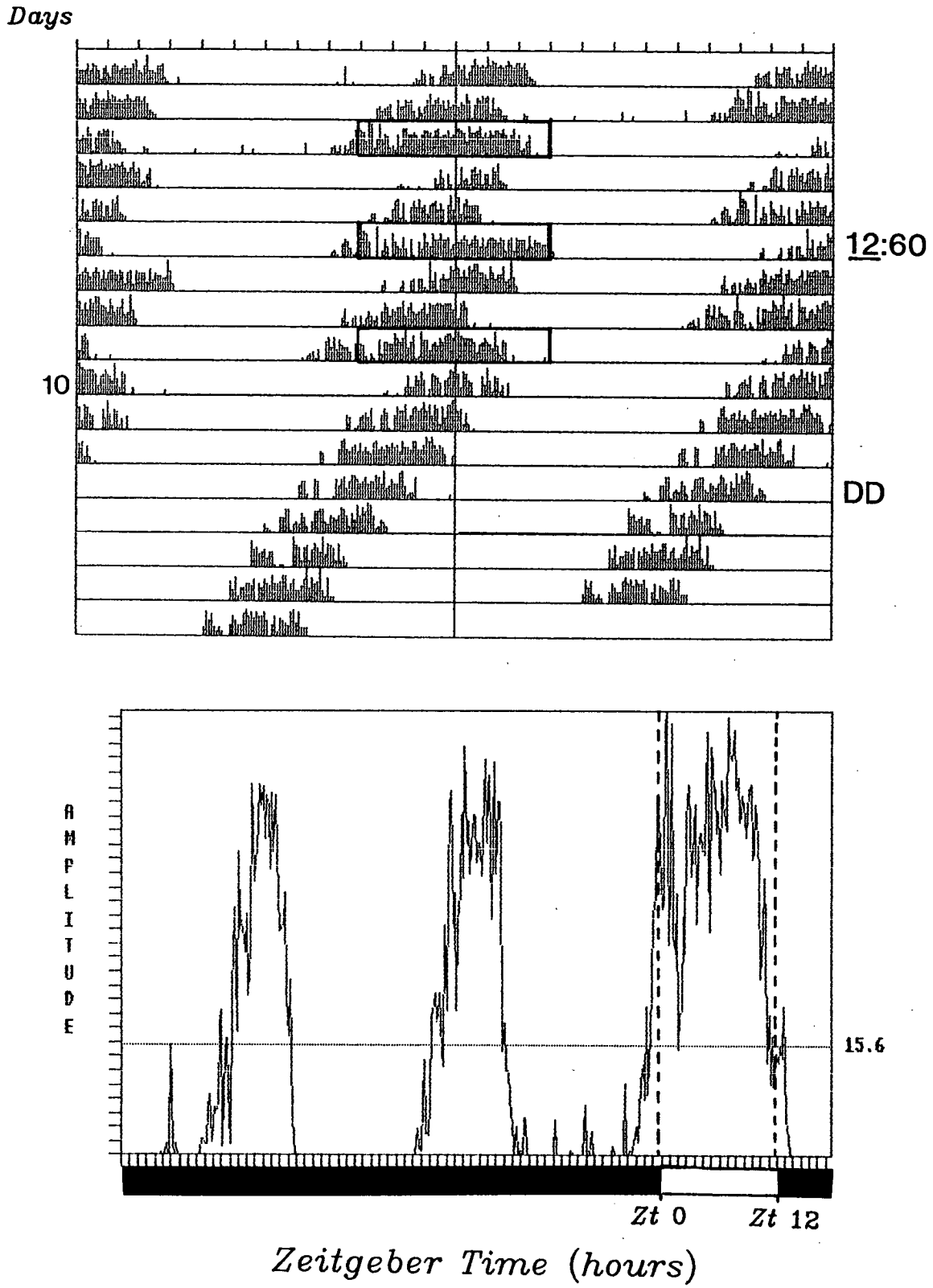

Figure 3.12. The entrained activity pattern in T72 (12:60). The activity profile clearly indicates how the light phase of the entraining cycle closely coincides with the period of the free-running activity rhythm.

supposition was confirmed in that those cycles which did produce a 24 hour-like pattern were 24 hour multiples, i.e. T48 and T72. Obviously, the most effective long entraining cycles would not be 24 hour multiples but rather multiples of the true period value, τ. In many ways these findings can also be related to the examples of locomotor rhythms entraining to cycles which are submultiples of 24 hours, i.e. 6, 8 and 12 hours (Bruce, 1960; Roberts, 1962; Nayer and Sauerman, 1971). This is not, however, particularly surprising, as such 'frequency demultiplication' is known as a basic property of most physical oscillators.

The observed patterns of entrainment were very consistent within each T-cycle, in only 4 cases out of 55 were the observed activities of an unusual nature. These were all at the shorter T-values. Figures 3.13 and 3.14 show rhythmic activity patterns which appeared to be unaffected by the entraining cycles, T20 and T36 respectively, and were in all appearances free-running with an average period of about 24 hours. Figure 3.15 is more interesting because while the rhythm was free-running it was also interacting with the T30 cycle each time the two systems crossed over. This was more clearly seen in the periodogram analysis where two periodicites could be discerned (see Fig. 3.15). It should also be noted that the intensity of the light pulses will affect the ability of the T-cycles to entrain locomotor rhythms. This will usually operate by the lower light intensities resulting in less effective entrainment, thus while the illumination in these experiments was of a generally high level it was not too bright to prevent certain individuals from free-running across the entraining cycle.

Two other parameters of the entrained rhythms were also analysed. These were the duration of the active phase and the amplitude of the rhythm (Table 3.3 and 3.4 respectively). The comparable results for T24 which appear in these tables came from the 12:12 data in the previous section. Although no obvious trends were apparent in terms of activity duration, an analysis of variance was able to reveal significant differences within the data ($F=10.690$ with 6 and 48 d.f.). However, the majority of this variation was due to the results for T20 and T30, for their removal from the analysis eliminated the significance of the results ($F=1.930$ with 4 and 34 d.f.). At first this may seem rather peculiar but the explanation probably lies in the fact that these two cycles alone had very noticeable extra activity associated with the entrainment. In the case of T20 this was after lights-out, and for T30 just before lights-on.

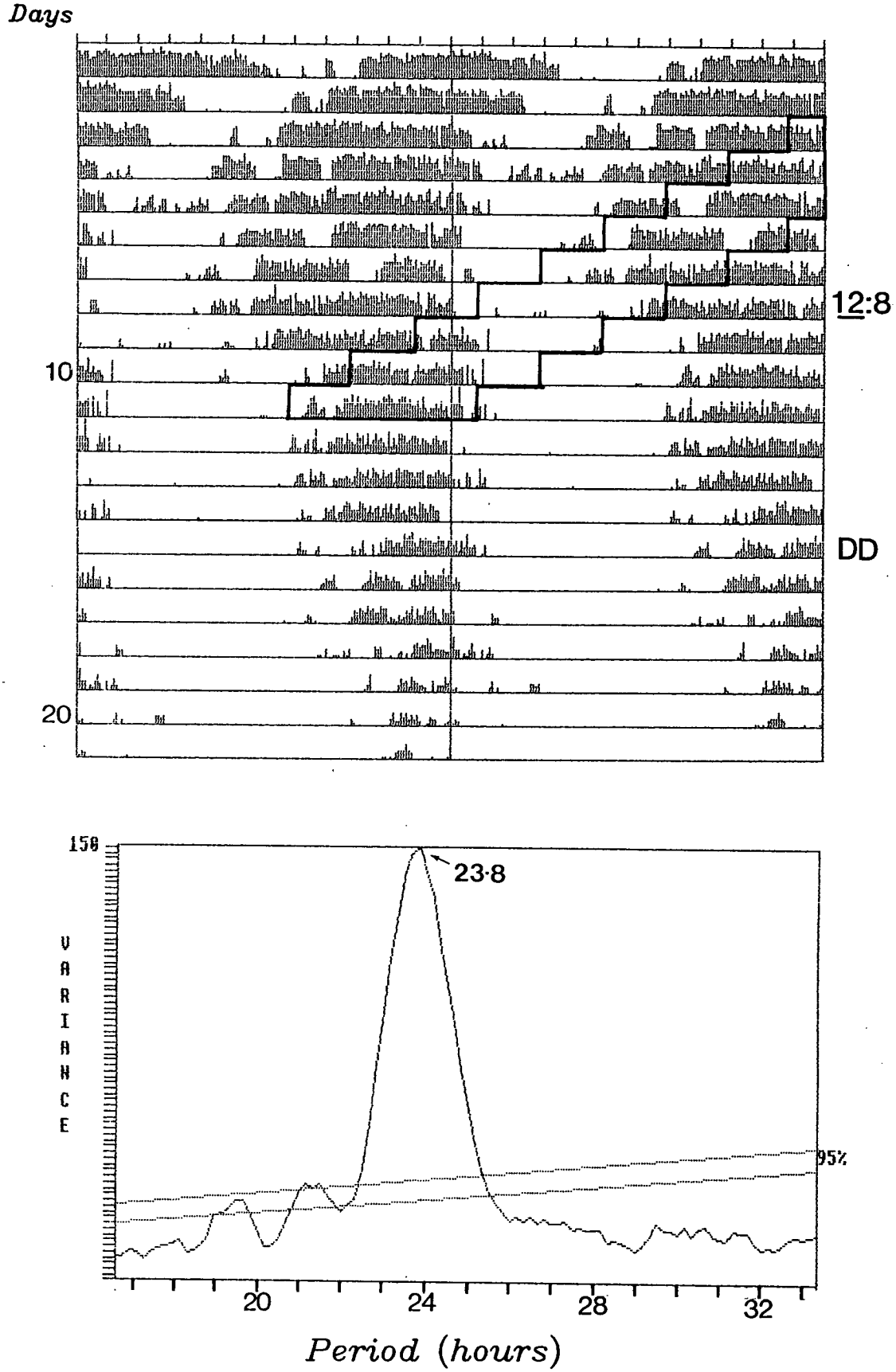

Figure 3.13. Unsuccessful entrainment to T20. The periodogram indicates that the average period during the time of exposure to the light/dark (LD) cycle was about 24 hours.

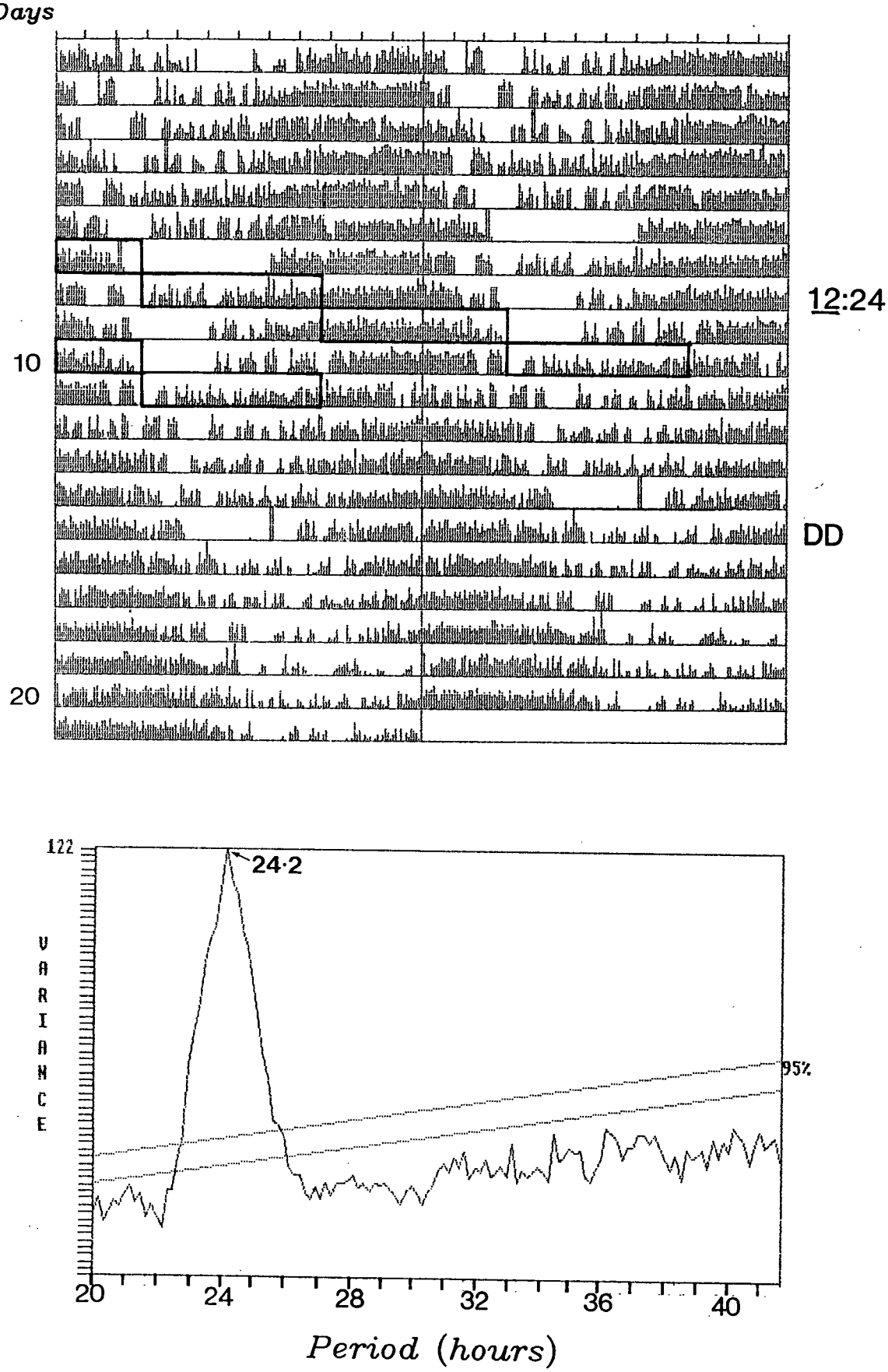

Figure 3.14. A further example of unsuccessful entrainment, this time in T36. At this T-value the periodogram again indicates the existence of a period close to 24 hours.

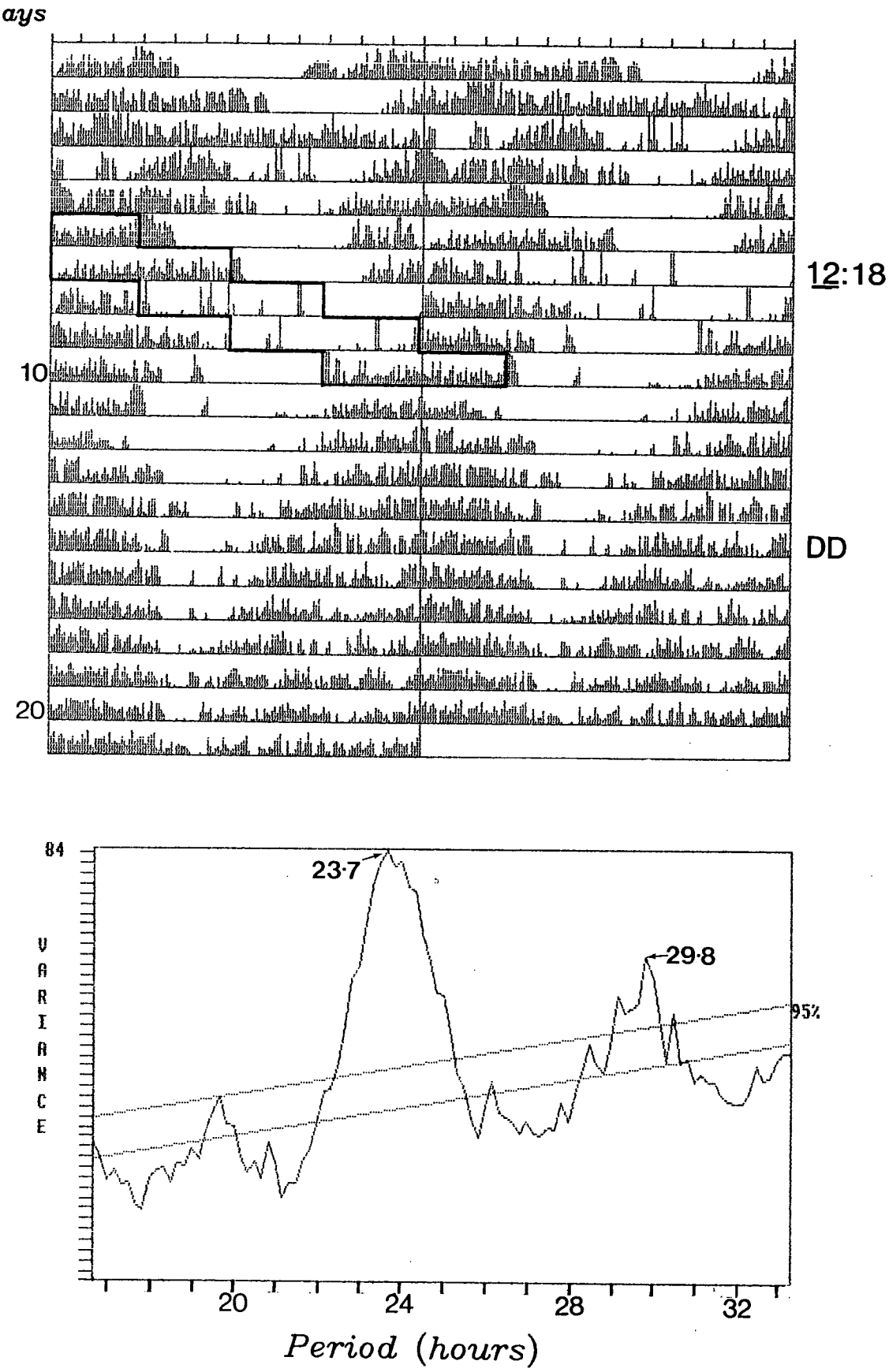

Figure 3.15. An unusual form of entrainment to T30. Although the rhythm appears to be free-running across the LD cycle, the periodogram clearly indicates some entrainment was occurring.

Length of entraining cycle, T (hours)	*Mean duration of activity in each cycle (hours±s.d.)*	*Number of observations*
20	16.48±1.44	7
24	14.57±1.33	5
30	19.17±1.81	9
36	13.83±0.83	9
48	15.17±1.89	7
60	13.53±2.34	8
72	15.43±1.83	10

Table 3.3. Comparisons of the average length of α for seven different T-cycles. All cycles had 12 hour photophases. Note that the T24 result came from Table 3.1.

Length of entraining cycle, T (hours)	*Mean amplitude of activity in one cycle (units±s.d.)*	*Number of observations*
20	15.24±4.71	7
24	14.03±1.28	5
30	10.00±3.79	10
36	10.71±4.55	9
48	16.40±6.14	7
60	22.68±15.09	8
72	11.98±5.88	10

Table 3.4. An analysis of the average amplitude of activity over various T-cycles. The T24 (12:12) result came from Table 3.2.

The amplitudes also proved to be significantly different on an analysis of variance ($F=3.033$ with 6 and 49 d.f.), although there appeared to be no obvious pattern to this. As T60 had an especially high value (and a correspondingly large standard deviation) it was considered possible that much of the variation came from this source. Indeed, when the T60 results were removed from the analysis the variance ratio was reduced to a non-significant value ($F=2.280$ with 5 and 42 d.f.), although a large amount of variation was clearly still present in the data. Therefore it was assumed that while the average amplitudes were generally very variable between the different cycles, the T60 observations may be aberrant data resulting from substantial individual variation. It was also noted that in T48, T60 and T72 (as had been seen in T36) the activity during the light was at a lower level than that seen in the dark. There was, however, no clear explanation of why this should be so.

These results of entrainment to non-24 hour cycles can perhaps be best summarized by recognizing that only a limited range of photoperiods can actually entrain the rhythm in the full sense of its meaning. Within the experimental photoperiods used here only T20 and T30 were within this range. The evidence for this comes in part from the excess activity associated with the fringes of the light phase in these T-cycles. These implied that the rhythm was being constantly 'pulled' into entrainment each time the light came on. As the true free-running period of the blowflies activity was found to be just less than 24 hours in T20 the light pulses must be phase-advancing the oscillator, and thus the observed rhythm, in each cycle. Thus the continual lagging of the activity behind the light pulse would explain the extra activity observed after lights-out. In T30 the opposite was true; the behavioural rhythm phase-advanced the entraining cycle and therefore one observed some activity before the lights come on.

In the longer T-cycles the locomotor rhythms free-ran to varying degrees before encountering the light again, thus the patterns produced were not examples of true entrainment, but rather a mixture of free-running and phase-readjustment. However, those entraining cycles which were multiples (or nearly so) of the period of the free-running rhythm did result in patterns which were indistinguishable from those seen in the entraining cycle which was closest to the natural period of the oscillator, i.e. T24.

Entrainment to 24 hour cycles

1. The blowflies entrained activity pattern was clearly diurnal in all photoperiods with the activity level gradually rising to a peak in the centre of the light phase.
2. Exogenous peaks were frequently imposed on this pattern at the LD transitions.
3. The duration of the active phase of the rhythm increased as the light length increased but this was not considered to reflect anything other than exogenous effects. Amplitude, on the other hand, showed no variation between different entraining photoperiods.
4. Activity in LL may be arrhythmic (see later sections).

Entrainment to non-24 hour cycles

1. Entrainment appeared to be quite limited in its range for light pulses of 12 hours duration and a light intensity of 700 lux. The only experimental photoperiods which were able to result in true entrainment were T20, T24 and T30.
2. In longer T-cycles free-running was seen to occur to varying extents between the light pulses. T-cycles which were multiples of the natural period of the oscillator produced patterns very similar to T24.
3. The duration of the active phase was the same throughout, except in T30 and T20 where the extra activity associated with the entrainment of the rhythm lengthened the average activity.
4. Amplitude of the rhythm was not considered to have varied significantly between the differing T-cycles.

Free-running After Short Entrainment

Having established the pattern of locomotion during entrainment to light/dark cycles, the next step was to investigate the behaviour of the locomotor rhythm when removed from that environment and allowed to free-run. This procedure is a crucial part of any circadian study for it is the most reliable indicator of the endogeneity of any activity rhythm. All of the previous work in this field has shown that this is best achieved by altering the light/dark regime to one of constant dark (DD) or constant light (LL). However, as the intensity of illumination used in the present experiments always resulted in apparently arrhythmic behaviour from the flies in LL, DD was chosen as the most appropriate environment in which to observe the free-run. Initially the flies were entrained for up to 8 days to a variety of 24 hour photoperiods before release into DD. These were 4:20, 8:16, 12:12, 16:8, 20:4 and LL. In all cases the flies free-ran in DD with distinct rhythmic patterns, all of which could be classified into one of three categories. These were:

Clear and constant period:	12.5%	(N=4)
Clear but changing period:	78.1%	(N=25)
Complex pattern :	9.4%	(N=3)

In all but a few examples the locomotor activity displayed very clear rhythmic elements, with most flies showing a change in their free-running period. This usually occurred 6-10 days after the last entraining cycle and was, in all cases, an increase from a period of less than 24 hours to one of 24 hours or greater. This period alteration was also very rapid, taking as little as 3 days for completion (Fig. 3.16). Those records which did not exhibit any period change did not appear to be associated with any particular photoperiod and the period of their activity was always less than 24 hours. The complex patterns were difficult to interpret, with one of them appearing to be arrhythmic and the other two displaying apparent combinations of several oscillating components. This area of locomotor rhythms will be discussed in greater detail in later section of this study.

The duration of the activity band seen in DD was clearly shorter than it had

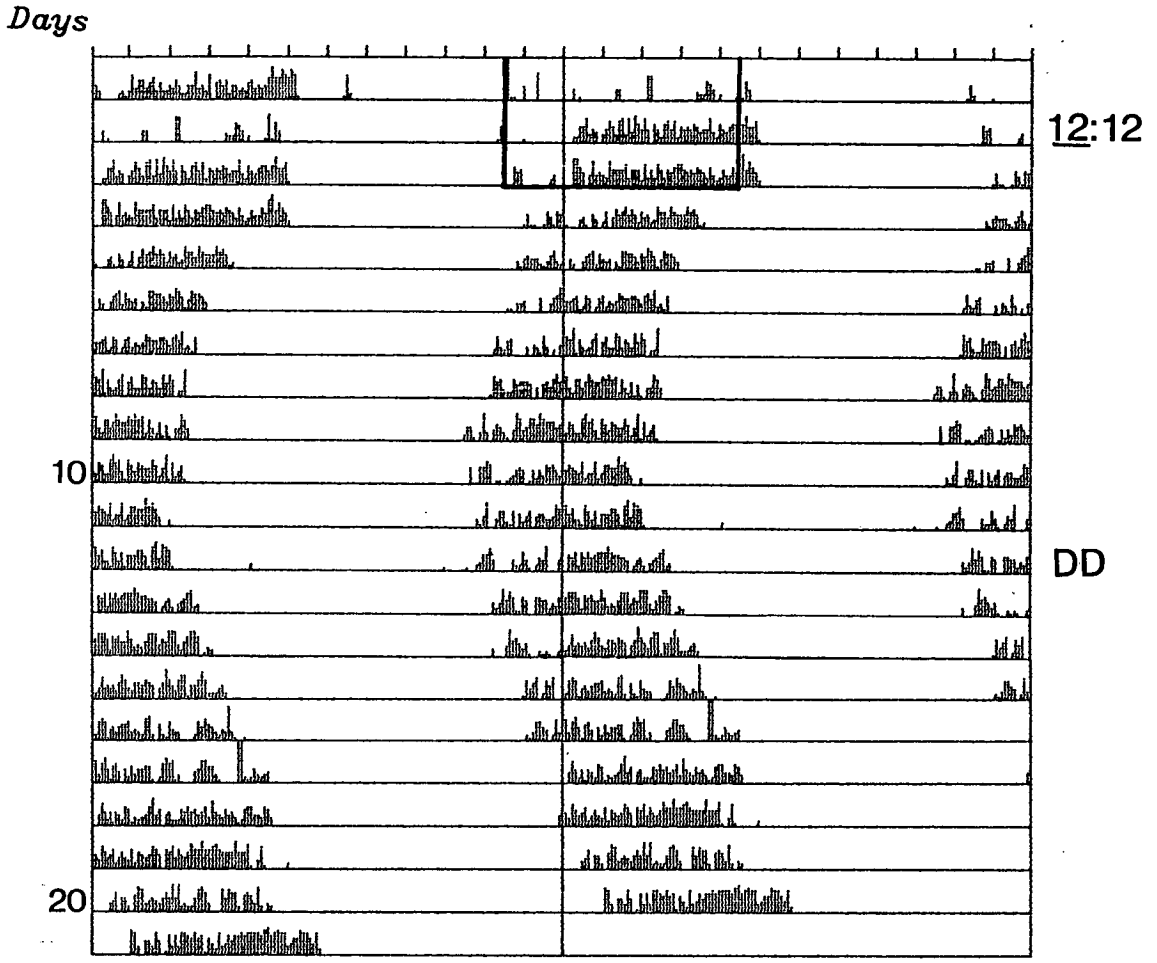

Figure 3.16. An example of the usual form of the *C. vicina* free-running rhythm in constant darkness (DD), in this case from a prior entraining cycle of 12:12.

been in the entrained state (see below) and thus it seemed likely that only part of the locomotion seen during LD cycles had given rise to the free-running pattern. Examination of the records indicated that much of this free-running activity may have derived from components at the lights-on portion of the entrained cycle (see Fig. 3.17), with a great part of the activity associated with the 'dusk' ceasing once the light cycles were stopped. However, this did appear to vary with the prevailing photoperiod, as the 12:12 regime in Fig. 3.16 clearly has the free-running activity arising from the dusk light/dark transition (see also the beginning of the free-runs in Figs. 3.1-3.5). This contradiction may stem from the fact that the initial peak of DD activity occurs roughly 12 hours after the end of the last light phase irrespective of the preceding LD cycle, thus in shorter photoperiods the dawn activity will appear to persist while long photoperiods will produce free-running activity from the dusk peak. However, if the first activity peak does occur at a fixed time after dusk then one must assume that the free-running activity is being phase-set by the dusk transition, and as such may have originated from that part of the entrained pattern.

It was also apparent that the short, intense bursts of activity seen in LD cycles at the point of the transition between the light and dark were not retained in the free-run, implying that these may have been largely exogenous (Fig. 3.17). Other than the single very large period change which was imposed on the system, the timing of the on-sets of activity were fairly consistent throughout the recordings, although the first peak of locomotion in DD occasionally had a slightly delayed start relative to the subsequent on-sets. This consistency in the on-sets of activity was a vital factor in allowing the periodogram to accurately calculate the periods seen in the free-run.

Although it was common for the blowflies to survive in this state for considerable lengths of time (1-2 months, as Fig. 3.18 shows) few individuals were able to sustain a consistently clear rhythm throughout. Any breakdown in the rhythmic patterns was seen as either a disintegration into arrhythmia or an increase in the amplitude of the activity to abnormally high levels. The latter was the least common of the two and usually occurred as the result of the flies consuming all of the available food reserves in their monitoring device. This was possible because the flies were never disturbed once a recording was started, and thus food was not replenished during a run. However, for the majority of the observed runs this was not an influential factor as the amounts of food available almost always outlasted the fly. The long-lasting records

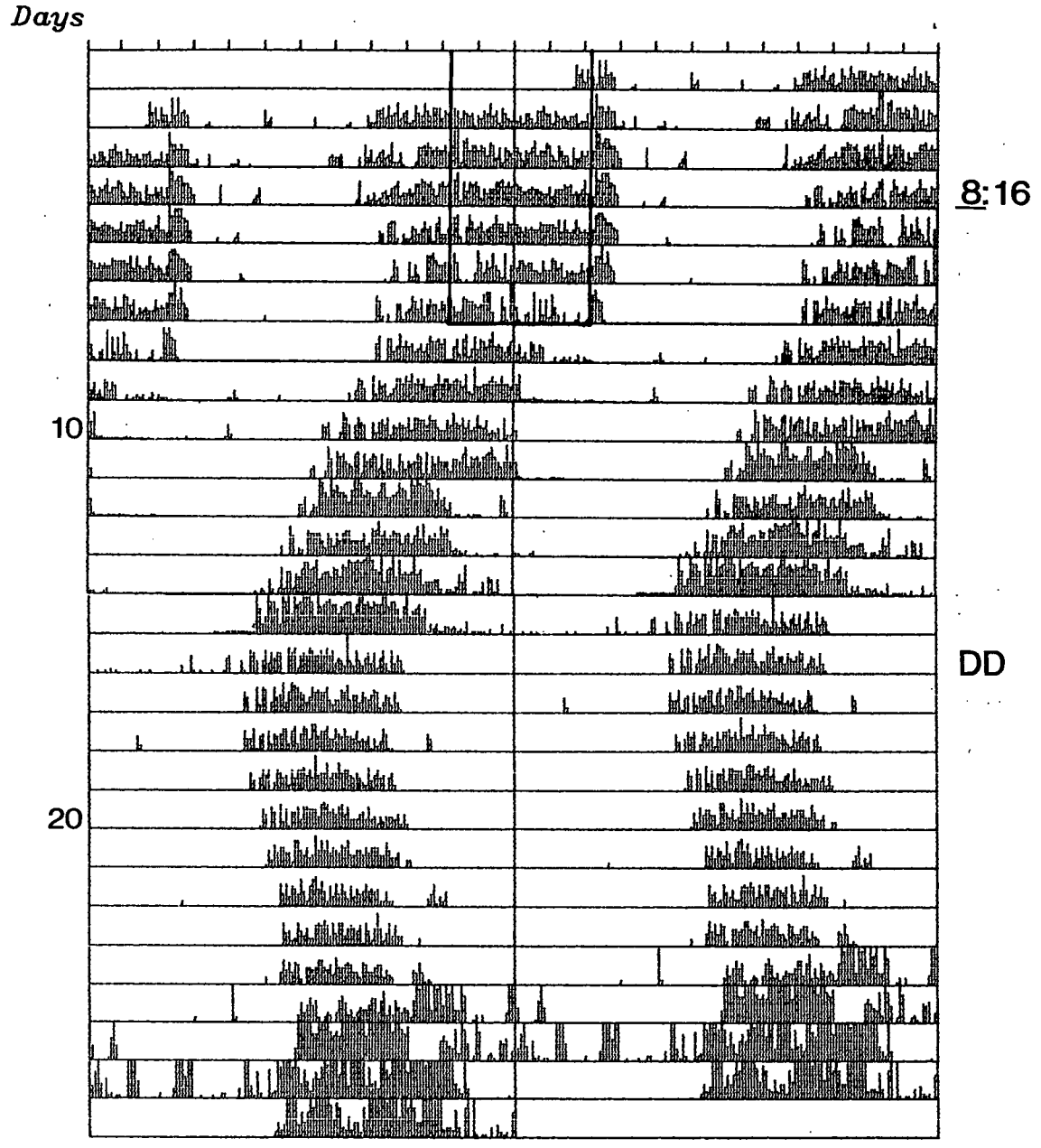

Figure 3.17. A further example of DD free-run. Note the activity seems to arise from the dawn transition of the 8:16 cycle.

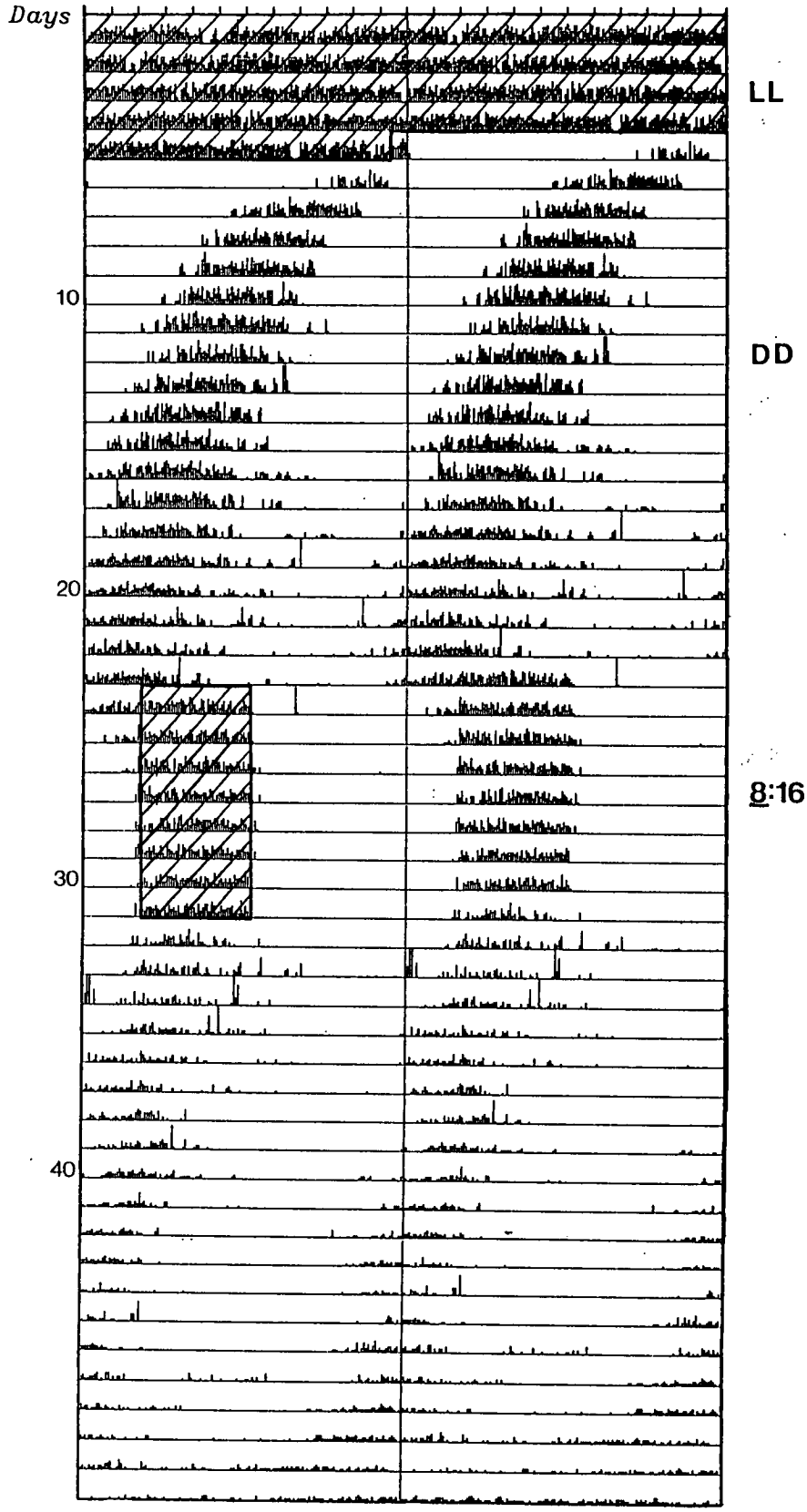

Figure 3.18. Showing a very long free-run in DD. Note that the rhythm can easily re-entrain to new LD cycles, even after extended periods in DD.

which did not succumb to these problems were able to show that free-running rhythms did not lose their ability to re-entrain, even after some time in DD (Fig 3.18). The transients which are normally associated with entrainment can also be clearly seen in this example. After several days in the LD cycle the rhythm was again able to reinitiate a free-run (albeit with reduced activity levels, see Fig. 3.18).

In order to analyse the records which showed a change in period it was deemed necessary to divide the free-running pattern into three segments: the section prior to the change in period, the portion immediately after the change and a further portion taken no less than 7 days after the change. For convenience these were termed 'Stage 1', 'Stage 2' and 'Stage 3' respectively, and are represented diagrammatically in Fig. 3.19. This figure also illustrates the areas of the free-run which are usually termed the active and inactive phases of the activity (α and ρ respectively). The first analyses undertaken attempted to ascertain whether any relationship existed between the preceding light/dark cycle, expressed in terms of the length of the light phase, and the free-running periods in DD (these are summarized in Table 3.5). Superficially there did appear to be some negative relationship between lightlength and the free-running period. An analysis of variance confirmed the existence of some variation in the data for Stage 1 periods ($F=3.954$ with 5 and 19 d.f.) but the full extent of this negative relationship was only revealed by a regression analysis (see Fig. 3.20). The other two stages did not reveal any significant variation between the separate experiments, thus indicating that this relationship did not survive the change in period (2nd phase, $F=1.887$ with 5 and 14 d.f.; 3rd phase, $F=1.076$ with 4 and 9 d.f.).

As it was not technically feasible to use more than 32 individual flies in each of the locomotion experiments it was always necessary to severely limit the sample size for each experimental group, so that the differing photoperiodic regimes could be recorded simultaneously. This was considered to be essential for ensuring that more meaningful comparisons could be made between the various LD cycles. Unfortunately, as can be seen from the Tables 3.5-3.7, this number was frequently contracted even further due to the death of the flies or to a complete breakdown in the rhythmic pattern from the start of the free-run (in itself a frequent precursor to death). The resulting small sample sizes combined with large inter-individual variation to prevent the mean period-values in Table 3.5 indicating (as clearly as each separate record was

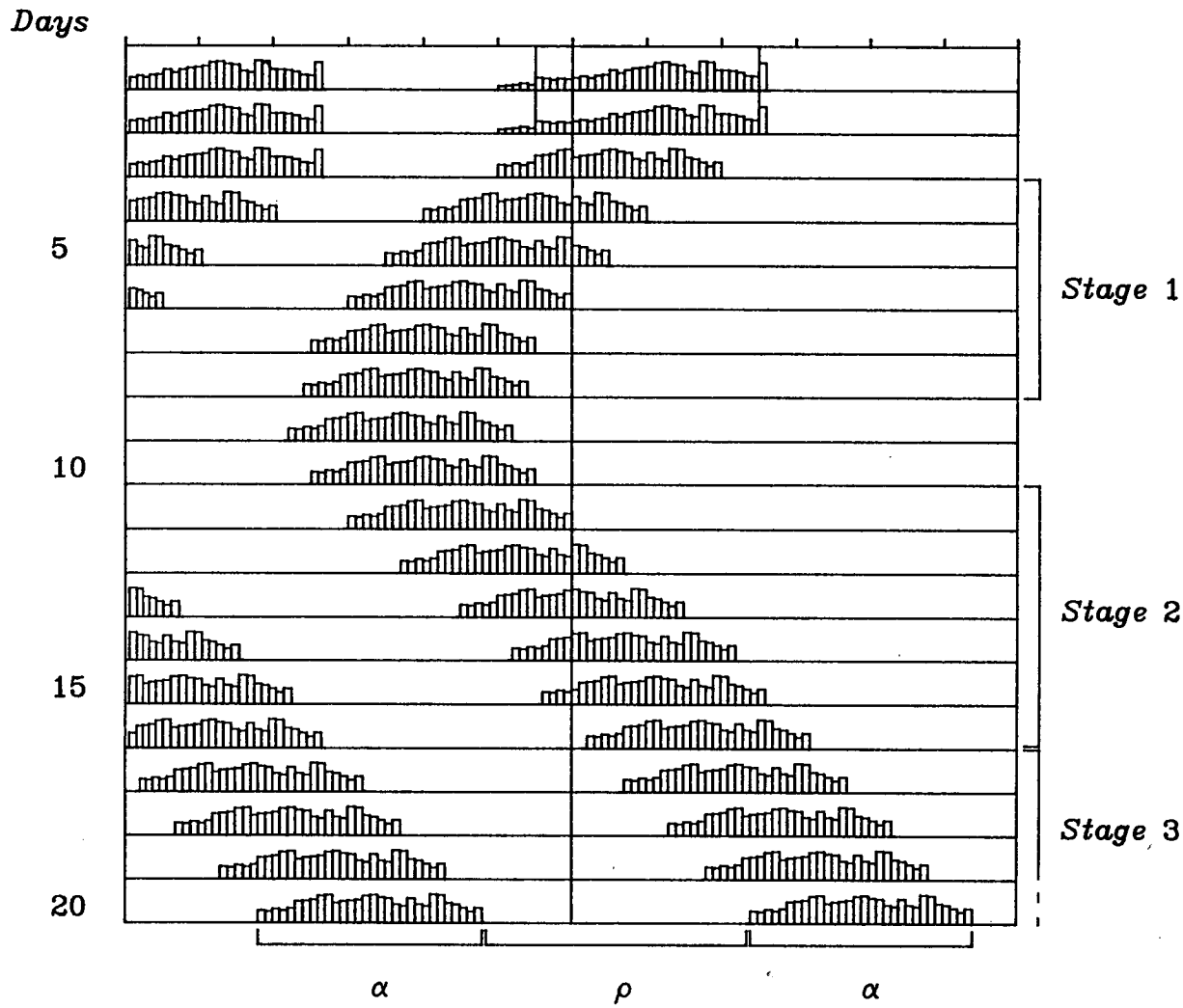

Figure 3.19. A schematic form of the free-run, designed to indicate the areas of the free-running pattern used for comparative analysis. The active and resting phases are also defined (with the terms α and ρ respectively).

Hours of light per 24 hours	*Mean free-running period (hours±s.d.)*						*Variance ratio (d.f.)*
	Stage 1	*N*	*Stage 2*	*N*	*Stage 3*	*N*	
4	24.17±1.03	4	24.33±0.67	3	24.89±0.38	3	0.75 (2+7)
8	23.30±0.98	5	23.93±0.40	5	24.21±0.67	4	1.62 (2+11)
12	23.39±0.67	3	23.67±0.94	2	23.25±0.35	2	0.19 (2+4)
16	22.45±0.43	7	23.27±0.45	5	23.89±1.00	3	7.37 (2+12)
20	23.05±0.75	3	24.42±0.59	2	--------	0	4.51 (1+3)
24	22.05±0.51	3	23.50±0.87	3	23.83±0.23	2	5.79 (2+5)

Table 3.5. The average period for the three free-running stages shown in relation to the length of the photophase in the prior entraining cycle. The right hand column indicates the results of an analysis of variance for these periods at each photoperiod.

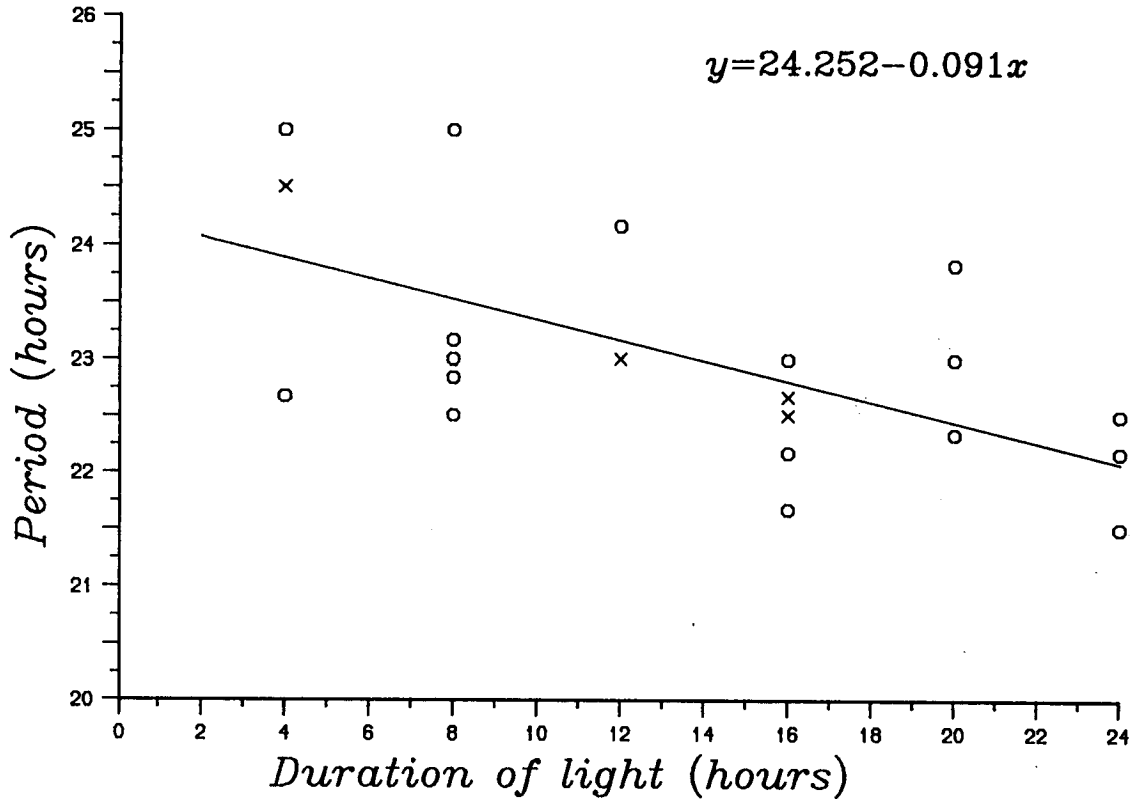

Figure 3.20. A scatter plot of the Stage 1 periods against the length of the photophase in the prior entraining LD cycles. There was a significant negative regression coefficient between the two parameters (t=3.798 with 23 d.f., $p<0.001$), as well as a significant correlation (r=0.621 with 23 d.f., $p<0.001$; goodness-of-fit, F=14.420 with 1 and 23 d.f., $p<0.005$). The circles represent single datum points while the crosses indicate the coincidence of 2 data points.

able to do) the manner in which the period lengthened with time (see the variance ratios in Table 3.5). However, combining the periods for each of the three stages over all of the experiments did indicate that the lengthening of the period was highly significant (F=8.620 with 2 and 56 d.f.).

The overall changes in period were also measured and analysed. No significant difference was found between the photoperiods in terms of the degree to which they lengthened the period, over either Stage 1 to Stage 2 where the largest changes were seen (F=1.326 with 5 and 14 d.f.) or Stage 2 to Stage 3 (F=1.105 with 4 and 19 d.f.). Despite this non-significance a trend in the degree of the change was apparent in the data, with longer light lengths seeming to result in larger changes (see Fig. 3.21). Combining the data for all of the photoperiods gave an average period change of +0.90±0.67 hours between the first two stages and +0.29±0.63 hours over Stage 2 to Stage 3.

As in the previous sections on entrained rhythms, both the duration and amplitude of the observed free-running activity bands were analysed. The results (Tables 3.6 and 3.7 respectively) indicated that activity duration was not affected by the preceding LD cycle (Stage 1, F=0.891 with 5 and 19 d.f.; Stage 2, F=1.330 with 5 and 14 d.f.; Stage 3, F=0.229 with 4 and 9 d.f.). Nor was there any significant change in this parameter over time (see Table 3.6 for the variance ratios). Similarly the amplitudes of the active phase did not vary between experiments (Stage 1, F=0.543 with 5 and 19 d.f.; Stage 2, F=1.690 with 5 and 14 d.f.; Stage 3, F=0.634 with 4 and 9 d.f.). Despite the fact that no significant trend was found in the amplitude of the activity over time, the data suggested that there was a slight decrease in this parameter as the fly aged. Unfortunately the effect was too slight and the sample sizes too small for this to be detected statistically. A further comparison of the average duration of activity when entrained (16.51±2.30 hours) with that seen in the first stage of the free-run (14.01±2.07 hours) revealed that the free-running pattern had significantly shorter active phases (t=4.138 with 49 d.f.). A similar comparison, this time between activity levels in Stage 1 of the free-run and those in the entrained state found no significant differences between the two situations (t =1.388 with 55 d.f.).

In conclusion therefore, the activity of the blowfly when released from 24 hour cycles into DD was characterized by a rhythm which seemed to emanate from the 'dawn' portion of the prior entrained pattern and free-ran with an

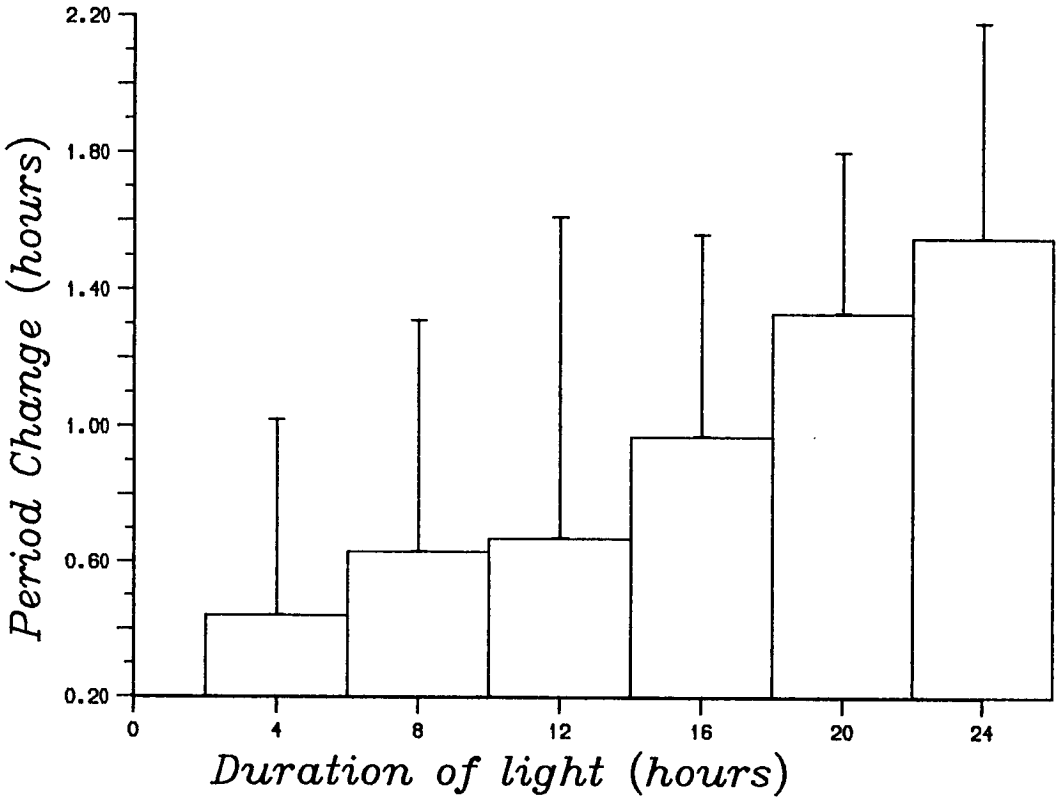

Figure 3.21. A histogram representation of the average change in period between Stage 1 and Stage 2 in relation to the duration of the photophase in the prior entraining cycle. The error bars show the standard deviations above the mean.

Hours of light per 24 hours	*Mean duration of activity per cycle (hours±s.d.)*						*Variance ratio (d.f.)*
	Stage 1	*N*	*Stage 2*	*N*	*Stage 3*	*N*	
4	13.67±1.67	4	14.67±0.60	3	13.05±3.43	3	0.40 (2+7)
8	13.57±2.28	5	15.40±3.24	5	13.50±3.55	4	0.61 (2+11)
12	14.22±1.33	3	11.08±2.47	2	13.75±2.24	2	1.73 (2+4)
16	14.43±1.62	7	14.57±0.93	5	13.94±3.56	3	0.10 (2+12)
20	12.33±3.44	3	13.50±1.41	2	--------	0	0.19 (1+3)
24	15.67±2.09	3	13.83±1.92	3	15.75±1.77	2	0.85 (2+5)

Table 3.6. As the previous table (Table 3.5), but representing the average length of the active phase for each free-running stage.

Hours of light per 24 hours	*Mean amplitude of activity per cycle (units±s.d.)*						*Variance ratio (d.f.)*
	Stage 1	*N*	*Stage 2*	*N*	*Stage 3*	*N*	
4	9.34±3.72	4	8.68±3.77	3	5.84±3.55	3	0.83 (2+7)
8	12.36±4.85	5	14.62±6.16	5	9.13±1.58	4	1.46 (2+11)
12	10.20±4.67	3	10.63±6.74	2	12.37±12.30	2	0.05 (2+4)
16	13.27±7.03	7	8.77±3.10	5	6.88±4.33	3	1.74 (2+12)
20	8.50±4.90	3	4.44±2.40	2	--------	0	1.10 (1+3)
24	12.30±3.32	3	13.82±6.57	3	9.48±0.58	2	0.52 (2+5)

Table 3.7. This table covers the same free-running patterns as were analysed in Table 3.5 and 3.6, but indicates the average activity level for each stage.

initial period of less than 24 hours which lengthened to 24 hours or greater. Although this initial period showed a significant negative correlation with the length of the light in the prior entraining cycle, the later free-running periods did not display such a relationship. Before and during this change of period, and for the subsequent recorded activity, no significant change was observed in the duration or the amplitude of the active phase.

These results immediately raised several questions, most notably whether this change in period was a spontaneous occurrence or whether it was the direct result of entrainment, i.e. was it an after-effect. It was of interest to note that in some of the recordings from long-lived individuals, as well as the initial lengthening of τ, there could also be a subsequent shortening of the period, although this could be as much as 25-30 days after the last light pulse (Fig. 3.22). As the following sections will indicate, the natural period of the unentrained rhythms was most probably less than 24 hours, therefore it may be concluded that this late shortening of the period probably represented the circadian system finally recovering from the last effects of entrainment. Thus, the long periods seen after the first change in τ must in themselves have been after-effects. Photoperiodic cycles, therefore, seem to be able to influence the free-running rhythms both in terms of their initial period and in the degree to which these subsequently lengthen (according to the number of hours of light in the entraining cycle). These effects were also seen to be long-lasting, the system frequently taking as long as one month after the end of the light/dark cycle to begin to recover.

In order to resolve the problem of the origin of the period lengthening it was necessary to conduct two further experiments. The first of these involved maintaining the flies in an entrained state for a greater length of time before allowing them to free-run. Thus, if the changes in period were truly after-effects one would expect results comparable to those seen for the early release from entrainment, i.e. a lengthening of the free-running period after 6-10 days. The second alternative was to free-run individuals which had not experienced light at any time during their lives and whose clocks would, therefore, not have come under the influence of any type of entrainment or purturbation due to light.

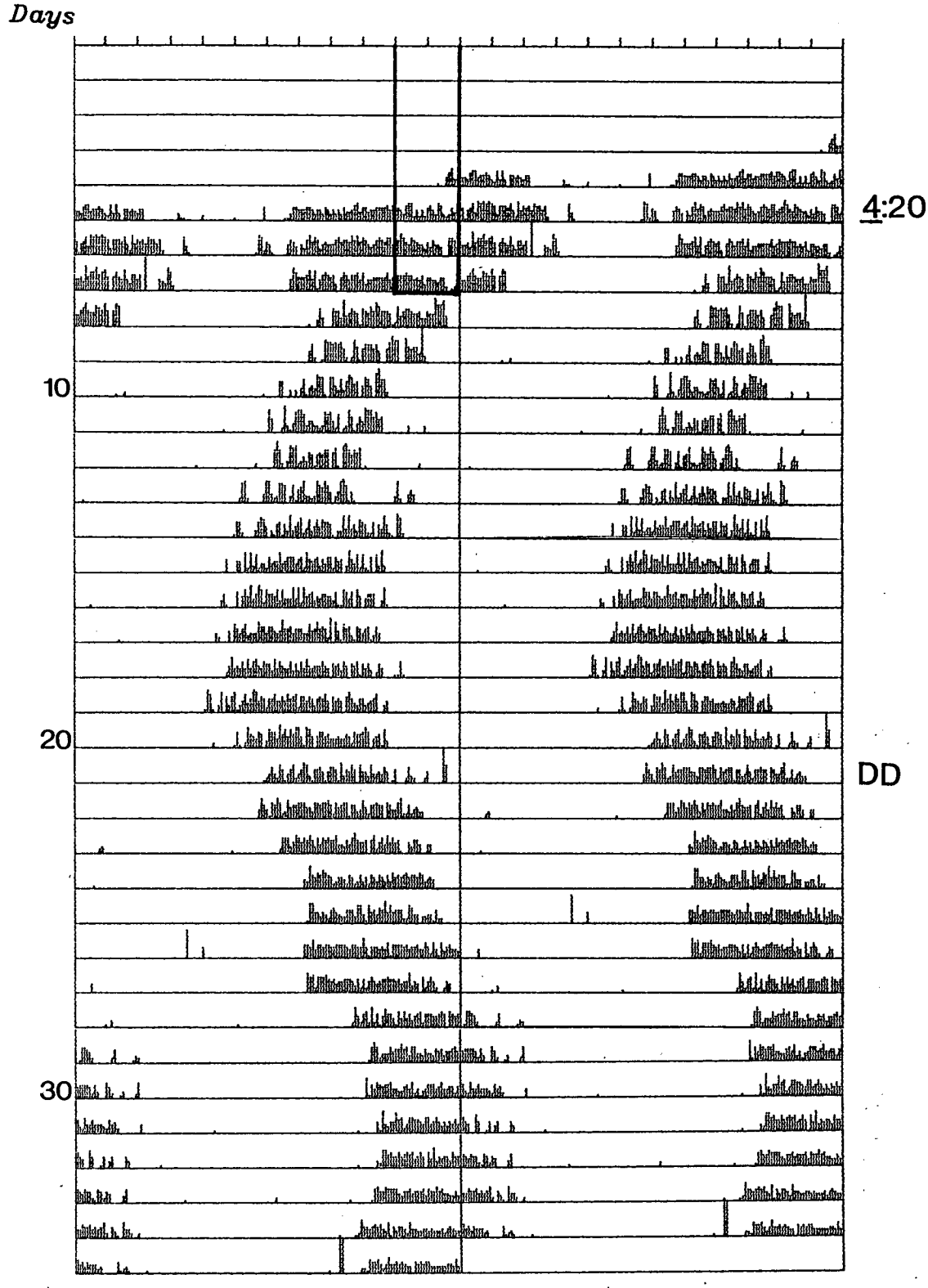

Figure 3.22. An example of a DD free-run in which the period of the rhythm appears to begin to shorten after an extended time in DD.

Free-running After Long Entrainment

For the first of these approaches a 12:12 LD cycle was chosen as the appropriate entraining cycle, with the flies being kept in this for 18-22 days before release into DD. The observed patterns in DD were of the same type as had been seen for the short-entrained rhythms, i.e.:

Clear and constant period:	21.43%	(N=3)
Clear-but-changing period:	71.43%	(N=10)
Complex pattern :	7.14%	(N=1)

Equally the percentage distribution of the activity types was not radically different from those earlier experiments, with the majority of the recordings showing a distinct lengthening in their free-running period. The results are presented in greater detail in Table 3.8 (a), (b) and (c) for the free-running period, the duration of the active phase and the amount of activity respectively. In contrast with the previous experiments an analysis of variance was able to show a significant lengthening in period over the three stages of the rhythm ($F=11.460$ with 2 and 20 d.f.). As one would expect most of the variation was between Stage 1 and 2 (t-test between Stage 1 and 2, $t=4.769$ with 17 d.f. is significant at 1%; t-test for Stage 2 to 3, $t=1.596$ with 6 d.f., not significant at 5%). The degree of the period change was found to be, on average, $+1.33\pm0.26$ hours. This proved to be significantly longer than the change seen after a short period of entrainment ($t=2.523$ with 23 d.f.) suggesting that the degree of the after-effect may be influenced by the length of time spent in entrainment. The duration of activity failed to show any significant change with time ($F=0.870$ with 2 and 20 d.f.) which was in accordance with the results obtained from the earlier short-entrainment experiments. Similarly the present experiments indicated a small drop in the amplitude of the active phase of the free-running rhythms over time but, as Table 3.8 indicates, this proved to be non-significant when subjected to an analysis of variance. The more sensitive t-test did however reveal that there was a substantial decline in amplitude between Stages 1 and 2 of the recordings ($t=3.368$ with 10 d.f.).

Further comparative analyses of the period, amplitude and duration of the activity were also made between the data from the short prior entrainment and the present long entrainment results. When the relationship between the Stage

(a)

Free-running pattern	*Mean free-running period (hours±s.d.)*						*Variance ratio (d.f.)*
	Stage 1	*N*	*Stage 2*	*N*	*Stage 3*	*N*	
Constant τ	24.72±0.48	3	--------		--------		--------
Changing τ	22.77±0.60	10	24.07±0.61	10	23.44±0.58	3	11.46 (2+20)

(b)

Free-running pattern	*Mean duration of activity per cycle (hours±s.d.)*						*Variance ratio (d.f.)*
	Stage 1	*N*	*Stage 2*	*N*	*Stage 3*	*N*	
Constant τ	13.83±2.96	3	--------		--------		--------
Changing τ	13.05±2.66	10	12.75±1.78	10	14.94±6.77	3	0.87 (2+20)

(c)

Free-running pattern	*Mean amplitude of activity per cycle (units±s.d.)*						*Variance ratio (d.f.)*
	Stage 1	*N*	*Stage 2*	*N*	*Stage 3*	*N*	
Constant τ	10.26±2.29	3	--------		--------		--------
Changing τ	7.35±2.77	10	6.65±2.81	10	3.27±0.80	3	2.75 (2+20)

Table 3.8. These tables represent, (a) the free-running periods τ, (b) the duration of α, and activity levels (c) observed after long-term entrainment. The tables also include the results of those individuals which showed no change to τ.

1 periods was evaluated (only the data from the 12:12 regime of short-entrainment being used) no significant difference was found (t=1.436 with 5 d.f.). For the analyses involving the length of active phase, Stage 1 data were averaged over all of the short-entrained results, as it had already been shown that this parameter was uninfluenced by these photoperiods. The conclusion from this was that no significant difference was present between long and short-entrained data over the initial stage of the free-run (t=1.020 with 20 d.f., for all photoperiods). When the amplitudes of the active phases were compared, however, the long-entrained individuals were seen to display markedly lower levels of activity than those which had only experienced a short time in entrainment (t=3.013 with 32 d.f.).

Although the short lifespan characteristic of many flies will necessarily adversely affect the degree of significance of any statistical test applied to the data, by reducing the sample size over time, some trends were supported by analysis. Among these was the fact that the changes in period associated with release from short entrainment were also seen after a far longer entraining period, leading one to conclude that such alterations in τ were most likely to be after-effects rather than the spontaneous behaviour of the oscillating system controlling activity. As well as having a marked increase in period these long-entrained rhythms shared other features with the short-entrained flies, having no change in the length of the active phase over the time of the free-run and only a slight concomitant decrease in amplitude (although this became more marked towards the end of each recording). The few constant period records proved to have a significantly greater τ than those seen in the majority of the observed runs (t=5.799 with 8 d.f.), although there was no associated difference in the duration or the amplitude of the activity (t=0.653 with 10 d.f. for duration, t=1.833 with 8 d.f. for amplitude). This implied that those individuals which had a constant period may have undergone an instantaneous lengthening of τ when released into DD. Alternatively, the period of the oscillator may become slightly longer as the flies age.

The only discernible variation found when direct comparisons were made between long and short lengths of entrainment was in the degree of the period lengthening and the overall amplitude of Stage 1 of the free-running rhythm; the former being smaller after a short time of entrainment and the latter being lower after lengthy experience of an LD cycle.

Free-running Without Prior Light Exposure

The second experiment which was undertaken to determine whether the lengthening of τ was an after-effect involved free-running individuals which had never been exposed to light during their lifetime. All of the blowflies which were used were cultured and handled in red light for at least 4 generations before their activity was recorded in DD. Although there is no direct evidence that *C. vicina* cannot see light of this wavelength, another Dipteran, *Sarcophaga argyrostoma*, was found to be insensitive to red light (Saunders, 1982a). Furthermore, there was no indication from the present experimental results that *C. vicina* adults were in any way influenced by red light. The experiment was run twice with only a minor variation in procedure. In the first run (Experiment 'A') the flies were placed in the recording device (under red light) as newly emerged adults and in the second (Experiment 'B') they were placed in the recorder as fully developed (intra-puparial) pharate adults. Thus in the second protocol, because the flies emerged in the recorder, their locomotion was disturbed as little as possible and it was ensured that the adults experienced nothing but constant darkness.

The results obtained were unequivocal. Although the locomotor patterns were in all aspects quite normal, with clear bouts of activity separated by periods of inactivity, in neither variant of the experiment was there any significant increase in period over time (Table 3.9a). Nor was there any difference between the two protocols in terms of the periods seen over any specific portions of the free-run (t=0.452 with 43 d.f. for the first 14 days, t =0.071 with 38 d.f. for the next 14 days). A typical example of the output obtained from these experimental regimes is shown in Fig. 3.23. While the results for the two protocols appeared to be very similar some interesting features did emerge from the data. For example, when the duration of the active phase was analysed over time in each of the two experiments, although it declined in both, only in experiment 'B' was this change significant. Equally, only experiment 'B' was able to show any significant decrease in the amplitude of the locomotion (see Table 3.9). However, comparisons made between the two experiments over selected parts of the recordings were unable to indicate the presence of any significant variation, i.e. over the first 14 days of the free-run a comparison of the duration of activity gave a t-value of 0.808 (with 41 d.f.), while one of the amplitudes resulted in t=1.458 (with 38 d.f.).

(a)

Experimental protocol		*Mean free-running period (hours±s.d.)*	*N*	*Two-tailed t-test (d.f.)*
'A'	*Day* 1–14	23.07±0.66	25	1.104 (43)
	Day 15–28	23.27±0.55	21	
'B'	*Day* 1–14	22.98±0.67	21	1.493 (36)
	Day 15–28	23.26±0.49	20	

(b)

Experimental protocol		*Mean duration of activity per cycle (hours±s.d.)*	*N*	*Two-tailed t-test (d.f.)*
'A'	*Day* 1–14	15.11±2.95	25	1.727 (40)
	Day 15–28	13.43±3.54	21	
'B'	*Day* 1–14	15.88±3.46	21	2.916 (36)
	Day 15–28	13.09±2.63	20	

(c)

Experimental protocol		*Mean amplitude of activity per cycle (units±s.d.)*	*N*	*Two-tailed t-test (d.f.)*
'A'	*Day* 1–14	13.53±7.72	25	0.892 (40)
	Day 15–28	11.82±5.15	21	
'B'	*Day* 1–14	17.52±10.36	21	2.212 (30)
	Day 15–28	11.81±5.59	20	

Table 3.9. A comparison of τ (a), length of α (b), and the activity levels (c) between two experimental protocols. Both regimes involved free-running flies without any prior exposure to light. In the first, 'A', the flies were placed in the recorder as adults, while in 'B' pupae were allowed to eclose in the recording devices. Note that the recordings were compared over days 1-14 and 15-28 of the free-run, rather than over the usual three free-running stages. This was necessary because of the stability of τ in these records (see Fig. 3.23).

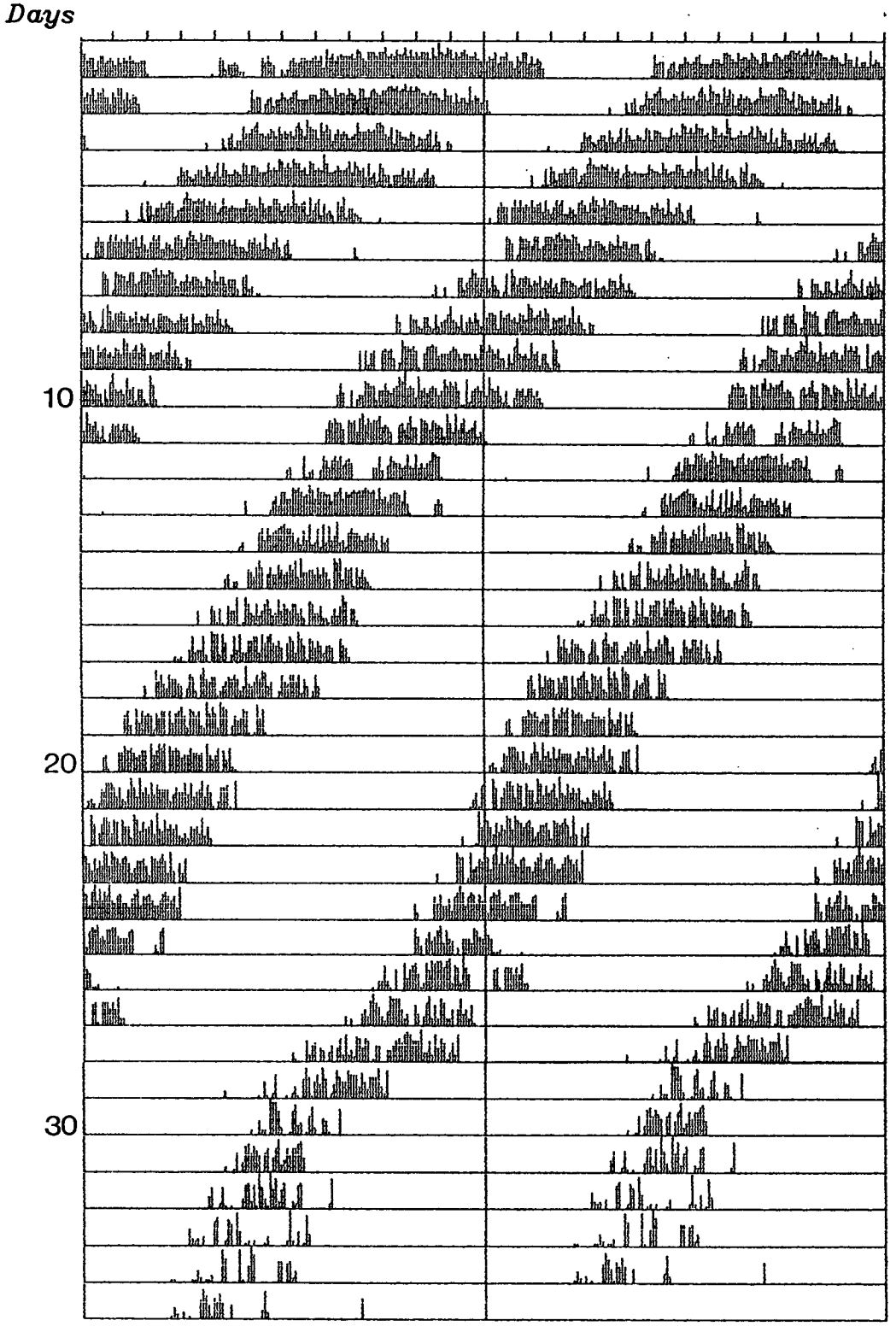

Figure 3.23. The adult free-running locomotor pattern produced by individuals which had not been exposed to light for several generations.

These results were important in several respects. Firstly they strongly suggested that the large period changes seen following entrainment were after-effects, and thus were consistent with the conclusions from the previous experiments. Despite this there was some indication of a slight lengthening of the period as the records progressed, implying perhaps that aging of the flies may result in small changes in τ, although this was too small to be significant (day 1-14 combined, τ=23.03±0.66 hours; day 15-28 combined τ=23.26±0.51 hours: t=1.835 with 82 d.f.). It was therefore possible to calculate an overall free-running period from the combined data of 23.14±0.60 hours. As the disturbances experienced by the activity rhythms described here were kept to a minimum it is probably reasonable to assume that this period was a good estimate of the natural period of the circadian pacemaker involved. Also the fact that the flies were able to produce such clear rhythms, even after having been bred without light for several generations, was evidence that, developmentally, light (or rather the transition between light and dark) was not necessary for setting the oscillator into motion. This in itself was an important observation and hence will be discussed in greater detail in a subsequent chapter.

Free-running From Non-24 Hour Cycles

The cycles in question were the same as those used for the entrainment study discussed previously, namely T20, T30, T36, T48, T60 and T72. In all cases they were made up of 12 hours of light coupled with variable amounts of darkness up to the required cycle length. These protocols were specifically chosen as obvious extensions to the previous experiments on the free-running patterns seen after T24 cycles, and it was hoped that their use might provide further insights into the way in which entrainment can influence free-running rhythms.

When the blowflies were entrained for 10 days before release into DD, the results were found to be indistinguishable from those described in the above sections, i.e. there was clear rhythmic behaviour which displayed a marked lengthening in period after roughly 7 days in DD (Table 3.10). As can be seen, only Stage 1 and 2 of the unentrained rhythm were considered for analysis. Some examples of the beginnings of these free-runs can be seen in Fig. 3.7-3.12. From these it can be seen that the first activity peaks occur 12 hours after the last light phase; this is the same as the situation found in the

Length of prior T–cycle (hours)	*Mean free–running period (hours±s.d.)*						*Two–tailed t–test (d.f.)*
	Stage 1	*N*	*Stage* 2	*N*	*Stage* 3	*N*	
20	23.08±0.61	6	24.00±0.59	6	--------	0	2.672 (10)
30	22.96±0.83	8	24.35±0.92	8	--------	0	3.172 (13)
36	23.19±0.83	8	24.52±0.86	8	--------	0	3.156 (13)
48	23.30±1.29	6	24.08±1.35	6	--------	0	1.021 (9)
60	22.36±0.69	6	23.05±0.95	6	--------	0	1.449 (9)
72	22.59±0.58	9	23.78±0.89	9	--------	0	3.341 (13)

Table 3.10. The periods obtained in the first two free-running stages after a variety of T-cycles. As only two results were obtained at each T-value the right hand column contains the results of a two-tailed *t*-test, rather than an analysis of variance.

free-run from 24 hour LD cycles.

Within each regime, only flies exposed to T48 and T60 failed to show a significant lengthening of their period in DD (see *t*-values in Table 3.10). However, a comparison of the combined data, over all of the T-cycles, for Stages 1 and 2 did show that a very large period change had occurred (t=5.515 with 83 d.f.). Furthermore no significant variation was found between the initial free-running periods seen after each T-cycle. This was true of both Stage 1 (F=1.326 with 5 and 37 d.f.) and Stage 2 (F=2.029 with 5 and 37 d.f.). Equally, the alteration in period induced by the prior entraining regimes was not dependent on the prior T-cycle (F=1.131 with 5 and 37 d.f.). Thus it was possible to state that the overall period change between Stage 1 and 2 was +1.09±0.73 hours, a value which was midway between the changes seen after long and short lengths of T24 entrainment. While these earlier regimes had shown a clear difference in their change of τ, the value produced after the exotic cycles proved not to be significantly different from either of them (t=1.007 with 55 d.f. for exotic versus short T24 entrainment; t=1.755 with 49 d.f. for exotic versus long T24 entrainment).

Analyses of the length of the active phases (Table 3.11) were unable to find any significant change, either over time (see Table 3.11) or between the separate protocols (1st phase, F=0.403 with 5 and 37 d.f.; 2nd phase, F=1.699 with 5 and 37 d.f.). Repeating the same types of analyses for the amplitudes of the locomotion proved to be equally unproductive, both over time (Table 3.12) and when T-cycles were compared (1st phase, F=0.336 with 5 and 37 d.f.; 2nd phase, F=0.860 with 5 and 37 d.f.).

Comparative analysis of the length and amount of activity seen in steady-state entrainment to non-24 hour cycles, and the subsequent free-runs from these cycles, showed no significant differences in terms of the amplitude of activity (T20, t=1.011 with 10 d.f.; T30, t=0.392 with 15 d.f.; T36, t=0.301 with 13 d.f.; T48, t=1.070 with 9 d.f.; T60, t=1.676 with 8 d.f.; T72, 0.042 with 15 d.f.), although the active phase length was sometimes significantly shorter during the free-run (T20, t=2.659 with 7 d.f.; T30, t=7.910 with 14 d.f.; T72, t=2.937 with 16 d.f.). The remaining T-cycles did not show any detectable alteration in the length of activity between entrainment and free-run (T36, t=0.856 with 8 d.f.; T48, t=2.069 with 8 d.f.; T60, t=0.496 with 11 d.f.). The most likely explanation for the results seen in T20 and T30 was thought to involve the

Length of prior	*Mean duration of activity per cycle (hours±s.d.)*						*Two-tailed*
T-cycle (hours)	*Stage 1*	*N*	*Stage 2*	*N*	*Stage 3*	*N*	*t-test (d.f.)*
20	13.08±2.83	6	13.69±3.54	6	--------	0	0.330 (9)
30	13.19±1.29	8	14.33±3.20	8	--------	0	0.958 (9)
36	12.98±2.71	8	15.50±3.98	8	--------	0	1.480 (12)
48	12.33±2.86	6	11.75±1.82	6	--------	0	0.421 (8)
60	14.19±2.57	6	14.75±2.06	6	--------	0	0.412 (9)
72	12.91±1.91	9	12.09±3.02	9	--------	0	0.685 (13)

Table 3.11. Further analysis of the free-run following a variety of T-cycles. In this case showing comparisons of the active-phase lengths (α).

Length of prior	*Mean amplitude activity per cycle (units±s.d.)*						*Two-tailed*
T-cycle (hours)	*Stage 1*	*N*	*Stage 2*	*N*	*Stage 3*	*N*	*t-test (d.f.)*
20	12.43±5.23	6	8.07±5.44	6	--------	0	1.416 (9)
30	9.33±3.49	8	8.39±3.57	8	--------	0	0.529 (13)
36	11.49±5.95	8	11.02±5.97	8	--------	0	0.156 (13)
48	12.07±8.11	6	15.11±14.44	6	--------	0	0.450 (7)
60	13.18±4.68	6	13.76±7.25	6	--------	0	0.165 (8)
72	12.06±7.82	9	10.33±7.21	9	--------	0	0.489 (15)

Table 3.12. As Table 3.10 and 3.11, but analysing the average amplitude of the free-running activity following entrainment to various T-cycles.

large amounts of excess activity associated with the steady-state entrainment to these cycles (due to constant phase-lagging of the rhythm to T20 and phase-leading in T30). As this extraneous activity was connected to the process of entrainment it should, therefore, be expected to disappear once the light cycles were removed. However, why T72 should have a substantially shorter length to its active phase in the free-run was more difficult to explain. The fact that T48 was also seen to have been very near to providing a significant result suggested that the peculiar 'entrainment' observed in T48 and T72 cycles (in which the patterns resembled T24 entrainment) may be involved. This was supported by the earlier evidence that T24 cycles always had a shorter length of activity in the free-run than during the entrained state. It would seem, therefore, that free-running from cycles which were close to T24, or whose entrainment patterns were the same as T24, resulted in a reduced overall length of the active phase in DD. Possible reasons for this will be put forward in the later discussion.

When the various parameters of the free-run from these non-24 hour T-cycles were combined and compared with the same free-runs from both long and short T24 cycles no significant differences were found (Stage 1 τ, $F=0.383$; Stage 1 amplitude, $F=2.705$; Stage 1 α, $F=1.402$; in all cases with 2 and 75 d.f.).

Summary

Free-running after entrainment to T24 cycles

1. Clear rhythms of activity were seen after both long and short entrainment times. In both cases the period of this free-run lengthened after 6-10 days in DD. There was, however, a significantly greater change observed after long entrainment. The duration of the light in the prior LD cycle may also affect the degree of the period change seen.
2. The initial periods of the free-runs were the same in the two experiments (12:12 being the comparative cycle). There was, however, a significant relationship between the length of light in the prior LD cycle and the Stage 1 periods in the short entrainment experiment; the longer the light, the shorter the Stage 1 period. The subsequent stages of this free-run did not show any relationship of this nature.
3. The activity associated with the lights-off LD transition appeared to be the main contributor to the free-running rhythm.
4. While the duration of the active phase of the rhythm did not appreciably alter throughout the free-run it was clearly shorter than that seen during entrainment. Also no difference was found in the free-running active phase length when the two entrainment protocols were compared.
5. Although the average activity was seen to decrease slightly over time, for most examples this did not prove to be significant when the data from long or short entrainment times were considered separately. However, the overall activity was significantly lower when the free-run commenced after the longer experience of LD cycles.
6. Most of the changes seen in the period of the free-runs were thought to be after-effects of prior entrainment to light/dark cycles. It was also evident that the influence of the prior LD cycle was long-lasting, being apparent in the free-run up to one month after the end of the last light pulse.

Free-running without exposure to light

1. The free-running period clearly did not change in these patterns. Therefore many of the observed patterns after entrainment were considered to be after-effects. The overall average period was found to be 23.14±0.60 hours when all the results were combined.
2. The average amplitude and length of the activity declined slightly over each recording but such changes only proved to be significant in those flies which had been least disturbed as adults.

Free-running from non-24 hour cycles

1. The free-run displayed the same features as those following T24 entraining cycles, namely a lengthening of period approximately 6-10 days after release into DD.
2. The different T-cycles had no effect on either the initial period seen in DD or on the degree of the subsequent lengthening of that period. The average value of this change was found to be midway between that seen after long and short times of entrainment to T24 cycles.

3. The duration and amplitude of the activity seen in Stage 1 of the free-runs was not significantly different between the various T-cycles.
4. When the amplitude of the free-run from a particular cycle was compared with that seen during entrainment to the same cycle, no significant differences were found for any of the cycles. However, the duration of the active phase in the free-run was not so invariable. In the case of T20, T30 and T72 the free-running α was seen to be substantially shorter than it had been in the entrained state.
5. Comparisons of the free-running first stage period, amplitude and activity-band length after non-T24 and T24 cycles (of both long and short duration) revealed no significant differences in any category.

Complex Free-running Activity Patterns

The patterns of locomotion which have been discussed so far have all been of a uniform type, with single clear rhythmic elements to the activity. However, a certain proportion of the runs displayed patterns which were highly complex in their form. These records all appeared to represent a disintegration, to varying degrees, of the commonly seen rhythmic elements of the system. The most frequently observed patterns were split rhythms, in which the main activity band divided into two or more separate bands. This usually appeared towards the end of the recordings, after the fly had experienced a previous LD cycle and had been in constant dark for some time (see Fig. 3.24). Although such rhythm-splitting has been seen in a wide variety of animal species, especially mammals, it has not been seen in the records of many insects.

The patterns seen in mammals were usually characterized by a very gradual splitting of the rhythm into its separate elements, many of which free-ran with clearly differing periods. It was also common for the two bands of activity to merge once again, although this could take some time. In insects, however, the bands of activity resulting from the split appeared in many cases to have the same, or similar, periods. Unfortunately, it was usually not possible to utilize the periodogram to disentangle the components involved in the records of *C. vicina* because the periods of the split elements were either too similar in their value (with the result that the periodogram peaks would overlap), or the levels of activity were too dissimilar, in which case the data with the largest amplitude would disguise the other data sets. This points up the inadequacies of the periodogram in isolating periodicities of low amplitude when other high amplitude data are present. Despite this there was a suggestion in Fig. 3.24 that the activity bands did not behave identically. It was also noted that the divided elements had similar activity-band lengths, although these appeared to be smaller than the previous undivided locomotion. In very few of the records was there evidence of a rejoining of the split elements, possibly because these phenomena usually occurred at the end of the recordings and the flies did not live long enough for the process to occur. In these records splitting occurred over a relatively short time, although it was by no means as instantaneous as the splitting displayed by some insects (e.g. the cockroach *Leucophaea maderae*, Wiedenmann, 1977a).

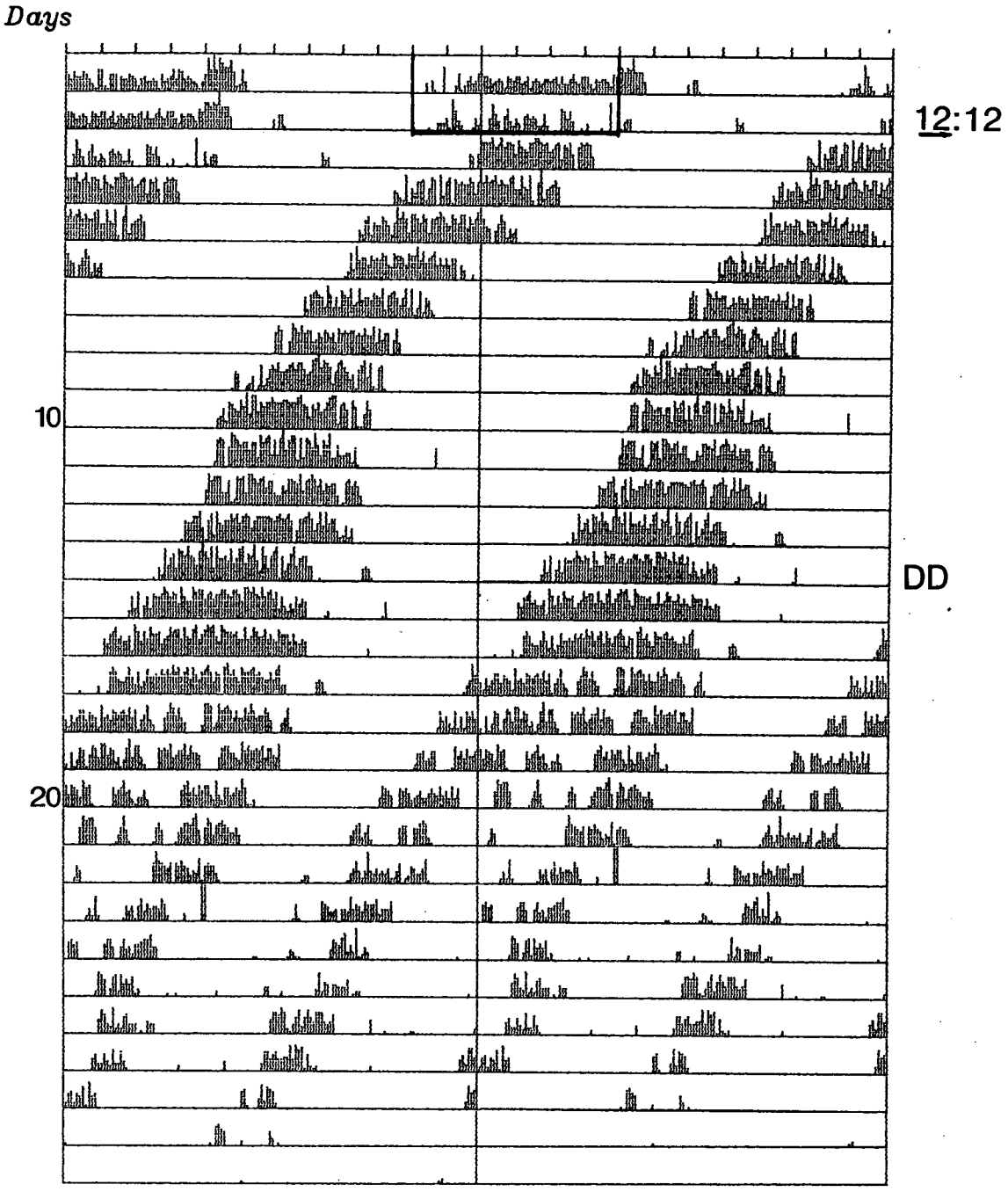

Figure 3.24. An example of a split rhythm in DD, seen after entrainment to a 12:12 LD cycle.

The regularity with which the properties of the splitting phenomenon were seen to vary between insects and mammals leads one to the conclusion that there may be a number of essential differences between the circadian systems which regulate locomotion in these two groups of animals. However, there were other less common forms to the split patterns seen in the blowfly which were remarkably similar to the mammalian examples (see Fig. 3.25). These were characterized by asymmetrical splitting, in which a main activity band was seen to have small bouts of activity breaking away and free-running separately. Furthermore, the main activity was not apparently altered during the process of the split. The elements of the system which split away frequently displayed a smaller length and amplitude to their active phase, as well as having periods which were longer than the main band of activity. The result of this was that the split components usually rejoined the main rhythmic element after free-running for a number of cycles. Figure 3.25 shows two such splits occurring shortly after one-another. It was interesting to note that although both of the splits appeared to arise from the end portion of the main activity band they were quite different in their free-running form. The first split rhythm was clearly the weaker of the two and had a period only slightly longer than the main activity, thus it took 11 days before it was able to rejoin the main rhythm again. The second split actually occurred before the first split element had remerged with the main activity, and having a much longer period than the earlier split it was able to rejoin the main band after only 7 days. This latter split was not only stronger than the preceding one, but it also had substantial effects on the active-phase length of the main periodicity. Despite this there was no evidence of the period of the main activity being radically affected by the split. Furthermore, it was interesting to note that in both examples the splitting and the reconstitution of the main band occurred fairly gradually.

These results from the blowfly are among the clearest examples yet seen of rhythm-splitting in the Diptera and they lend clear support to the models for a multioscillator structure to the circadian system controlling locomotion.

The next most frequently seen pattern of splitting was very different from the records previously described, being always associated with the beginning of the main activity band and appearing as short regular bursts of activity of $\frac{1}{2}$ to 1 hour in duration. This type of pattern was observed to have varying degrees of severity; in Fig. 3.26 the bursts of activity occupied much of the normally inactive phase of the free-run. Indeed, in this figure the main rhythmic

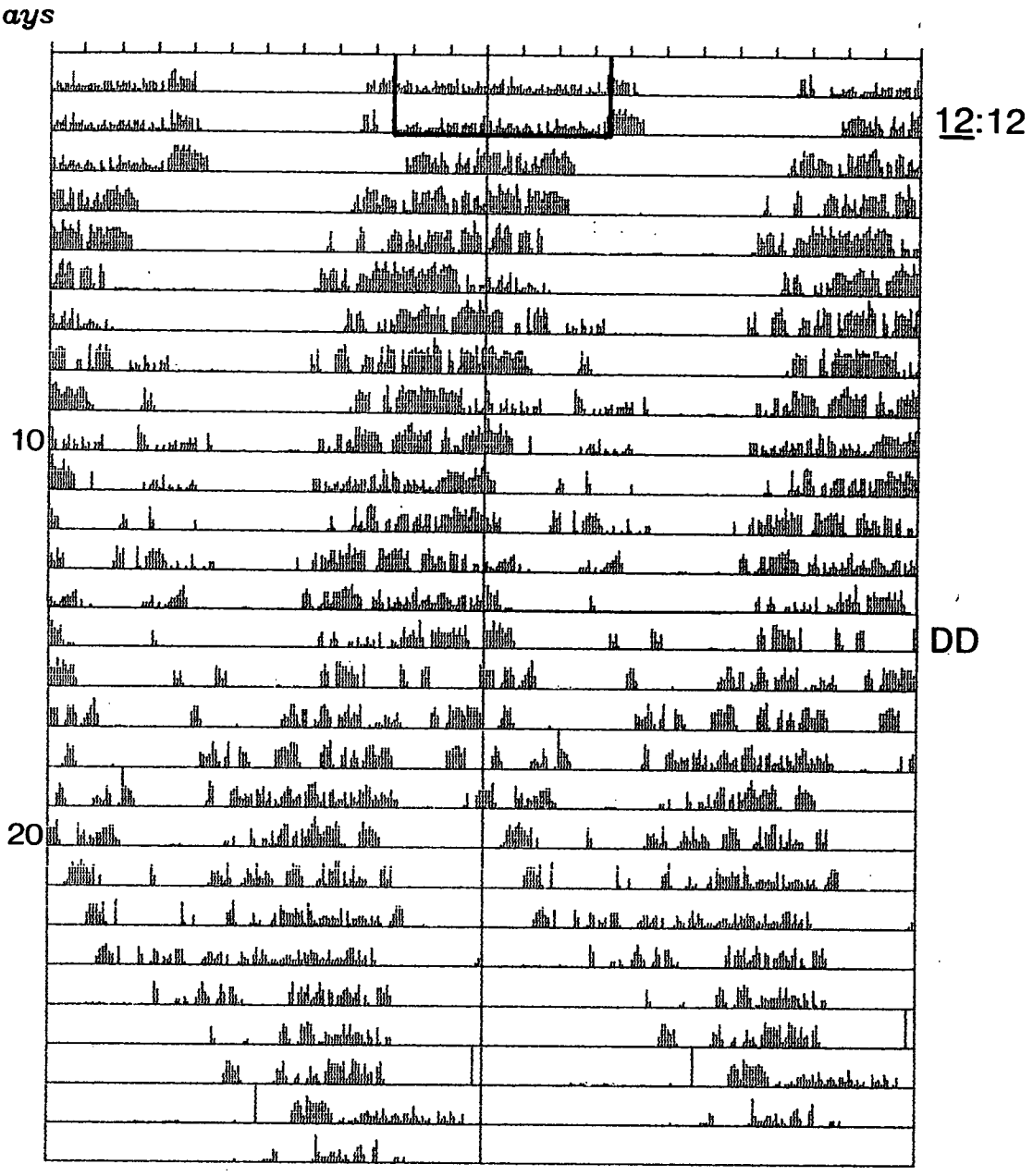

Figure 3.25. A particularly complex split pattern, observed after a 12:12 LD cycle. See text for a detailed description.

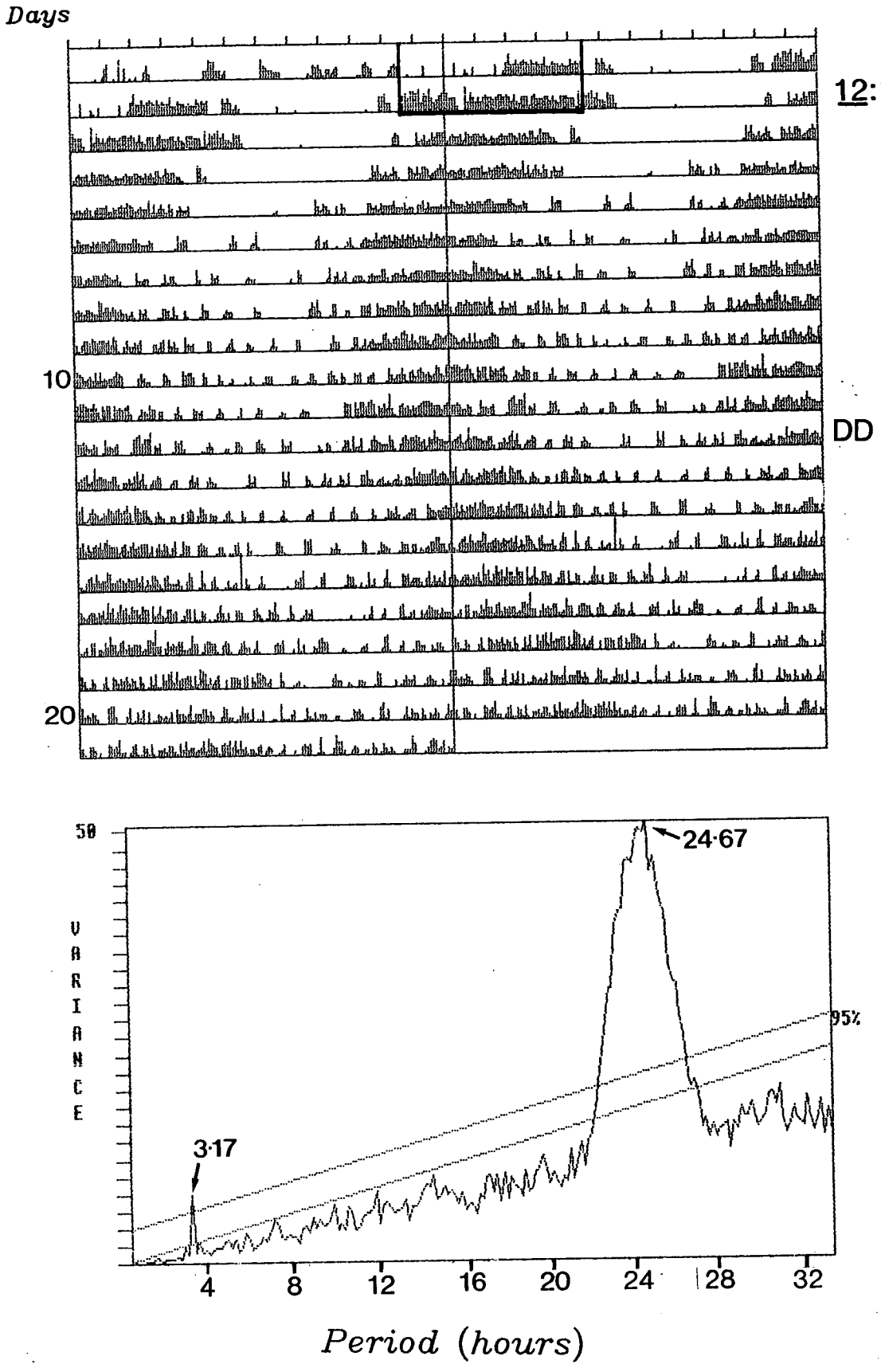

Figure 3.26. An attempt at showing the existence of ultradian rhythms in Stage 2 of a destabilized free-running locomotion pattern. As well as the expected periodicity of longer than 24 hours, there was also the indication of a further rhythmicity at 3 hours 10 minutes.

band appeared to be beginning to break down entirely after 19 days in constant dark. As it was considered possible that these activity bouts might have been examples of very short (ultradian) rhythms, the records were subjected to periodogram analysis. The results proved to be very revealing, as Fig. 3.26 shows. For as well as the main periodicity there appeared to be a second very short rhythmic element in the record, having a period of 3.17 hours. Although not all of the records having short activity bouts showed such a peak in the periodogram, in all of the cases where it was seen the period values were very similar to that shown here, being around 4 hours. It was equally evident that records which did not show any disintegration of the main band of activity did not have such an extra peak in the periodogram. The inference from this was that the small bursts of activity were not randomly distributed but may actually represent very short periodicities, or ultradian rhythms.

The presence of these short rhythms in constant darkness prompted an analysis of the arrhythmic patterns which had been seen in constant light, to ascertain whether similar periodicities were exhibited in this regime. Unfortunately, none of the LL analyses were able to show any distinct short period elements, implying that blowfly activity in LL was truly arrhythmic.

Summary

1. The complex patterns which were seen in DD were varied in their nature, and included clear rhythm-splitting and disintegration into short bursts of activity. Both of these were found to contain clear rhythmic elements, thus suggesting that the circadian system controlling locomotion may comprise a number of oscillators.

The results of the present experiments clearly showed that the activity patterns of individual blowflies were diurnal in natural (T24) light cycles. The active phases in these entrained states were always unimodal (the maxima being situated in the centre of the light phase) and of a fixed length, irrespective of the photoperiod involved. Although many other related Diptera have shown similar diurnal behaviour in the laboratory and the wild (e.g. Green, 1964a; Helfrich et al., 1985; Lewis and Taylor, 1965; Parker, 1962; Smith, 1983; Waddell, 1984), these patterns were not always unimodal. For example, mosquitoes, *Drosophila* spp. and tsetse flies have all been seen to display bimodal activity in natural LD cycles. Interestingly, some of the normally unimodal species could be induced to display bimodal patterns by entrainment to long light phases (*Calliphora stygia*, Waddell, 1984).

As it proved to be possible to produce apparent bimodal patterns in *C. vicina* an attempt was made to find which elements of the activity rhythm were responsible for this feature. It subsequently appeared that only two factors were necessary to produce this bimodality: firstly, an active phase length which did not alter appreciably as the photophase increased, and secondly, the presence, in all photoperiods, of short intense bouts of activity associated with the light/dark transitions (especially the dusk). Thus, as the photophase length increased these two features became more detached, until in 20:4 the main band of the locomotion had decayed away by the time the lights went off and the extra activity was seen. This therefore resulted in patterns of activity appearing to be bimodal.

Such observations must, however, be interpreted carefully, not least because experimental LD cycles are highly artificial, especially around the transitions between light and dark. In those cases where it was possible to produce more gradual transitions much of this extra activity was seen to be dramatically reduced (Jones et al., 1972a, b). When it was not possible to do this the problem was resolved by allowing the activity to free-run. In the present study, and for all of the other examples taken from the Cyclorrhapha, any bimodal patterns seen during the steady-state entrainment promptly disappeared when the LD cycles were stopped, e.g. *Calliphora stygia* (Waddell, 1984), *Drosophila pseudoobscura* (Engelmann and Mack, 1978), *D. robusta*

(Roberts, 1956), without there being any evidence of the two peaks of activity being able to free-run independently. These facts strongly implied that the bimodality seen in *C. vicina* and the other Cyclorrhaphan species was an artifact of the LD cycle.

In contrast to the patterns seen in the Cyclorrhapha the bimodal behaviour of the mosquitoes (Nematocera) did persist in constant conditions, implying that it was a real feature of their circadian system. For this reason many researchers have proposed mosquito locomotor models which involve at least two oscillators (or groups of oscillators) separately phase-set by dawn and dusk (Jones, 1982; Clopton, 1984a, b, 1985). However, these differences between the two Dipteran sub-orders are probably not as great as they appear, for many recent experiments (including those in the present study; see below) have shown that the Cyclorrhaphan system is highly complex, and is probably constructed from a number of oscillators. Furthermore, the strength of the coupling between the oscillators would have to be far greater than the phase-setting effects of the light if one were to provide a suitable explanation for the patterns which were seen in T24 LD cycles.

There was, however, no clear reason why the circadian system of mosquitoes should display such a different pattern in constant dark. The fact that it appeared to be the smallest of the Cyclorrhapha which produced the bimodal patterns, albeit only in LD cycles, suggested that there may be some size-related selective advantage to this type of behaviour, perhaps stemming from some aspect of the surface-area/volume ratio (e.g. susceptibility to desiccation). Thus, one might argue that although many small Diptera have found it necessary to adopt bimodal behaviour patterns, only the mosquitoes have been able to incorporate this into the properties of their circadian system. This may be because the lifestyle of mosquitoes exposed them to stronger selection forces over evolutionary time than the other Diptera. Although an extensive study carried out in the south of England (Lewis and Taylor, 1965) found that there was no relationship between the time of flight and insect size, this may not affect any predictions for more extreme climates where the flight pattern might be dependent on a variety of size-related features.

The activity patterns which were observed in steady-state entrainment to non-24 hour cycles provided little direct information to the discussion on the structure of the circadian system controlling locomotion. However, the results

of these experiments were interesting because they enabled one to see how it may be possible for T-cycles which were very much longer than T24 to produce entrainment patterns resembling that cycle. Thus if enough T-cycles could be examined one could construct a resonance profile for locomotor behaviour, in which there would be peaks at those T-cycles which were equal to, or multiples of, the period of the free-running rhythm. These results of non-24 hour entrainment closely mimic the way in which one imagines the photoperiodic peaks and troughs to be produced in the Nanda-Hamner protocol, a regime which is very widely utilized for illustrating the presence of circadian elements in the production of seasonal morphs. For this reason these T-experiment results are especially significant in the discussion of the overall circadian system(s) which control both locomotor behaviour and photoperiodic effects.

Irrespective of the particular light/dark cycle to which the fly had become entrained, once that cycle was stopped and the activity was released into constant darkness, the locomotor behaviour was seen to continue with a period which was less than 24 hours. Thus, it was evident that the system controlling the activity of *C. vicina* was endogenous. Not only did the rhythm prove to be highly persistent (lasting for several months in constant darkness), but its period was relatively stable when not influenced by any prior light cycles. There was also some evidence that there may have been a slight increase in τ and a slight decrease in the amplitude of the active phase as the fly aged. Relatively stable free-running periods have also been seen in cockroaches (Page and Block, 1980). This was in contrast to the case for three rodent species (Pittendrigh and Daan, 1974) where a strong age-related shortening of the free-running period was found.

As well as being endogenous, the activity pattern of *C. vicina* could clearly be initiated without exposing the fly to light at any time during its development. In the present experiments all of the flies showed clear rhythmic activity with, on average, a period of 23.14 hours. This was in complete contrast to a recent study on the pattern of locomotion in *Drosophila melanogaster*, in which only 33% of the flies had clear rhythmic elements to their locomotion. Over half of the remaining flies appeared to have arrhythmic behaviour, with some of these having ultradian elements to their activity (Dowse and Ringo, in press). There was no obvious reason why this difference existed, although it may be significant that the blowflies used for the present

experiments were only recently taken from the wild, while the *D. melanogaster* has probably been in culture for some time. Alternatively, Dowse and Ringo proposed that the lack of rhythmicity in *Drosophila* may have resulted from the constituent oscillators being out of phase in the absence of light. Does this then indicate that the clock in *C. vicina* has more tightly coupled oscillators than *Drosophila*? Whatever the answer to this problem may be, it is clear that other insects and mammals also have 'self-starting' clocks (Page and Block, 1980; Konopka, 1981).

While the absence of light resulted in stable rhythms, it was equally evident that the presence of prior entraining photoperiods resulted in alterations to the rhythms observed in DD. The first obvious effect on the free-run was the manner in which the first activity peak was slightly delayed, a feature which has been seen in some other insects, e.g. the mosquito *Anopheles gambiae* (Jones, 1973). Also it was clear that the length of the active phase was shorter at all stages of the free-run than it had been during the LD cycles, a feature of the free-run which had not been noted before. This may be explained if the pacemaker controlling blowfly activity was constructed from several tightly coupled oscillators which could be influenced slightly by light to dark transitions. Thus in steady-state entrainment the length of the activity band would 'stretch' due to the influence of the transitions (although not sufficiently to show true bimodality), but as soon as the rhythm was removed from an entraining cycle the mutual coupling of the oscillators would pull them together, thereby resulting in a shorter active phase. Further confirmation of this came from the experiments in which the activity rhythm was entrained to non-24 hour T-cycles. During these experiments the active phase of the free-running rhythm was clearly shorter than it had been during entrainment, but only after those T-cycles which were close to, or multiples of, T24. The other T-values produced no change in the length of the active phase from the entrained state to the free-run. As the length of the free-running active phase was found to be the same between the T-cycles it was therefore concluded that non-modulo T24 cycles displayed active phases in their locomotion which were similar to the free-running state. Thus, the transitions between the light and dark may only be able to exert their full effect when they have a certain phase relationship to the activity rhythms, the optimum position being produced in modulo T24 cycles.

It should be pointed out that there were several unusual features associated

with the initial free-run of *C. vicina*. The first was the observation that the free-running activity was always phase-set by the dusk light/dark transition, and that as a result the activity 'appeared' to arise from the dawn or the dusk depending on the prevailing photoperiod. In reality the phase-setting by the lights-off transition implies that the activity arises from the dusk activity. This finding is in agreement with the results from other related insect examples, in which the dusk activity appeared to persist in DD. Secondly, it was clear that the largest, and most consistent, exogenous responses during light cycles were produced by the dusk transition. This is exactly the opposite of the results which have been seen in other diurnally-active insects. No suitable explanations have been found to explain why this should be the case for the activity rhythms of *C. vicina*.

Throughout the remainder of the free-run the observed patterns were, to a large extent, after-effects of the prior entrainment. In most of the examples in the present study the period of this free-run was initially short but lengthened substantially after 6-10 days. Similar lengthening of the free-running period after a short time in constant conditions has been seen in a number of other insect species, e.g. the Cool-weather mosquito *Culiseta incidens* (Clopton, 1984a, b, 1985); the house fly *Musca domestica* (Helfrich et al., 1985); the blowfly *Phormia terraenovae* (Aschoff and von Saint Paul, 1982), although it is likely that many more examples of this type might have been found if the various researchers had allowed the free-runs to continue for greater lengths of time. In the case of *C. vicina* it was evident that the prior entraining cycle influenced the free-run in several ways. Firstly, the initial (Stage 1) period of the free-run was found to be correlated with the length of the light in the preceding cycle, such that longer light phases resulted in shorter periods. The other two stages did not, however, show this relationship. Secondly, increasing the length of time which the insects spent in entrainment resulted in a greater change in the free-running period, although no relationship was found between the length of the prior photophase and the degree of the increase in τ. Increasing the duration of entrainment did not influence the periods seen. Aschoff and von Saint Paul conducting a similar study with *Phormia terraenovae* concluded that the lengthening in period was not an after-effect as it occurred after both LL and LD cycles. The results from the present study suggested that this change was most certainly an after-effect in *C. vicina*, and furthermore, LL could not be considered to be appreciably different from LD as both produced

the same type of pattern when the rhythm was released into DD. Their study also suggested that the lengthening was not age-related. The results presented here confirm that this was probably also true for *C. vicina*.

One of the most extensive investigations yet seen for insect locomotor rhythms was carried out by Christensen and Lewis (1982) on the New Zealand weta *Hemideina thoracica*, a large nocturnal Orthopteran. Many aspects of this study were considered to be relevant to the present experiments on the activity of *C. vicina*, not because the same results were obtained, but rather because the model that was subsequently proposed for the control of locomotion seemed especially applicable to the blowfly (see below). Indeed many features of the free-runs which they observed were diametrically opposed to the results from the blowfly. For example, *H. thoracica* had an initially long free-running period which shortened, the reverse of the situation seen in *C. vicina*. Furthermore, while non-24 hour T-cycles produced predictable effects on the initial free-running period in *H. thoracica*, they appeared to have little influence on the initial period in *C. vicina*. Also, the length of the active phase was usually longer after entrainment to cycles in which $T=\tau$ for the weta, while *C. vicina* was seen to have its shortest free-running active phase when T was close to, or modulo, τ.

As the weta was clearly a nocturnal insect and the blowfly was equally clearly diurnal, it was tempting to attribute many of these differences to the contrast in their lifestyles. However, the very fact that the behaviour of one appeared to be mirror-imaged in the behaviour of the other suggested that perhaps they could have similar circadian systems which responded to the same environmental signals in contradictory ways. For example, the lights-off would function as the terminator to the blowfly's rhythm but would initiate the weta's activity.

Inevitably the after-effects of entrainment must eventually disappear from the free-running record, although depending on the species involved this can take months to occur. In general though, the lifetime of the blowfly was so short that the rhythms of many individuals did not survive to a point at which it could be categorically stated that they had recovered from entrainment. Fortunately, on several occasions some individuals were seen to maintain clear rhythms for considerable lengths of time, and in many of these the period of the rhythm was seen to shorten again, an indication perhaps of recovery from

the after-effects of entrainment. Surprisingly, this has not previously been observed in any insect species, although it has been seen in sparrows (Eskin, 1971). *Hemideina thoracica* did display very large spontaneous period changes but these did not seem to be able to stabilize towards any mean value (Christensen and Lewis, 1982).

It is now generally accepted that after-effects of the type described here cannot be explained by single oscillator models. Thus, it has been proposed that several oscillators must control the activity rhythms of many animal species (Pittendrigh, 1974; Pittendrigh and Daan, 1976c). However, the most convincing evidence of the presence of a multioscillator system has usually been found in those rhythms which show a breakdown in the structure of their circadian systems, commonly called rhythm-splitting. *Calliphora vicina* was no exception to this rule, and displayed a variety of split rhythms on many occasions. These records are, to date, the clearest examples of Dipteran rhythm-splitting, although similar examples were seen in *H. thoracica* (Christensen and Lewis, 1982). The blowfly's split rhythms were also comparable to the weta, in that the divisions occurred fairly gradually, and there could be an occasional rejoining of the split elements. In the majority of the examples the rhythm was seen to divide into two elements, with both of the new activity components appearing to have the same period, but a shorter active phase, than the pre-split rhythm. Occasionally, however, there could be more than two elements visible at once; one particular example (Fig. 3.25) actually showing three free-running simultaneously and all with differing periods.

These examples all clearly implied that multioscillator models must be the only suitable means for describing and explaining the activity patterns seen in the blowfly. The actual form that this takes is, however, more open to debate. Although it may be along the lines suggested by Pittendrigh and Daan (1976c) a more recent model based on the activity seen in the weta (Christensen and Lewis, 1983; Christensen et al., 1984) seemed to be more applicable to the blowfly.

This model differed from all previous attempts in that it linked together a number of feedback oscillators which had known responses to light and temperature. Their detailed analysis of the model revealed that it was the linkage factor which exerted most control over the way in which the population

of oscillators free-ran and responded to perturbations (this linkage factor being equated with the strength of the coupling between the oscillators). Therefore at low linkage values each of the oscillators would be seen to oscillate with its own period thus producing an arrhythmic combined output. Higher linkage factors, however, were able to produce patterns which were similar to their observed results. Analyses of the simulated free-runs showed that as the linkage factor increased more of the oscillators were recruited into a single group of mutually entrained oscillators, which in turn were responsible for producing the main activity band. Potentially this would still leave a certain number of oscillators (both fast and slow) continuing to free-run across the main pattern of activity. Thus alterations in the free-running pattern could be explained by these 'rogue' fast and slow oscillators crossing the path of the single main group of oscillators, and thereby producing the changes which were seen in the period and active phase.

By this means it was possible to construct a scheme which could explain the after-effects of entrainment as they appeared in *C. vicina*. In order to do this, however, it was necessary to make certain assumptions on the nature of the circadian system in the blowfly:

1. When the insects were not experiencing any environmental perturbation the linkage factor would be strong enough to ensure that almost all of the constituent oscillators were mutually entrained. This should give rise to a rhythmic pattern with a fairly stable period. Furthermore, as the natural period of the free-run in the blowfly was about 23 hours it must be concluded that the short-period oscillators would be the dominant rhythmic elements in the system.
2. Any LD cycles which the insects experienced would result in a break-up of the system, allowing some of the short-period oscillations to free-run independently, although this could take several days to occur once the rhythm was released from entrainment. Once this process was complete, however, the relatively weaker long-period oscillations would become the main influence on the period of the overall activity. This would result in the lengthening of the period which was seen after 6-10 days.
3. Eventually, however, the short-period oscillations which had broken away would cross the path of the main activity band. The result of this would be a shortening of the observed activity period, such that the period of the main band would return to a value close to its unperturbed period.

Differing types and lengths of entrainment may then exert their influence on the free-run by allowing greater or lesser numbers of oscillators to break away. Thus, the longer the light phase of the prior photoperiod the greater will be the initial period of the free-run, as more of the short-period oscillators try to

break away, and the greater will be the lengthening in the period. Similar explanations can also be proposed for the occurrence of rhythm-splitting. In this case the removal of some short-period oscillators, as a result of the split, may disrupt the coupling of those which remain in the activity band, with the result that several independent oscillators (or groups of oscillators) are seen. Also in some rarer circumstances it may be the long-period oscillations which break away, but being weaker than the short period rhythms they produce little change to the main band of activity, both as they split and when they remerge (e.g. Fig. 3.25).

Theoretically, this type of model is not very different from the morning and evening oscillator model first proposed by Pittendrigh and Daan (1976c), if one assumes that the long and short-period elements are arranged as two populations of coupled oscillators. Therefore, during the entrained state the short-period group could be phase-set by the dawn, and the long-period group phase-set by the dusk. Waddell (1984) used a derivative of just such a model to predict the behaviour of the free-running rhythm in *Calliphora stygia*. Furthermore, it is possible that these oscillator populations may only be the 'slaves' to the true pacemakers in the system, if the hierarchical circadian model (Pittendrigh, 1967; Pittendrigh and Bruce, 1957, 1959; Pittendrigh et al., 1958) proves to be correct.

In conjunction with the features described above, there were other aspects of the activity of the blowfly which also indicated a multioscillator structure to the circadian system, ultradian rhythmicities. While their presence in *C. vicina* is not completely confirmed, they certainly seem to be present in another Dipteran, *Drosophila melanogaster*. This particular species has been found to possess several alleles to the *per* locus which affected the output of the clock controlling locomotor behaviour (Konopka and Benzer, 1971). It was two of these (*per* $^{-}$ and *per* ^{o}) which were found to have ultradian rhythmicities, resulting from an inoperative *per* gene. In the former the gene had been completely deleted, and in the latter it was present but appeared not to function (Dowse et al., 1987). Similar genetic variability in the expression of locomotor behaviour has also been found in rats, where ultradian rhythms were clearly associated with the male members of one strain, i.e. LEW/Ztm (Wollnik and Dohler, 1986). This ultradian trait was also found to be highly inheritable (Wollnik et al., 1987).

It was considered interesting that the periods of the ultradian rhythms found in *C. vicina* (about 4 hours) were remarkably close to the lower range of periods found in *D. melanogaster* (periods ranged from 4 to 22 hours). These in their turn were similar to the periods of many intra-cellular molecular mechanisms (e.g. glycolytic oscillations, protein synthesis, enzyme activities), which have been found in cell suspensions, embryonic or differentiated tissues and in isolated organs. It has also been suggested that the periods of these ultradian rhythms may differ between certain organs, between specialized cells within organs, or for certain physiological functions (Jerebzoff, 1987). Thus, one would have to hypothesize a system comprising many disparate oscillations under the control of a number of cellular ultradian pacemakers. This has led some researchers to suggest that the circadian system may be structured around a population of coupled ultradian oscillators, and that the period of the free-running rhythm is dependent on the tightness of the coupling between these oscillators (Dowse and Ringo, 1987; Dowse et al., 1987).

It is, as yet, impossible to know the exact form which the circadian system takes in the control of the locomotion of the blowfly. However, a picture does emerge of a highly complex endogenous biological clock involving a number of oscillators, possibly arranged in two mutually coupled populations. Of the various models so far devised Christensen and Lewis' appears to be the most applicable to the rhythms in the blowfly. This has advantages over most of the other models, in that it can readily explain much of the lability which is seen in insect free-running rhythms, as well as being able to make predictions on the types of locomotor behaviour which may arise from any alterations to the parameters which control the system.

CHAPTER 4

The Effects of Temperature and Geographical Origin on Activity

Introduction

For the purposes of the experiments in both this and subsequent chapters it was decided to establish some form of control free-running rhythm. This was felt to be necessary because the recording apparatus could only accommodate a relatively small number of individuals at any one time (30 in these experiments), and although this may have sufficed for the experimental flies it left too few recorders available for any meaningful controls to be run with each experiment. Whenever possible some controls were run concurrently with the experiments to ensure that the recording environment had not changed appreciably. Fortunately, in all these cases the controls (some of which will be mentioned) were found not to differ significantly from the full controls discussed here. It was also essential to choose an appropriate photoperiod from which to free-run the flies. One of 12 hours light: 12 hours dark was picked, firstly, because it produced reliable numbers of clear free-running patterns, and secondly it was known to be a strong diapause-inducing photoperiod. This latter feature was important as it allowed clearer comparisons to be made between the photoperiodic experiments which will be discussed later and the present locomotor study. As these photoperiodic experiments also involved an American strain of *C. vicina* the controls were repeated using this strain. This enabled aspects of the locomotor system in the U.S. strain to be compared with its own photoperiodic results, and allowed comparisons to be made with the locomotor patterns seen in the Scottish strain. Although some work has been done on the variation in the properties of the photoperiodic system between different geographical populations (see later), no comparative work has been undertaken for the locomotor patterns of such insects. Thus, these first experiments were aimed at finding whether any discernible differences existed between the locomotor patterns of the two strains.

It has been known for some time that temperature can have many dramatic effects on locomotor rhythms, as well as being able to act as an entraining agent (Roberts, 1962; Saunders, 1982a). Thus, as both of the above experiments

were carried out at 20°C, it was considered instructive to repeat the experiment a third time using the Scottish strain, but this time at the temperature of 24°C. Despite these effects, however, it is generally accepted that once the rhythm has been released from the entraining influence of any regularly fluctuating Zeitgeber (be it light or temperature) the ambient temperature can only exert a minimal influence on the rhythm. This must be so if one assumes that the biological clock controlling the activity functions to measure time; for any clock which was too easily affected by the ambient temperature could not be an effective time-measuring device (Pittendrigh, 1960).

Such temperature-compensation has been noted in the free-running periods of a number of insects, most specifically cockroaches, e.g. Roberts (1960). For example, one individual *Leucophaea maderae* was shown to shorten its period from 25 hours 6 minutes to 24 hours 17 minutes when the temperature was raised from 20°C to 30°C. In order to provide some quantitative measure to these period changes circadian biologists have adopted a parameter which is extensively used to describe the changes that occur in the rates of chemical reactions, the Q_{10} value. This is used to indicate the degree to which a particular physiological process is accelerated with each 10°C temperature rise. In a perfectly compensated system the Q_{10} value would be 1, although most chemical reactions tend to have a value of about 2, i.e. every 10°C temperature rise doubles the reaction rate. In the example above the Q_{10} value was about 1.04, which was indicative of a highly temperature-compensated control system for the locomotor activity. Interestingly, Wiedenmann (1978, cited in Saunders, 1982a), working with the same species, found a Q_{10} of 0.97. Furthermore, Caldarola and Pittendrigh (1974) found that in this species the free-running period appeared to have a non-monotonic function with respect to the ambient temperature, i.e. the period was longest at 17°C, shortest at 20°C, but lengthened once more when the temperature was raised to 30°C. They had also previously shown (Pittendrigh and Caldarola, 1973) a Q_{10} of 0.97 for this species: a value which exactly agreed with Wiedenmann's result above. Q_{10} values of 0.82 and 1.15 have also been produced for the New Zealand Weta *Hemideina thoracica* (Gander, 1976 cited in Saunders, 1982a).

Thus, the second set of experiments described here were an attempt to discern how the features of the free-running rhythms discussed in the previous

chapters were affected by such a change in the ambient temperature.

Materials and Methods

All of the flies used in these experiments were reared as stated in the General Methods. They were all placed in a 12:12 regime as pupae and emerged into this photoperiod. One to two days after emergence they were put into the recording devices, where they were exposed to the same 12:12 cycle. After 4 further days in this photoperiod all of the flies were released into constant darkness. While both strains were exposed to 20°C for the control experiments, the Scottish strain was also maintained in 24°C.

Both the U.S. and the 24°C Scottish results were then analysed in comparison to the Control group (Scottish strain at 20°C). It should be noted that no locomotor activity records are included in the figures for this chapter. This was because the observed runs were too similar to previous figures to merit inclusion.

Results

Table 4.1 contains the results obtained from the Scottish strain at 20°C. As was expected there was an increase in period between the first and second stages (t=6.804 with 42 d.f.) but no demonstrable alteration thereafter (2nd to 3rd stages, t=1.876 with 45 d.f.). This was reflected in the values obtained for the average change in τ seen between the different stages of the free-run; Stage 1 to Stage 2 showing a lengthening of 1.27±0.96 hours while Stage 2 to Stage 3 had only 0.47±0.86 hours. It was evident that most of the records showed clear rhythmic behaviour in constant darkness, with almost all of these showing the characteristic lengthening of the period:

Clear and constant period:	10%	(N=3)
Clear but changing period:	83.33%	(N=25)
Complex patterns:	6.67%	(N=2)

As can be seen, these proportions were very similar to the percentage distributions found in the earlier chapters. Although analyses on the duration of the active phases showed that there was little change throughout the time of

Free-running pattern from a 12:12 regime	*Stage 1*	*N*	*Stage 2*	*N*	*Stage 3*	*N*
Mean free-running period (hours±s.d.)	22.71±0.48	28	23.98±0.87	28	24.45±0.87	21
Mean duration of activity per cycle (hours±s.d.)	13.45±2.25	28	14.27±2.84	28	14.30±2.90	16
Mean amplitude of activity per cycle (units±s.d.)	14.61±6.44	28	13.05±6.54	28	9.26±4.79	16

Table 4.1. The results from the three free-running stages in the Scottish strain at 20°C. Note that while the period increased between Stage 1 and 2, the activity levels showed a marked decline over the duration of the recordings.

the recordings (Stage 1 to Stage 2, t=1.198 with 51 d.f., Stage 2 to Stage 3, t =0.038 with 38 d.f.), the amplitude of the rhythm did show a declining trend. This was first noticed when the amplitudes were subjected to an analysis of variance test (F=3.861 with 2 and 69 d.f., significant at 5%). It would appear from Table 4.1 that this decline was a gradual process, although the fact that the change from one stage to the next became increasingly significant as the records proceeded inferred that the amplitude may have been decreasing more rapidly as the flies aged (1st and 2nd phases, t=0.896 with 53 d.f., not significant; 2nd and 3rd phases, t=2.202 with 41 d.f., significant at 5%). Thus, these results agreed with the previous chapters in terms of the features of free-running rhythms after entrainment, i.e. having a period which lengthened markedly after 6-10 days, but did not change subsequently and a stable active phase length. Although the amplitude did eventually decline, this was well after the period increase and may therefore have been a separate process unrelated to the circadian system, perhaps being an ageing effect.

The U.S. strain behaved in an almost identical manner to the Control Scottish flies (see Table 4.2), having substantial increases in period early in their free-runs (Stage 1 to Stage 2, t=7.804 with 45 d.f.) and little change thereafter (Stage 2 to Stage 3, t=0.063 with 45 d.f.). Inevitably, this was correlated with a large average change in period between Stage 1 and Stage 2 (1.81±0.88 hours) and almost no lengthening between the second and third stages (0.04±1.13 hours). The distribution of the various types of pattern seen in the observations was as follows:

Clear and constant period:	0%	
Clear but changing period:	92.6%	(N=25)
Complex patterns:	7.4%	(N=2)

Again most of the results showed a significant lengthening of the period. The active phase length and the amplitude of the activity did not, however, significantly change with time (F=1.741 with 2 and 71 d.f. and F=1.766 with 2 and 71 d.f. respectively), although there was a suggestion that the amplitude did decline slightly. Equally, the Scottish and U.S. strains were indistinguishable with regard to these two parameters at each of the three stages which were analysed: α having t-values of 0.572 (50 d.f.), 0.157 (49 d.f.) and 0.155 (36 d.f.) over Stages 1 to 3 respectively and the amplitude having t-tests resulting in

Free-running pattern from a 12:12 regime	*Stage 1*	*N*	*Stage 2*	*N*	*Stage 3*	*N*
Mean free-running period (hours±s.d.)	23.07±0.74	25	24.89±0.88	24	24.91±1.09	25
Mean duration of activity per cycle (hours±s.d.)	13.09±2.32	25	14.39±2.72	24	14.16±2.80	25
Mean amplitude of activity per cycle (units±s.d.)	13.62±4.18	25	12.34±4.58	24	11.06±5.57	25

Table 4.2. The free-running data produced by the U.S. strain at 20°C. As with the Scottish flies, the period increased noticeably between Stages 1 and 2, while the activity levels dropped over the three stages.

0.670 (45 d.f.), 0.458 (46 d.f.) and 1.097 (38 d.f.) for the same stages.

Also the Stage 1 free-running periods appeared to be the same in both groups (t=2.064 with 41 d.f.) although the U.S. strain had a consistently longer average τ. This effect became more noticeable (and significant) in the Stage 2 periods, where the U.S. strain had, on average, periods which were nearly an hour longer than the Scottish flies (t=1.569 with 43 d.f.). This significant result reflected the substantially greater change in period which was seen between Stages 1 and 2 for the U.S. flies (t=2.102 with 49 d.f.).

Repeating the experiment once more with the Scottish strain at 24°C gave the results seen in Table 4.3. Although only the first two stages were considered for analysis the lengthening of the period was very evident (t=4.770 with 43 d.f.). While no changes were observed in the duration of the active phases (t=0.260 with 43 d.f.), the amplitude was seen to decrease substantially over time (t=2.144 with 40 d.f.). A comparative analysis of these results with the previous Control (Table 4.1) revealed several interesting facts. Firstly, although the Stage 1 periods were not found to be significantly different (t =0.270 with 44 d.f.), the Stage 2 periods were clearly not the same in the two groups (t=2.165 with 46 d.f.), with the longest periods being in the group at 20°C. These results were an obvious reflection of the average period change which was observed in the two groups, in which the controls showed a lengthening of 1.27±0.96 hours while the higher temperature group had an increase of only 0.97±0.68 hours. Unfortunately these changes in τ were not in themselves significantly different (t=1.302 with 45 d.f.), although the resulting periods were clearly not the same.

The analysis of the distribution of activity types in these results (given below) failed to reveal any noticeably differences from previous observations.

Clear and constant period:	8.33%	(N=2)
Clear but changing period:	79.17%	(N=19)
Complex patterns:	12.5%	(N=3)

When the amplitude and the duration of the active phases were compared at the two temperatures, they were both significantly smaller at all points in the free-run in the higher temperature group (Stage 1 α, t=2.415 with 48 d.f.;

Free-running pattern from a 12:12 regime	*Stage 1*	*N*	*Stage 2*	*N*	*Stage 3*	*N*
Mean free-running period (hours±s.d.)	22.67±0.60	23	23.53±0.62	23	-------	
Mean duration of activity per cycle (hours±s.d.)	12.00±2.02	23	11.65±1.94	23	-------	
Mean amplitude of activity per cycle (units±s.d.)	10.64±6.30	23	7.32±4.72	23	-------	

Table 4.3. A repetition of the Scottish strain's free-run, but in this case the ambient temperature was 24°C. Note the very sharp fall in the amplitude of the activity between the two stages.

Stage 2 α, t=3.597 with 45 d.f.; Stage 1 amplitude, t=2.104 with 48 d.f.; Stage 2 amplitude, t=3.628 with 46 d.f.). Moreover, it was also evident that the significance of the difference between the two temperature groups increased for both parameters as the recordings progressed. It was also noted that the amplitudes seen in the second stage of the 24°C runs were indistinguishable from those seen in the third stage of the 20°C records. These results perhaps implied that the two parameters were not responding to the rising temperature in a purely exogenous manner, for if they had one might have expected one or both to have shown an increase in their values. Rather, it appeared that the temperature was speeding up some deeper physiological processes. The importance of these results will be considered more fully in the next section, but in essence it seemed that these changes in duration and amplitude were reflections of some time-dependent physiological mechanism, for example aging.

One further analysis was carried out, which compared the Stage 1 periods with those obtained from flies which had never experienced light (Chapter 2). These flies had shown no apparent after-effects of entrainment and thus their periods were considered to be good estimates of the true free-running period of the whole oscillating system. In all three experiments the first stage periods were not significantly different from this earlier experiment (Scottish 'Control', t =1.595 with 38 d.f.; U.S., t=0.395 with 43 d.f.; Scottish at 24°, t=1.642 with 40 d.f.).

Summary

1. The U.S. strain was very similar in its free-running pattern to the Scottish Control strain. No variation was found between the two groups in terms of the active phase length and amplitude.
2. The Stage 1 periods were also equally indistinguishable, although the Stage 2 periods were significantly longer for the U.S. strain.
3. The Scottish strain maintained at a higher temperature showed a marked decline in the levels of activity with time. Furthermore, both active phase length and amplitude were smaller than had been seen in the low temperature group. These changing values may represent an acceleration in certain physiological processes as the temperature increased.
4. The Stage 1 periods at both temperatures appeared to be the same, although the Stage 2 periods were longer at 20°C.
5. The Stage 1 periods from all three experiments were identical to those found in flies which had never been exposed to light.

It is always difficult in any observations of labile free-running rhythms to establish which aspects of the records, if any, most closely resemble the stable pattern of the oscillating system. The results from previous chapters have indicated that the patterns seen in flies which have not been exposed to light were probably closest to this situation. Thus, a comparison of these patterns to the free-runs seen after LD cycles should help to show the least disturbed portions of this activity. In terms of the period of the free-run it was clear that only the Stage 1 periods resembled the probable true period of the oscillator, this being about 23 hours. The periods exhibited by the subsequent stages were, therefore, assumed to reflect a destabilized system.

The differences which existed between the Stage 1 periods and the subsequent stages could be explained if one assumed a hierarchical structure to the circadian system, as has been proposed by Pittendrigh (1967), Pittendrigh and Bruce (1957, 1959) and Pittendrigh et al. (1958). In this scheme the top of the hierarchy would be dominated by a number of temperature-compensated oscillators. They in their turn would control, by a temperature-dependent coupling, the expression of a number of non-temperature-compensated 'slave' oscillators. The system as a whole would, however, operate in a temperature-compensated manner, due to the controlling effect of the pacemakers. Thus, the periods which were seen in the first stage of the free-run would be a representation of the nearly stable system before entrainment after-effects had had time to exert their full influence on the activity pattern. One would therefore expect the Stage 1 periods to be uninfluenced by the change in temperature, as the system at that time should still be capable of some degree of temperature-compensation. The observed results would seem to agree with this.

Furthermore, it would be supposed that the altered periods in the second stage of the free-run were the result of a breakdown in the circadian system, perhaps due to a disruption in the coupling factors between the oscillators. Thus, it could be hypothesized that during this stage the after-effects of the entrainment had weakened, or removed, the influence of these pacemakers with the result that the periods of the slave oscillators had become more apparent. As these periods will not display the same degree of

temperature-compensation as was seen in the first stage of the free-run, the average period of this stage of the output will be altered by changes in temperature. It would also be expected that there would be a negative relationship between period and temperature, with lower temperatures slowing down the various slave oscillators and thereby producing longer periods. The results of the present experiments were found to agree with this hypothesis both in terms of the periods seen and also in terms of the observed direction of the period changes.

By a similar model one could propose explanations for the differences seen in the two geographical strains of *C. vicina*. In this case these would be the result of differing selection pressures on the fly populations at the two localities. Thus, one may suppose that the genes responsible for the pacemakers at the top of the hierarchy would be highly conserved parts of the genome, whereas the genes encoding the slave oscillators would be susceptible to local environmental selection pressures. Thus, as the stable system in the two fly strains should be an expression of the main pacemakers rather than the slaves, the Stage 1 periods (to some extent a reflection of the pacemaker) should be expected to be similar in the two strains. Equally, the Stage 2 periods might be expected to be different as they would be the overt expression of slave oscillators which had been fashioned over many generations by local environmental selection pressures. The results observed in the present experiments appeared to be a confirmation of this, as the Stage 1 periods were indistinguishable between the two strains, while the Stage 2 periods were clearly very much longer in the U.S. strain.

Although explaining the direction of this variation in Stage 2 periods is difficult due to the lack of research on activity behaviour of insect species from differing geographical localities, some clues may be found in the studies which have been undertaken on the photoperiodic responses of insects. This work will be reviewed in a future chapter, but suffice it to say that it was generally found that the more northerly the strain of a particular species, the greater was its critical daylength, and also the shorter was the theoretical period of the rhythm which controlled this response. While it may not be applicable to compare photoperiodic effects with locomotor behaviour it was interesting to note that the U.S. strain of *C. vicina* (which had the longer Stage 2 period) came from 36°North while the Scottish strain came from 55°North. Thus, the results obtained here conform to the clines in circadian properties which one might

expect to see as the latitude changed.

The variation in the other two parameters of the free-run (the amplitude and duration of the active phase) present further problems. It was clear that the two geographical strains were identical with regard to both of these features at all points in their free-runs. However, once the temperature was raised both amplitude and active phase length declined markedly. Furthermore, the amplitude of the activity (which had been seen to decrease slightly in all of the locomotor experiments in this and previous chapters) was seen to decrease to a greater degree with time then in any other experiment, implying that this feature of the free-run was involved with some physiological processes which naturally altered with time, but which were also accelerated by the increase in temperature. A suitable explanation could be found for the reduction in the active phase length if one assumed that it reflected some aspect of the degree of coupling between the slave oscillators. Thus, as this coupling was thought not to be temperature-compensated the active phase length would be expected to change as the temperature altered. However, if this was the case one might suppose that the coupling of these oscillators would increase in strength with increasing temperature, which would have delayed or reduced the after-effects seen in DD. Unfortunately, there was no evidence that this was the case. Whatever the effects on the free-running patterns, it was clear that most of the processes involved were not temperature-compensated, and thus it may be supposed that they were situated at a fairly superficial level in the system controlling the expression of the activity.

Similar declining amplitudes have also been observed in the cockroaches *Periplaneta americana* (Brady, 1967) and *Blaberus* (Harker, 1964 cited in Brady, 1967). Brady noted that the declining amplitude of activity only occurred in infra-red recorders, and that transferring individuals to running-wheels re-established their activity at higher levels. Thus, he reasoned that although both types of recorder were highly artificial, the infra-red detectors provided a far less stimulating environment to the insects' locomotor behaviour, with the result that locomotor activity damped out. However, as he had also shown that disturbing the cockroaches could produce higher levels of activity, one might suspect that the act of transferral between the recorders may have acted as a stimulant to the insects' activity. Such reductions in responsiveness to environmental stimuli have also been observed in behavioural studies on species from almost every animal phylum. In these cases it has been generally

referred to as 'habituation' (see Manning, 1981). However, it was difficult to see how increasing the ambient temperature could produce such large changes to the amplitude of the activity if it were simply a matter of habituation. An alternative explanation might be that some processes associated with physiological aging were responding to the change in temperature. However, a great deal of further work will have to be done before any certainty can be attached to any of these theories.

CHAPTER 5

The Effects of Chemical Treatment on Activity

Introduction

One of the many approaches to the study of biological clocks has been to subject rhythmic organisms to chemical disturbances in order to alter the workings of their clock, and through the interpretation of these alterations, to allow insights to be gained into its mode of operation. Many of these substances have been either specific inhibitors of macromolecular synthesis, narcotizing agents, growth stimulants or sublethal doses of metabolic poisons. However, of all of these substances, deuterium oxide (heavy water) has been found to provide the most consistent and reproducible effects.

Its influence was first noted by Bruce and Pittendrigh (1960) on the free-running phototaxis rhythm of the unicellular flagellate *Euglena gracilis*, where it lengthened the period by 5-6 hours. Subsequently, Suter and Rawson (1968) reported that deuterium oxide (D_2O) in the drinking water of the deer mouse *Peromyscus leucopus* increased its free-running activity period, this effect being both reversible and dose-dependent. Similarly, lengthened periods were observed with D_2O in the activity patterns of the mouse *Mus musculus* (Palmer and Dowse, 1969; Dowse and Palmer, 1972; Daan and Pittendrigh, 1976b), another deer mouse species, *P. maniculatus*, the golden hamster *Mesocricetus auratus* (Daan and Pittendrigh, 1976b), the laboratory rat and hamsters (Richter, 1970), the African waxbill *Estrilda troglodytes* (Palmer and Dowse, 1969) and pigeons (Snyder, 1969 cited in Enright, 1971a). Enright (1971a) found the same feature with the tidal activity rhythm of the marine isopod *Excirolana chiltoni* when they were bathed in D_2O. Several insects have also displayed lengthening of their periods of free-running activity with the application of this substance, e.g. *Drosophila pseudoobscura* (Pittendrigh et al., 1973) and *Leucophaea maderae* (Caldarola and Pittendrigh, 1974). The latter of these studies also found a dose-dependent response to D_2O, where greater concentrations of deuterium resulted in larger degrees of lengthening of the cockroaches' locomotor period.

The ubiquity of such lengthened periods in response to D_2O led many

authors to predict that heavy water may be a useful tool to understanding the physical nature of the cellular oscillations responsible for circadian rhythmicity. For this reason it would be helpful to summarize the known effects of deuteration on biological systems. Pittendrigh et al. (1973) suggested three categories for this:

1. **Primary isotopic substitution**. This is where the deuterium atom substitutes for hydrogen at the reaction site in a molecule, resulting in a change in the energy of the bond to be broken in subsequent reactions. In other words, the activation energy of the bond is raised and any reactions involving it will proceed more slowly at a given temperature.
2. **Secondary isotopic substitution**. The substitution of deuterium for hydrogen occurs at sites not directly involved with the reaction, with the result that the effects are less predictable but not necessarily negligible. In terms of biological function this is probably most important in relation to the stability of protein structures. Furthermore, it is now well known that hydrogen is especially important for stabilizing the structure of the α-helix and the β-pleated sheet, both of which contribute to the tertiary and quarternary structure of proteins (for review see Stryer, 1981). Substitutions of deuterium for hydrogen tend to strengthen these stabilizing bonds.
3. **Solvent effects**. This reflects the manner in which the properties of D_2O, when used as a solvent for cellular reactions, affect various intracellular processes. These influences occur for a variety of reasons: D_2O is more viscous than water; the increased mass of the molecule slows its diffusion; the dissociation constant for D_2O is different from that for H_2O (i.e. pD is not equivalent to pH) resulting in many changes to the electrochemistry of the system, e.g. different ion mobilities. Solvent effects also alter the hydrophobic bonds which develop in the α-helices and the β-sheets which stabilize the tertiary structure of proteins. In these cases, nonpolar side groups on the amino acid chains rotate away from the water in which they lie and thereby come into close proximity with each other. Their mutual attraction, by van der Waals forces, creates a bond which stabilizes the folded configuration. Again D_2O is thought to strengthen these interactions when used instead of water as the protein solvent. Such strengthening of protein bonds is thought to be responsible for the increased tolerance to high temperatures shown by some species when their body fluids are deuterated (Pittendrigh and Cosbey, 1974).

The result of many of these effects of D_2O is to slow down virtually every biological process to which it is applied (Pittendrigh et al., 1973). The variety of these influences on cellular processes also provide a great number of possible explanations for the observed rate-depression of biological rhythms. For example, the rates of diffusion of many cellular constituents will be retarded by the increased viscosity and the greater mass of the solvent molecule, while the altered electrochemical environment will change the mobility of some ions. Furthermore, increased reaction energies will result in a slowing of reaction-rates.

Pittendrigh et al. (1973) have indicated that deuterium can also impair both enzymatic systems and the regulatory mechanisms which assure optimal reaction-rates in the normal unperturbed situation. Equally, the alterations which D_2O produces in the stability and flexibility of proteins could affect their efficacy as enzymes and the properties of many cellular membranes.

Given that D_2O slows most biological processes, one should not be surprised that it lengthens the period of most of the circadian oscillators which have been exposed to it. This does not, however, provide very specific explanations of the physical nature of biological oscillations, but rather suggests a myriad of possible mechanisms for the rate-depression. In an attempt to counter this Pittendrigh et al. (1973) put forward the hypothesis that the influence which D_2O has on biological systems had many similarities with the effects of low temperatures. This had first been seen with the flagellate *Euglena* (Bruce and Pittendrigh, 1960) where both low temperatures and D_2O caused an extreme lengthening of τ and a general damping of the system.

However, it is known that biological oscillators cannot afford to be too greatly perturbed by any factors which are present in the environment of the organism, otherwise they could not provide an accurate time-measuring system: Pittendrigh and Caldarola (1973) were able to show that a wide variety of temperature changes could only produce slight changes to the period of the activity in the cockroach *L. maderae*. Thus, it was especially interesting to note that different aspects of circadian systems could be affected to quite differing amounts by D_2O treatment. Enright (1971a) presented a review of a variety of rhythmic events in many species, some of which were known to have oscillators as the basis to their rhythmicity and others which did not. It was clear that the former were affected by D_2O to a far lesser extent than those rhythmicities which were not oscillatory.

Pittendrigh et al. (1973) extended this work to temperature-compensated and temperature-dependent aspects of the circadian system gating the emergence of adult *Drosophila pseudoobscura* from their puparia. The pacemaker which controlled this system was entrained by light cycles and assumed, in the steady state, a unique phase relationship to the light (which was almost invariant with temperature). The observed rhythm of gated emergence was, however, effected by a second oscillator driven by the light-sensitive temperature-compensated pacemaker. It was clear that the

relative phase relationship of this second oscillator to the pacemaker, and thus to the light, was temperature-dependent. In the experiments described it was found that the period was only slightly lengthened by D_2O and was largely invariable within a 10°C temperature range. On the other hand, the phase relationship of the rhythm of eclosion to the light cycle was strongly temperature and D_2O-dependent. Thus, as well as two related aspects of the same circadian system displaying quite differing responses to the same environmental disturbances, it was found that temperature and D_2O produced similar effects on these different parts of the system.

However, as has been stressed, circadian systems must be homoeostatically protected against frequency changes in order to function as reliable clocks. Thus, results which suggest that D_2O acts in an equivalent manner to temperature may only be manifestations of such a general homoeostasis. This difficulty can be overcome by comparing species which vary in their temperature-compensation of period, such that some pacemakers have negative, and others positive, temperature-coefficients. Thus, although D_2O normally acts to lengthen period, the expectation would be that where the action of temperature is to increase frequency, D_2O should shorten τ. While no experiments of this type have been carried out, an alternative was described by Caldarola and Pittendrigh (1974) based on the non-monotonic response of τ to increasing temperature in the cockroach *L. maderae*. The shape of this curve predicted that a temperature of 20°C should result in an increase in free-running period if D_2O was substituted for the drinking water, while at 30°C one would expect τ to decrease. Unfortunately, the observed results did not support this as the free-running period increased at both temperatures. However, it was seen that the lengthening effect of D_2O was highly temperature-dependent with 20°C having a greater lengthening effect than 30°C at the concentration used (25%). Thus, they concluded that D_2O was only affecting selected parts of the homoeostatic mechanism and in doing so it could not be wholly equivalent to a lowering of the temperature. Further work on the marine dinoflagellate *Gonyaulax polyedra* (McDaniel et al., 1974) also rejected the 'low-temperature equivalence' hypothesis.

Despite the claims implicit in the arguments above, that the period of the rhythm is homoeostatically protected from a variety of environmental perturbations, an increasing number of chemical compounds have been found to influence this aspect of circadian rhythms: one of the most important of

these being lithium salts. These have been found to lengthen the period of biological oscillations in a wide variety of organisms, e.g plants (Engelmann, 1972, 1973; Engelmann et al., 1976), man (Johnson et al., 1980; Engelmann et al., 1982), other mammals (Hofmann et al., 1978; Reinhard, 1985; Schull et al., 1988) and insects (Hofmann et al., 1978; Schmid and Engelmann, 1987). A review of the literature on the effects of lithium may be found in Engelmann, 1987).

In general the lengthening effects of Li^+ on insect rhythms have been found to be far smaller than those of D_2O. *Drosophila* showed a lengthening of 0.4 hours in its locomotor activity rhythm when given Li^+ in its drinking water (Mack, 1980, cited in Engelmann, 1987), while the eclosion rhythm of *D. pseudoobscura* was found to be unaffected by LiCl when present in the larval medium (Reinhard, unpublished, cited in Engelmann, 1987). Cockroach locomotor rhythms were also found to be lengthened by 0.1 hours to 0.18 hours in Li^+ concentrations of 10mM to 100mM (Hofmann et al., 1978). Reinhard (1983, cited in Engelmann, 1987) also found that the range of entrainment was broadened by Li^+.

Although the effects of Li^+ were consistent and reproducible it was not clear how this element produced its effects. In an attempt to explain some of these Engelmann (1987) used a derivative of a model which had been first used to simulate the activity patterns seen in the New Zealand weta *Hemideina thoracica* (Christensen and Lewis, 1982, 1983). This model involves a number of mutually coupled oscillators, each of which synthesize a hypothetical substance within a membrane-bound system. This substance is also gradually lost by passive diffusion from the system. The difference between a reference concentration and the actual concentration of the substance outside the membrane (plus a time delay factor for the crossing of the membrane) make up a feedback loop which controls the initial synthesis rate. Thus, the actual concentration of the substance will oscillate as the synthesis rate is adjusted to equilibrate any differences between the reference point and the concentration outside the membraned system. The time between the peaks in this oscillation (its period) will depend upon the time delay in the feedback loop.

It had been noted that the phase response curves of Syrian hamsters were asymmetrically affected by chronic Li^+ treatment, such that the phase advances

were reduced while the phase delays were unaltered (Engelmann, 1987). The model described above was able to simulate this if it was assumed that Li^+ increased the time delay in the feedback loop and decreased the sensitivity of the oscillator to light pulses. Time delay is the parameter of the model which is largely responsible for determining the period of the oscillation, and thus an increase in its value will produce a lengthening in the period. As has been indicated lengthening of period is a known property of Li^+. A decrease in light sensitivity on the application of lithium has only been shown in one case, that of the cockroach (Rauch, Reinhard and Engelmann, unpublished, cited in Engelmann, 1987). Thus, it would seem that the model of Christensen and Lewis may provide a useful basis for further study into the effects of Li^+ on circadian rhythms.

The results of these simulations lead one to interpret the effects of Li^+, on a cellular level, as changes to the properties and transporting abilities of the membrane. Thus, lithium is known to interfere with many cellular functions which rely on ions or membrane transport, e.g. neurones (for review see Engelmann, 1987). These effects on cellular processes seem to partially depend on ionic substitutions with other cations, e.g. Na^+, K^+, Ca^{++} and Mg^{++}. This must inevitably alter the properties of excitable cells, change neurosecretion and membrane transport processes and affect the activity of some enzymes, such as adenylate cyclase (Engelmann, 1987). Interestingly Rb^+ ions seem to be able to reverse some of the effects of Li^+, but how or why this occurs is not known (Schmid and Engelmann, 1987; Engelmann, 1987).

Another outcome of these effects is that a variety of biochemical rhythms are seen to be affected by lithium, e.g. neurotransmitters and hormones. Furthermore, it has been found that experimental disruption of these same biochemical processes can influence the circadian rhythms of several organisms. Eskin (1982) found that treating the eye of *Aplysia* with the amino acid precursors of neurotransmitters (tryptophan and tyrosine) produced lengthening of the period by about 1.7 hours. Furthermore, the effect was seen to be much greater in L-tryptophan than in D-tryptophan, suggesting a biochemical rather than a physio-chemical site of action for the amino acids. Also, Earnest and Turek (1985) found that the neurotransmitter acetylcholine was an important constituent in the mechanism by which light regulates circadian rhythms in the golden hamster *Mesocricetus auratus*. Little work of this nature has been carried out on insects, but it has been shown that

azadirachtin, a compound which interferes with the insect ecdysteroid levels (Ludlum and Sieber, 1988), can shorten the period length and induce rhythm-splitting in the locomotor rhythm of the cockroach *Leucophaea maderae* (Han and Engelmann, 1987)

As many of the results of deuteration on the cellular milieu appeared to be very similar to those effected by lithium (and as both acted to slow down biological oscillations), it was surmised that they might produce their effects by a similar means. While D_2O was seen to affect almost all cellular processes, lithium tended to exert its influence at the level of the membranes, implying that the most significant area of D_2O's influence may be at the level of membrane function and stability.

Consequently, any experimental treatments which also disturbed the equilibrium of the cellular membranes could potentially affect biological oscillations. For this reason many protein inhibitors have been found to produce a range of effects on the rhythms controlled by such oscillations. For example, Feldman (1967) found that cycloheximide, an inhibitor of protein synthesis, lengthened the period of the phototaxis rhythm in *Euglena*, while Takahashi and Turek (1987) found that anisomycin, another protein synthesis inhibitor, induced dramatic phase changes in the activity rhythm of the golden hamster *Mesocricetus auratus*. Alterations in the phase response curve of *Aplysia* were also found when the animal was exposed to cycloheximide (Jacklet, 1977). Further experiments have also shown alcohols to affect biological rhythms (Enright, 1971b; Brinkmann, 1976). The influence of these alcohols also indicated that membrane stability may be important to the proper functioning of the oscillatory system.

It is clear, therefore, that most of the evidence points to membrane properties as being key factors in biological rhythms. Thus any disturbance to the membrane systems will inevitably affect the oscillatory output of the organism, the usual result of this being an increase in period.

One suitable model has already been mentioned to explain some of these results (Christensen and Lewis, 1982, 1983). This had, as one of its main components, a time delay factor, which was viewed as a representation of some sort of membrane within the system controlling the oscillation. Alternative models have also been proposed based on more detailed

biochemical knowledge (Njus et al., 1974). Although these are not within the scope of the present discussion it was clear that they also incorporated membranes as crucial elements to the working of the clock.

The experiments which follow attempted to observe the responses of the blowfly's locomotor rhythm to a variety of chemical treatments, and to ascertain whether they concurred with the findings from the other species described above.

Materials and Methods

All of the flies which were used in these experiments were bred in the manner described in the General Materials and Methods, and were allowed to eclose into a 12:12 light cycle. After 1-2 days the blowflies were placed in individual recorders, where they were exposed to the 12:12 cycle for another 3-6 days. At this point the light cycle was stopped and the flies were placed in continuous darkness, where they remained for the duration of the experiments.

In each case the chemical treatment was given to the flies in their drinking water, in the concentrations outlined below. It was clear that the flies were actually drinking these solutions, as they continued to live for sometime after the commencement of the experiments; flies which are deprived of any water source will die within 4-6 days. In the majority of the experiments the insects had continuous exposure to the particular chemical, although in one case the treatment was for short duration. The chemicals used were deuterium oxide (D_2O), lithium chloride (LiCl), ethanol and acetone. Deuterium oxide (99.8% pure) and ethanol were given as percentage concentrations, such that 100mls of a 5% solution contained 5mls of the chemical plus 95mls of distilled water. In both cases the concentrations used were 5%, 10%, 15% and 20%. On the other hand, the lithium and acetone were given as molar solutions, lithium having to be first dissolved. Acetone was provided as 0.8M and 0.08M while LiCl was given in concentrations of 0.1M, 0.05M, 0.01M and 0.005M.

One further D_2O experiment was undertaken in which the flies were exposed to a 5% solution for a limited time only, starting on the fifth day of the free-run and ending on the eleventh day. At this point the D_2O was replaced by fresh distilled water. As in the previous experiments the prior photoperiod was 12:12. As a form of internal control a number of flies were

subjected to the same conditions but maintained continuously on water.

The final experiment involved exposing both larval and adult stages to LiCl. This was achieved by using meat supplements in the larval medium, which had been first dried and then soaked in 0.005M LiCl for 24 hours. These cultures were then kept in the usual light and temperature regime. The resulting adults emerged into a 12:12 light cycle, where they remained for two days before being placed in the recorders. After a further two days at this photoperiod the flies were free-run in DD.

Results

Continuous Exposure to Deuterium Oxide

The flies in these experiments were presented with four differing concentrations of D_2O (5%, 10%, 15% and 20%) and the free-running patterns analysed over the first and second stages of the recordings.

Superficially the patterns seen in DD were identical to those previously described for flies exposed to water, with an initially short period lengthening after about 6 days in darkness. Furthermore, the overall distribution of the pattern types appeared similar to the controls in the previous chapter:

Clear and constant period:	13.5%	(N=5)
Clear but changing period:	83.8%	(N=31)
Complex patterns:	2.7%	(N=1)

Although the Stage 1 periods showed no significant variation as the concentrations of D_2O increased ($F=2.205$ with 3 and 33 d.f.), the Stage 2 periods were clearly related to the strength of deuterium present ($F=4.305$ with 3 and 31 d.f.). A regression analysis revealed a significant positive relationship between these Stage 2 periods and D_2O, with increasing concentrations of D_2O resulting in longer periods (see Fig. 5.1). Furthermore, the slope of this linear regression indicated that each 10% rise in D_2O resulted in c. 1.80% increase in the period of the rhythm. Comparisons made for both stages between the deuterated flies and water controls (from previous chapter) indicated that the periods were significantly longer at all points in the free-run in those flies

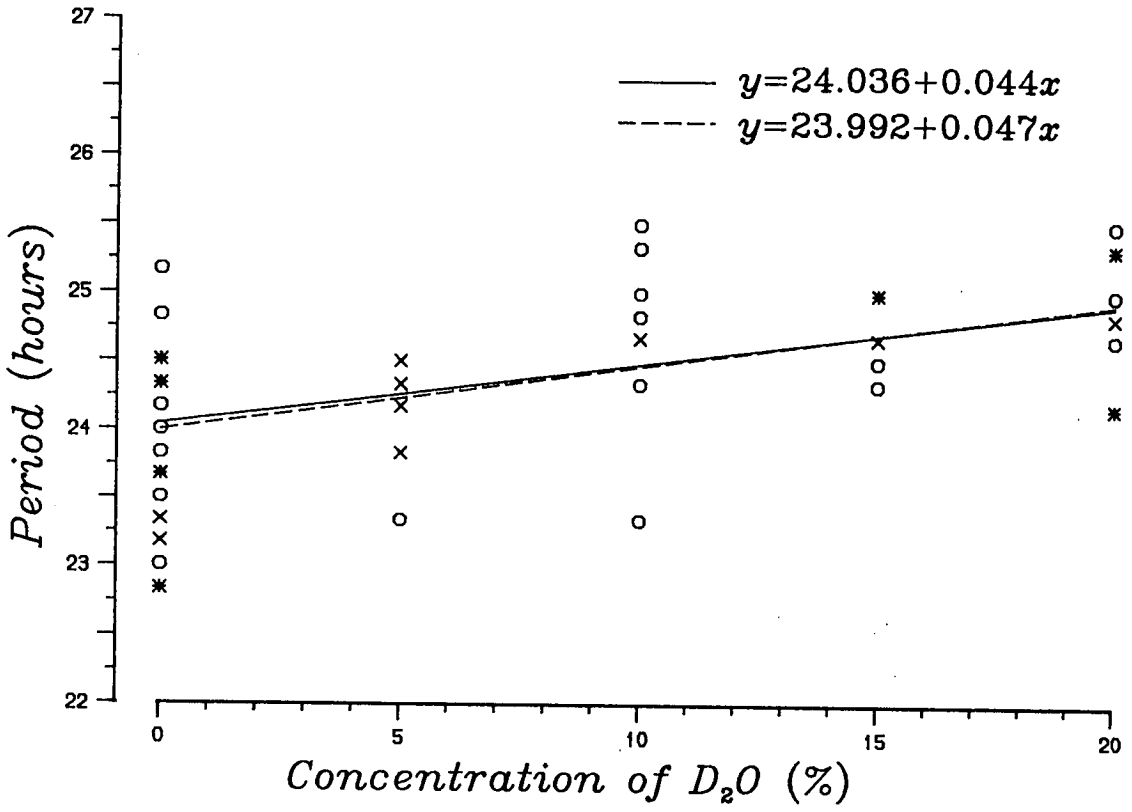

Figure 5.1. The relationship of Stage 2 free-running periods to increasing concentrations of Deuterium Oxide (D_2O).

Solid line. This represents the regression of the present D_2O data alone. The regression coefficient was found to be significant (t=3.125 with 33 d.f., $p<0.005$), as was the correlation coefficient (r=0.478 with 33 d.f., $p<0.01$; goodness-of-fit, F=9.768 with 1 and 33 d.f., $p<0.005$).

Dashed line. Inclusion of the water data from Chapter 4 at the 0% point on the x-axis had very little effect on the regression equation, with a significant correlation coefficient still being found (r=0.478 with 61 d.f., $p<0.001$; goodness-of-fit, F=18.064 with 1 and 61 d.f.). The circles represent one datum point, the crosses two and the asterisks three or more data points.

which had experienced D_2O (Stage 1, t=4.938 with 59 d.f.; Stage 2, t=3.301 with 47 d.f.). Interestingly, the average change in τ between the first and second stages was not found to differ significantly when D_2O was used instead of water (t=0.603 with 58 d.f.). The average periods, active-phase lengths and amplitudes for each stage are contained in Table 5.1.

Consideration of the active-phase length revealed no differences between the combined data and the water controls (Stage 1, t=1.024 with 60 d.f.; Stage 2, t=0.453 with 60 d.f.) although both stages showed significant variations as the concentration of D_2O was altered (Stage 1, F=7.097 with 3 and 33 d.f.; Stage 2, F=3.622 with 3 and 31 d.f.). With a regression analysis the slope of the relationship was seen to be negative for both stages, with increasing strengths of D_2O resulting in shorter active-phase lengths (Fig. 5.2). It was also noted that the second stage active-phase lengths appeared to be more affected by the changing D_2O concentrations than Stage 1 values, as evidenced by the steeper gradient for the former stage. However, this difference was not found to be significant (see Fig. 5.2). Further analysis on the amplitude of the activity found no variation either between the concentrations within each stage (Stage 1, F=0.801 with 3 and 33 d.f.; Stage 2, F=1.978 with 3 and 31 d.f.) or between the present experiment and the water controls (Stage 1, t=0.193 with 61 d.f.; Stage 2, t=0.605 with 60 d.f.).

Therefore, it was seen that D_2O lengthened the periods in all parts of the free-run, with the response being dose-dependent during the second stage of the free-run. The active-phase length was also clearly negatively dose-dependent over both stages.

Short Exposure to Deuterium Oxide

In these experiments, in addition to the treated flies, there was a group of control flies maintained in identical conditions but kept continuously on water. These controls did not differ from the large control group in the previous chapter (Stage 1 τ, t=0.222 with 14 d.f.; Stage 2 τ, t=1.202 with 32 d.f.; Stage 1 α, t=1.216 with 27 d.f.; Stage 2 α, t=2.009 with 32 d.f.; Stage 1 amplitudes, t =1.242 with 24 d.f.), although the Stage 2 amplitudes were seen to be far lower in the small internal control than had been observed in the prior controls (t =3.713 with 33 d.f.). It was not known why this should be, although the value at the second stage for the present control (6.66±3.38 units) did appear to be

Free–running pattern from a 12:12 regime	*Stage 1*	*N*	*Stage 2*	*N*	*Stage 3*	*N*
Mean free–running period (hours±s.d.)	23.45±0.73	37	24.60±0.55	35	-------	
Mean duration of activity per cycle (hours±s.d.)	12.89±2.08	37	14.62±3.30	35	-------	
Mean amplitude of activity per cycle (units±s.d.)	14.30±6.31	37	14.16±7.97	35	-------	

Table 5.1. The data on the parameters of the free-run in flies continuously exposed to D_2O. The results were averaged for each stage over all of the concentrations used.

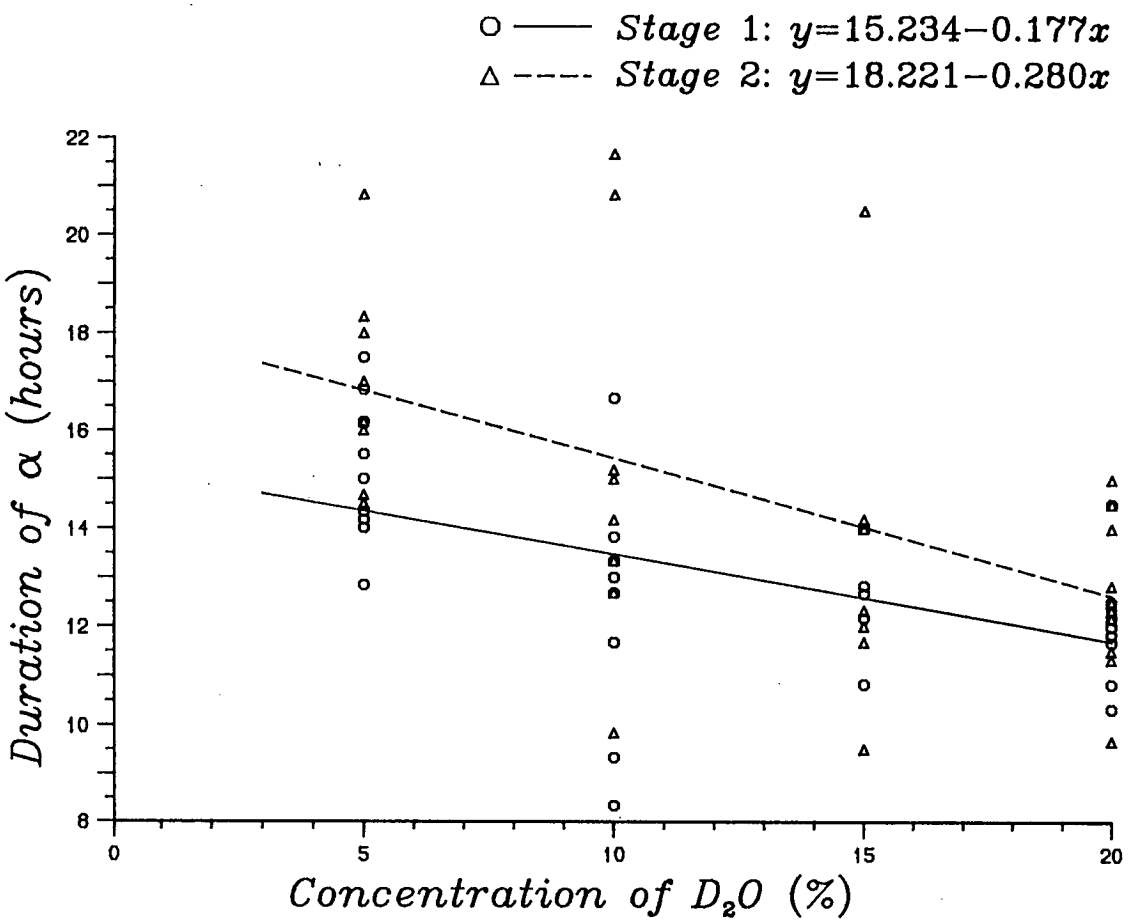

Figure 5.2. The relation between the active phase length (α) and the concentration of D_2O.

Solid line. Data from Stage 1. There was found to be a significant regression coefficient (t=3.540 with 35 d.f., $p<0.005$) and correlation coefficient (r=0.513 with 35 d.f., $p<0.01$; goodness-of-fit, F=12.532 with 1 and 35 d.f., $p<0.005$).

Dashed line. Stage 2 data. Here also there was a significant negative regression (t=3.378 with 33 d.f., $p<0.005$) and correlation coefficient (r=0.507 with 33 d.f., $p<0.01$; goodness-of-fit, F=11.411 with 1 and 33 d.f., $p<0.005$). A comparison of the two regression coefficients revealed that the relationship of α to D_2O was not significantly different between the two stages (d=1.063, $p>0.1$).

unusually low in comparison to most of the previous experiments.

The D_2O pulses were given after the initial lengthening of τ, largely because it was known that the period of the free-run tended not to change from this point onward. Furthermore, it also appeared that the homoeostatic control of the pacemaker may have been weaker at this point in the free-run, thus allowing any effects of D_2O to be more easily expressed. Examination of the records of such deuterated flies clearly indicated that their periods were lengthened (e.g. Fig. 5.3), although this was not large enough to prove significant when compared to the internal water control (H_2O τ=23.89±0.83, D_2O τ=24.60±0.52; t=2.046 with 11 d.f.). When D_2O was once more replaced by water the subsequent periods were found not to be significantly different from the internal control (t=1.977 with 13 d.f.). This lack of significance was probably due to large individual variations in response, for while some flies showed marked lengthening effects with D_2O others appeared to be completely unaffected. Furthermore, no significant differences appeared in either the length or amplitude of the activity when the water and deuterated runs were compared over the specific portion of the records (t=0.965 with 9 d.f. and t =1.564 with 8 d.f. respectively). Equally, both of these parameters were indistinguishable from the controls after the D_2O treatment (α, t=1.432 with 10 d.f.; amplitude, t=1.113 with 12 d.f.).

Thus, it was apparent that in some cases D_2O effected a small lengthening of the period immediately after its application, although once the water was replaced the period was seen to shorten within 1-2 cycles. The rapidity of these responses implied that D_2O was probably exerting its effects as a free molecule, rather than being substituted for hydrogen in various macromolecules.

Ethanol

Ethanol was chosen as an appropriate alcohol to use on the blowflies' activity rhythm as it had been previously shown to have detectable effects on the period of activity in other species. Its application in the present instance resulted in free-running patterns similar to the water controls, although there was a tendency for the rhythmic pattern to be slightly 'messy', particularly at the end of the recordings, with extra activity occurring in the subjective night of the free-run (see Fig. 5.4). Furthermore, there was no indication that

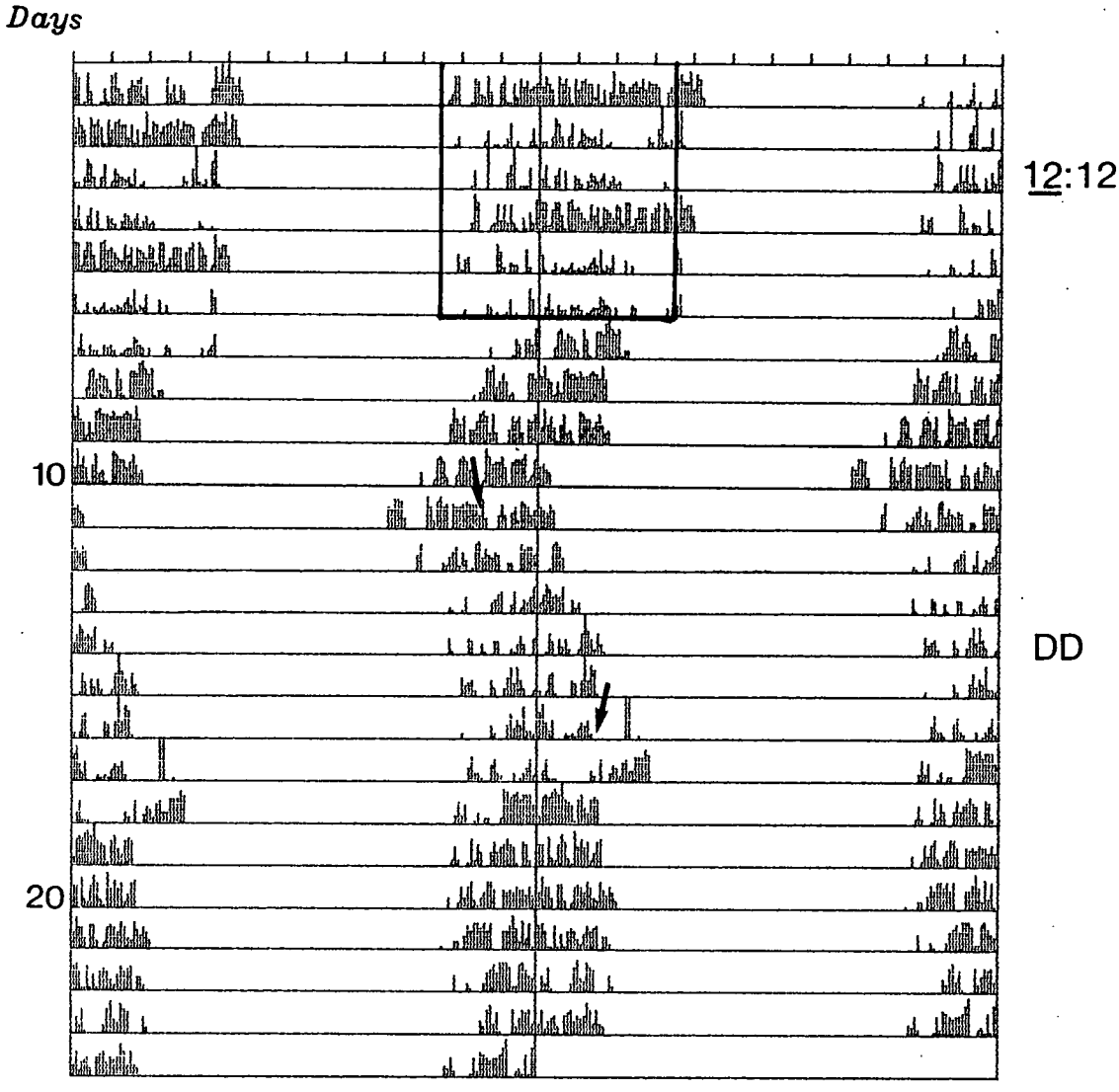

Figure 5.3. The activity pattern produced by short term application of 5% D_2O in the drinking water of the adult blowfly. The duration of the exposure to the chemical is marked by the two arrows. This clearly shows that D_2O can exert its effects very rapidly.

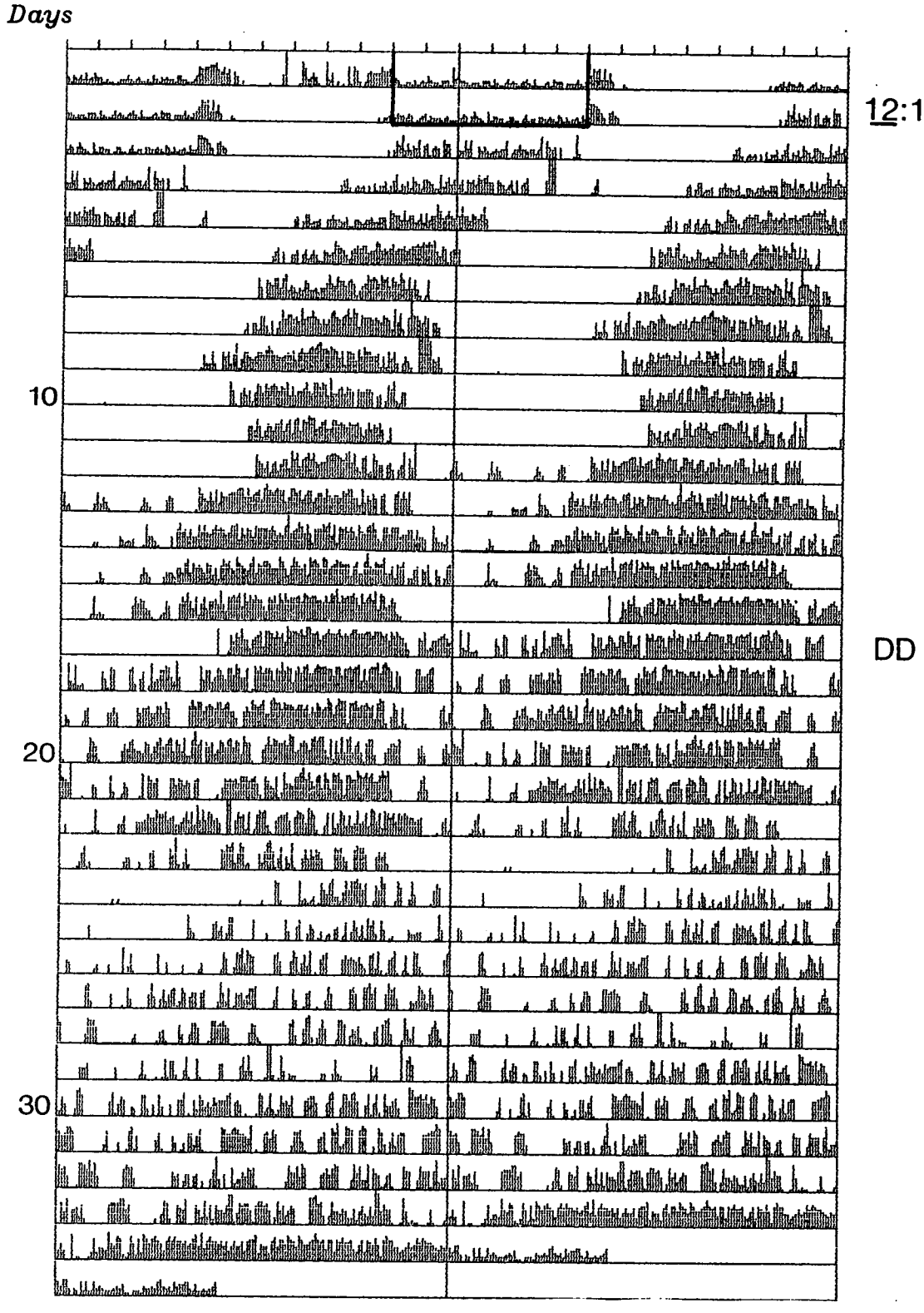

Figure 5.4. Continuous exposure to ethanol (in this case 10%) tended to induce a breakdown in the activity pattern, although the main rhythmic elements could still be discerned. This disintegration did not appear to be dose-dependent.

increasing the alcoholic concentration increased the likelihood of such destabilization. Fortunately, the disintegration of the pattern did not hamper their analysis, as the main rhythmic elements were still very distinct in the early parts of the observations.

The activity patterns which were seen fell into the following percentage groups:

Clear and constant period:	7.4%	(N=2)
Clear but changing period:	85.2%	(N=23)
Complex patterns:	7.4%	(N=2)

In common with the D_2O treatment, the distribution of these activity types matched those seen with the water alone. Analysis of the three parameters of the free-run (average values at each stage given in Table 5.2) firstly revealed no significant variation in the periods of the first two stages with changing concentration of ethanol (Stage 1, $F=0.959$ with 3 and 24 d.f.; Stage 2, $F=1.232$ with 3 and 21 d.f.). The average periods for each stage (Table 5.2) were also found not to differ from previous controls (Stage 1, $t=0.160$ with 50 d.f.; Stage 2, $t=0.809$ with 44 d.f.). The same invariance was found for the length of the active phase, both between the differing concentrations (Stage 1, $F=0.488$ with 3 and 24 d.f.; Stage 2, $F=0.904$ with 3 and 21 d.f.) and on comparison to the water controls (Stage 1, $t=1.792$ with 53 d.f.; Stage 2, $t=0.473$ with 48 d.f.). Equally, the amplitude of the rhythm showed little variation between concentrations (Stage 1, $F=1.491$ with 3 and 24 d.f.; Stage 2, $F=1.893$ with 3 and 21 d.f.) and with the controls (Stage 1, $t=1.354$ with 53 d.f.; Stage 2, $t=0.843$ with 47 d.f.).

As well as these results being non-significant there did not appear to be any trends within the data which may have implied that low sample sizes were masking some of the effects of the ethanol. Therefore, it was evident that while ethanol did precipitate a breakdown in the free-running pattern towards the end of the recordings (albeit in a non-dose-dependent manner) it had no appreciable effects on the three parameters of the free-run which were analysed.

Free–running pattern from a 12:12 regime	*Stage 1*	*N*	*Stage 2*	*N*	*Stage 3*	*N*
Mean free–running period (hours±s.d.)	22.73±0.63	28	24.14±0.53	25	--------	
Mean duration of activity per cycle (hours±s.d.)	14.50±2.15	28	13.95±2.08	25	--------	
Mean amplitude of activity per cycle (units±s.d.)	17.11±7.36	28	14.76±8.01	25	--------	

Table 5.2. The results of continuous adult exposure to ethanol. Again the data have been averaged for each stage over all of the treatment concentrations.

Acetone

The patterns produced under this treatment were distinctive in several respects. Firstly, although the period changed in the normal manner after 6-10 days into the free-run it was seen to 'fracture' as it lengthened (see Fig. 5.5). Furthermore, comparisons of the degree of lengthening in these experiments and the controls found that acetone induced significantly greater lengthening (pooled acetone data, t=2.291 with 43 d.f.), a feature which can also be discerned in Fig. 5.5. Although the pattern break-up resulted in active phases interspersed with short intense bursts of extra locomotor activity, the main rhythmicities were still strong enough to allow analysis. Similar patterns have also been seen on rare occasions in the presence of water. Interestingly, the disintegration of the rhythm rarely advanced further than the fractured stages, and there was no evidence of separate free-running rhythmic elements, as had been seen in previous split rhythms.

Analysis revealed that there was no variation between the two concentrations of acetone used (0.8M and 0.08M) in terms of the period, duration or intensity of the activity in either stages of the free-run (Stage 1 τ, t =0.834 with 12 d.f.; Stage 2 τ, t=1.760 with 14 d.f.; Stage 1 α, t=0.272 with 15 d.f.; Stage 2 α, t=0.175 with 15 d.f.; Stage 1 amplitude, t=0.190 with 14 d.f.; Stage 2 amplitude, t=0.104 with 15 d.f.). Equally, the slight break-up of the pattern did not appear to unduly affect the characteristic lengthening of the rhythm, with all of the flies at both concentrations showing a significant increase in period (0.8M, t=5.739 with 15 d.f.; 0.08M, t=6.044 with 11 d.f., total N=18).

Comparison of the averaged acetone data (Table 5.3) with water controls showed that there was no significant difference in the active-phase lengths of the two treatment conditions (Stage 1, t=1.202 with 43 d.f.; Stage 2, t=0.918 with 43 d.f.). Equally, the Stage 1 periods were indistinguishable from the controls (t=1.931 with 33 d.f.). The Stage 2 periods, on the other hand, were found to be significantly longer in the group experiencing acetone (t=3.990 with 43 d.f.). When the amplitude of the activity was analysed it was clear that acetone had induced substantial increases over the previous controls (Stage 1, t =2.467 with 25 d.f.; Stage 2, t=3.373 with 26 d.f.).

Evidently, acetone did produce some marked changes to the free-running

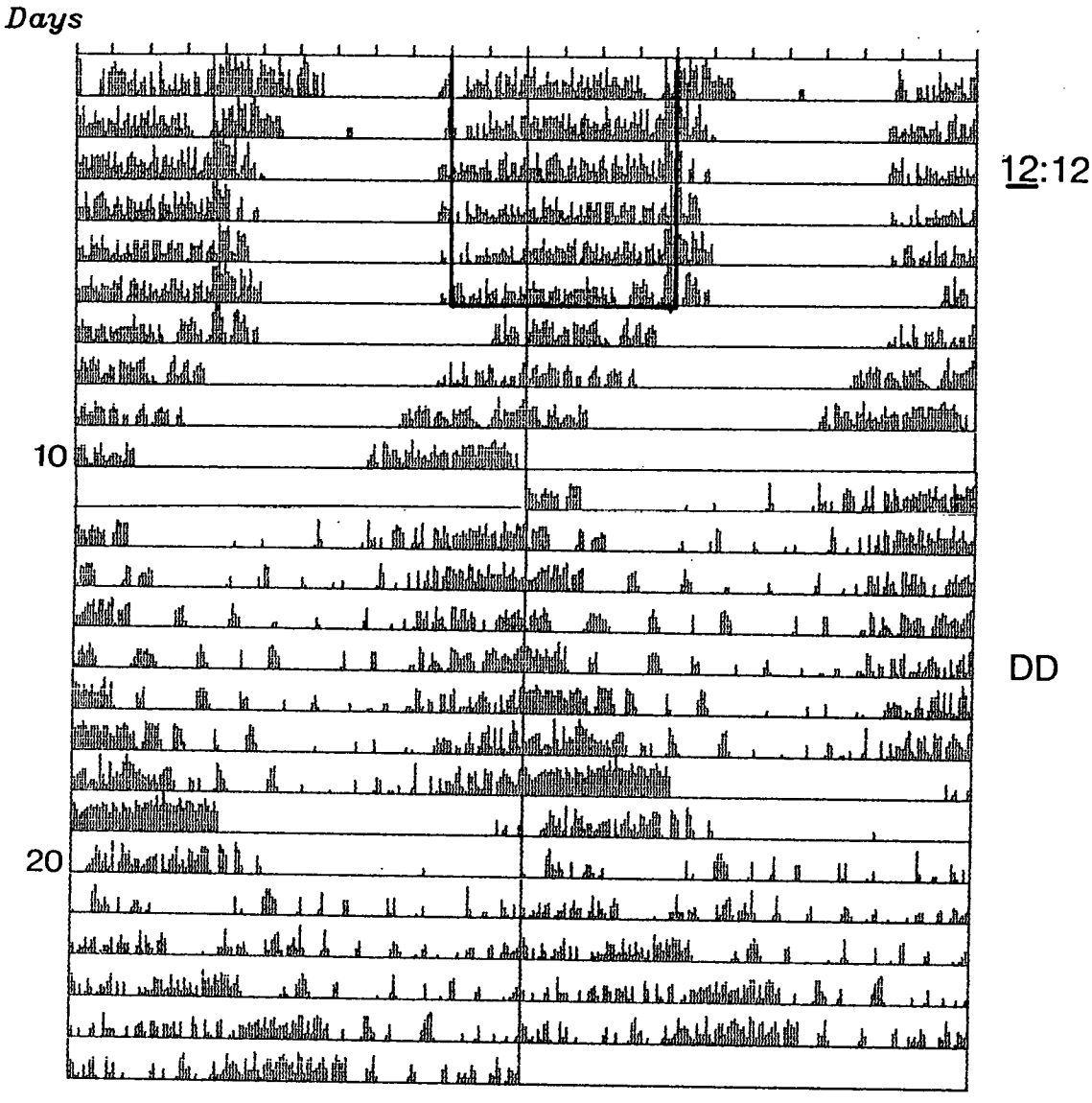

Figure 5.5. This represents the effects of continuous 0.8M acetone treatment. Note that the pattern only begins to disintegrate during the lengthening of the period, which develops as an after-effect to the previous entrainment. Although it is hard to distinguish from the figure, acetone also increased the amplitude of the overall activity. The data from day 11 was lost due to a computer crash.

Free-running pattern from a 12:12 regime	*Stage 1*	*N*	*Stage 2*	*N*	*Stage 3*	*N*
Mean free-running period (hours±s.d.)	23.05±0.66	18	24.92±0.72	18	--------	
Mean duration of activity per cycle (hours±s.d.)	12.68±1.99	18	14.99±2.44	18	--------	
Mean amplitude of activity per cycle (units±s.d.)	22.89±13.28	18	23.96±12.67	18	--------	

Table 5.3. The results of continuous exposure to acetone. As with the previous tables, the data have been averaged over the two treatment concentrations. It should also be noted that the amplitude of the activity was greatly increased by the presence of acetone.

pattern of the blowfly, especially with regard to the amplitude of the activity. Furthermore, the lengthening of the period which occurs as an after-effect of entrainment was seen to be exaggerated by the presence of acetone. This effect, and the fractured pattern, suggested that acetone was perhaps influencing the coupling between components of the circadian system.

Adult Exposure to Lithium Chloride

Four differing LiCl concentrations were used in these experiments, namely 0.1M, 0.05M, 0.01M and 0.005M. Of these the 0.1M solutions proved to be fatal after only a few days exposure. The majority of the surviving flies at the other concentrations showed disrupted patterns in their free-run, although the usual lengthening of τ could still be seen in almost all cases.

Analysis found that the differing molarities of LiCl did not produce significantly different periods or lengths to the active phases (Stage 1 τ, $F=0.123$ with 2 and 16 d.f.; Stage 2 τ, $F=0.621$ with 2 and 12 d.f.; Stage 1 α, $F=1.138$ with 2 and 16 d.f.; Stage 2 α, $F=2.704$ with 2 and 12 d.f.). Although the first stage amplitudes were equally invariable with increasing concentrations of LiCl ($F=1.023$ with 2 and 16 d.f.), those in the second stage were significantly different with differing concentrations of LiCl ($F=8.020$ with 2 and 12 d.f.). This may, however, have been an aberrant result as it seemed that the significance was entirely due to an unusually low value at 0.005M LiCl.

An internal water control run with the experimental flies appeared in all respects identical to the previous water controls (Stage 1 τ, $t=0.678$ with 9 d.f.; Stage 2 τ, $t=0.406$ with 26 d.f.; Stage 1 α, $t=1.249$ with 26 d.f.; Stage 2 α, $t=0.672$ with 30 d.f.; Stage 1 amplitude, $t=1.473$ with 19 d.f.; Stage 2 amplitude, $t=1.485$ with 20 d.f.). Comparison of the LiCl treatment (see Table 5.4 for the average values) with this internal control indicated that the periods seen in LiCl, although on average longer, were not significantly different from those flies which had only experienced water (Stage 1, $t=1.872$ with 18 d.f.; Stage 2, $t=1.681$ with 17 d.f.). The same picture emerged on analysis of the active-phase length and the amplitude of the activity (Stage 1 α, $t=0.818$ with 20 d.f.; Stage 2 α, $t=1.225$ with 16 d.f.; Stage 1 amplitude, $t=0.570$ with 21 d.f.; Stage 2 amplitude, $t=0.832$ with 17 d.f.).

Thus, although LiCl was seen to produce marked disruptions to the free-running pattern this was not reflected in the any of the parameters of the

Free–running pattern from a 12:12 regime	*Stage 1*	*N*	*Stage 2*	*N*	*Stage 3*	*N*
Mean free–running period (hours±s.d.)	23.17±0.91	19	24.68±0.96	15	--------	
Mean duration of activity per cycle (hours±s.d.)	14.46±2.15	19	15.94±3.15	15	--------	
Mean amplitude of activity per cycle (units±s.d.)	16.59±9.52	19	14.07±9.44	15	--------	

Table 5.4. The results of continuous adult exposure to lithium chloride. Once again the data presented here were averaged over the three treatment concentrations.

rhythm which were analysed. This is, in some respects, surprising as LiCl has been previously found to produce substantial lengthening of the period in a large variety of species.

Larval exposure to Lithium Chloride

Culturing the larvae in the presence of LiCl (albeit at a relatively low concentration, 0.005M) produced highly abnormal patterns of locomotion in the resulting adults (see Fig. 5.6 and 5.7). Although some of the flies did show period changes they were clearly different from the observations made with water. Unfortunately, the presence of such a wide degree of individual variability made any meaningful quantitative analysis impossible and necessitated the use of qualitative descriptions of the patterns. Thus, using the previous groupings, it was found that the activity types could be divided as follows:

Constant period:	48.15%	(N=13)
Changing period:	22.22%	(N=6)
Complex patterns:	29.63%	(N=8)

Of the changing periods, some were clearly lengthenings while others displayed a shortening of τ. Many of the complex patterns showed a normal lengthening pattern which broke down just after the period increased, in most of these (7 out of the 8) the rhythm was seen to split into two separate elements (Fig. 5.7a). Interestingly, the shape and position of the split was fairly consistent between the individual flies, always occurring several days after the period lengthened and requiring 2–3 days for completion. Finally, analysis of the constant period rhythms revealed a wide spectrum of responses, from those with periods of less than 24 hours, through periods of about 24 hours, to τs longer than 24 hours (see Fig. 5.6). It was also evident that the relative distribution of the activity pattern types was quite different from any of the prior experiments, although the significance of this observation was uncertain.

In conclusion, it appeared that exposure of the larvae to low levels of LiCl produced dramatic effects on the adult locomotor activity. Inevitably, this raised many questions on the relationship of the larval clocks to the adult oscillatory system, some of which will be considered in the following discussion.

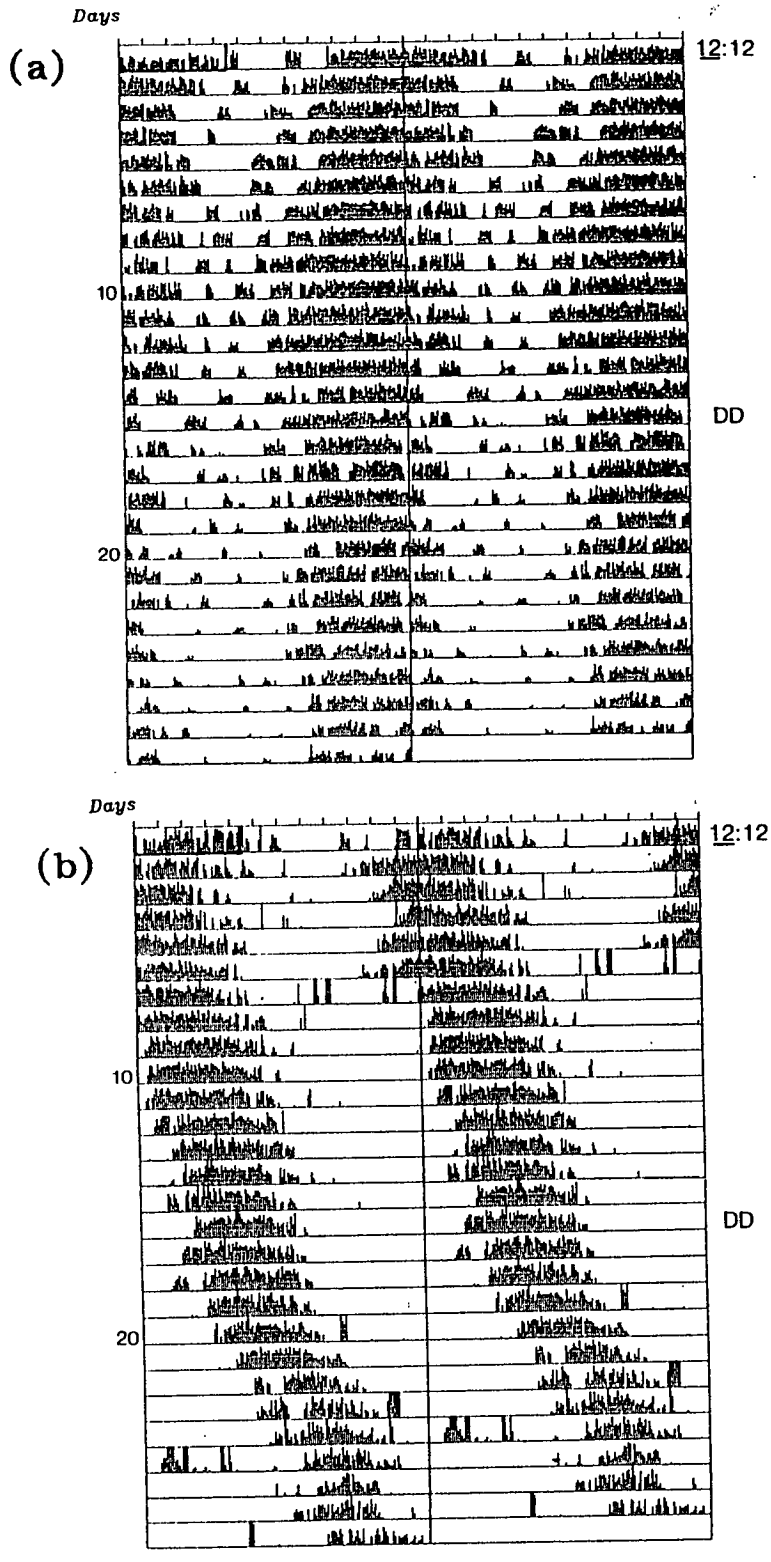

Figure 5.6. Two activity records produced by blowflies which had been bred in the presence of 0.005M lithium chloride (LiCl). (a) A pattern with a constant period close to 24 hours. (b) A rhythm with a period longer than 24 hours.

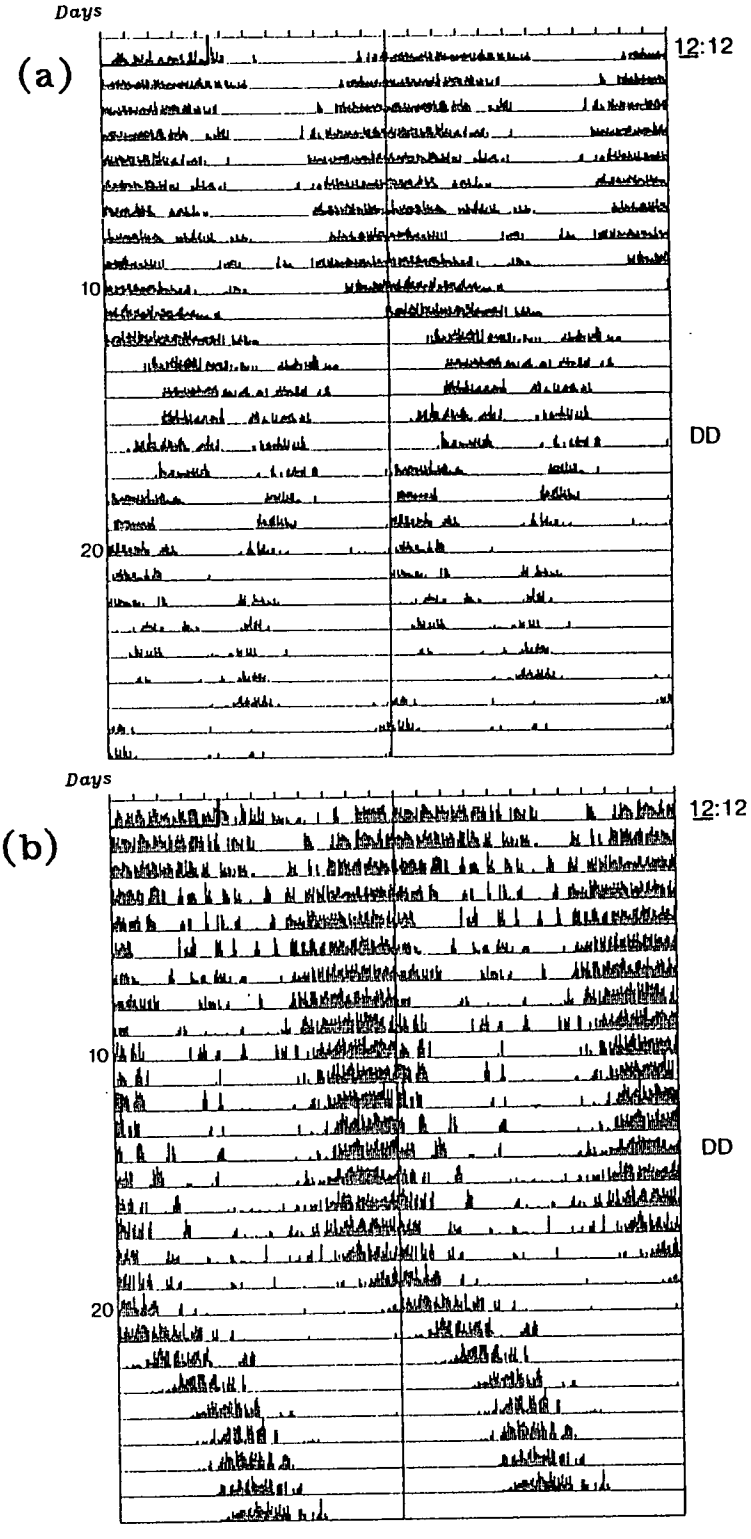

Figure 5.7. Two further examples of the effects of larval exposure to 0.005M LiCl. (a) This record contains a clear example of rhythm-splitting. This effect was seen in a number of the LiCl experiments, with the split usually occurring several days after the lengthening after-effect. (b) An unusual form of the entrainment after-effect in which the full lengthening is only seen after 10 days of intermediate period transients.

Summary

1. While continuous D_2O exposure produced significant lengthening of the free-running period, this was only found to be dose-dependent during the second stage of the free-run. Also, despite the pooled data for α and amplitude being indistinguishable from the water controls, α clearly had a negative relationship to increasing D_2O concentrations, in the first two stages of the free-run.
2. A short exposure time to low D_2O concentrations had slight effects on the period and none on α or amplitude. These effects appeared to occur instantaneously and were equally quickly overcome when the water was replaced.
3. Ethanol, administered continuously, produced no detectable effects on the free-running rhythm, although there was a tendency for the pattern to disintegrate with the treatment.
4. Acetone also disrupted the rhythmic pattern, although this only seemed to occur specifically at the point where the period lengthened. The degree of the period lengthening, and thus the subsequent Stage 2 periods, were also increased by the acetone treatment.
5. Although acetone produced no dose-dependent effects, and α was unaffected by its presence, the amplitude of the activity was clearly increased by acetone application.
6. Lithium chloride produced marked effects on the free-running activity pattern, especially when experienced during the larval stages of development. Despite this, neither the period nor the active-phase length, or the amplitude of the activity were affected by the concentrations of LiCl used here.

Discussion

It has been hypothesized in previous chapters that the periods exhibited in the first part of the free-run immediately after entrainment (Stage 1 in the present nomenclature) closely resembled the true unperturbed period of the oscillator (or compound oscillatory system). The periods during this stage were usually less than 24 hours and appeared to be temperature-compensated, implying that this point in the free-run represented a relatively stable oscillatory system. On the other hand, the second part of the free-run (Stage 2) was characterized by periods equal to, or greater than, 24 hours, periods which did not appear to have a temperature-compensating mechanism. Thus, this portion of the free-run was considered to reflect a partially destabilized circadian system. The interpretations of the present experiments, in which the circadian system was artificially affected by chemicals, was considered in the light of these hypotheses.

As indicated in the introduction, deuterium oxide was known to lengthen the period of the rhythmic patterns in a number of species, sometimes in a dose-dependent manner. The present experiments found that both stages of the free-run were significantly lengthened by D_2O, although the details of this lengthening differed. Thus, the first stage was uninfluenced by changing D_2O concentrations, while Stage 2 displayed a positive relationship between its period and the level of D_2O present in the drinking water. The slope of this correlation (1.8% increase in τ for every 10% rise in the D_2O concentration) was found to be very similar to other studies (reviewed in Enright, 1971a) where values of about 2% for each 10% rise in D_2O were found. Enright viewed these near identical dose-dependent curves for D_2O, among very different organisms, as reflecting some fundamental similarity in the mechanism of their rhythms. While this may be the case, the present study was more concerned with why such dose-dependent effects were only present in the second stage of the free-run.

Although D_2O does increase period the changes induced have always been found to be quite small. For this reason, Pittendrigh and Caldarola (1973) intimated that the oscillator may be homoeostatically protected from greater disturbance by environmental factors, including D_2O. Thus, the well known temperature-compensation may simply be a special example of a more general

homoeostatic mechanism. Subsequently the 'low temperature equivalence' theory (Pittendrigh et al., 1973) attempted to explain the action of D_2O in terms of its similarity to the effects of low temperatures.

The present results indicated that while D_2O generally depressed the frequency of the oscillation in all parts of the free-run, dose-dependent effects were only observed in Stage 2. The effects of temperature were also only seen during this second stage. Thus, assuming that the first stage of the free-run was homoeostatically protected while the second stage was not, D_2O must be affecting the rhythm on several different levels. Firstly, it appeared to be acting as a general rate-depressor, possibly due to some of its differing properties as a solvent, an effect which lower temperatures did not exhibit. Secondly, although the homoeostatic system could not prevent a small increase in period due to the D_2O, it was able to limit the changes which occurred as the D_2O concentrations increased. Similarly, the system had limited the effects of temperature on the Stage 2 period.

Therefore, it may be that D_2O application *per se* does not relate to low temperatures, but rather the change in the degree of its influence (brought about by changing concentrations) equates to the effects of changing temperatures.

It therefore appeared as if some specific action of D_2O had changed the 'reference' period used as the basis for the homoeostatic machinery, without altering its ability to protect the rhythm from further changes. Thus, when the circadian system is relatively stable (as it appeared to be during Stage 1) changing the temperature or increasing the strength of D_2O has no detectable effect on the period of the rhythm. However, once the system is destabilized (as it probably was during Stage 2) the homoeostatic mechanism breaks down, with the result that the rhythm's period is found to respond to environmental fluctuations of temperature and D_2O. Therefore, although the present experiments with *C. vicina* found decreasing temperature and increasing D_2O concentrations both acted to slow the Stage 2 rhythm, such similarity of action may only be a reflection of the general homoeostatic system. The fact that D_2O altered all stages of the free-run, while temperature did not, suggested that this may be the case.

Although the period of the rhythm appears to be largely controlled by the

pacemaker (and thereby is also tightly regulated in the slave oscillatory system), the active-phase length and the amplitude of the activity may be more flexible in their control, and could be influenced by downstream, non-temperature-compensated, processes (Pittendrigh and Bruce, 1957, 1959). Thus, it was not surprising to find that active-phase length displayed a relationship to changing D_2O concentrations in both stages of the free-run, with increasing strengths of D_2O inducing a shorter α. As the previous chapter had shown that increasing temperatures also reduced the active-phase length (and the amplitude) of the activity, the action of D_2O cannot be directly equated with the lowering of the temperature.

The mode of action of acetone had many similarities to D_2O, in that the period was only altered in Stage 2. However, the effects of acetone differed from D_2O in that acetone produced a far greater degree of period lengthening between the first two free-running stages, while the same change was found to be indistinguishable between water and D_2O. The main effect of acetone was, however, to increase the amplitude of the rhythm. Unfortunately, as this was the first time that acetone had been found to affect circadian rhythms there was no literature on what its action might be. Although, it has been found as the ultimate product of fatty acid breakdown in mammals it is uncertain whether this relates to its effects on insect locomotor activity rhythms. However, as *C. vicina* breeds in an environment of decaying meat, it is possible that acetone may be encountered in nature. Interestingly, a series of preliminary experiments clearly indicated that feeding meat to the adult flies greatly increased the amplitude of their activity. Thus, acetone may be implicated in this process. Although this effect of meat-feeding has been previously noted for some insect species, e.g *Lucilia cuprina* (Bartell et al., 1969) and *C. vicina* (Sanderson and Charnley, 1983), it is unknown what ecological advantages are gained from such increases (see Curio, 1976).

Short term application of a 5% solution of D_2O also produced small, though on average non-significant, increases in the period. Despite this, effects could be discerned in the individual records, where some flies showed clear lengthening with D_2O while others were unaffected by the treatment. Thus in hindsight stronger D_2O solutions may have been more effective in producing clear period increases. Those insects which did have lengthened rhythms were seen to respond almost instantaneously to the application of the deuterium. Equally, the replacement of water for D_2O resulted in a very rapid shortening of

the period to a value indistinguishable from the controls. The obvious conclusion from this is that the body fluids of the blowfly were exchanged very rapidly, and D_2O's effects changed correspondingly. Daan and Pittendrigh (1976b) found similar rapid lengthening with D_2O in four rodent species.

Such time constants of uptake and loss of D_2O in these various species tends to exclude secondary isotopic substitutions (of deuterium for hydrogen) as the means by which period lengthening occurs, for deuterium incorporation as organically bound hydrogen is a very slow process, especially in the brains of mammals (Katz et al., 1962), the recognized site of the pacemaker. On the other hand, D_2O in solution rapidly crosses the blood/brain barrier (Daan and Pittendrigh, 1976b) and is probably instantaneously present in the fluid around the pacemaker, where it can immediately induce changes as a result of its solvent properties. In the rodent species investigated by Daan and Pittendrigh the changes in period took several days to complete, perhaps due to the presence of quantities of metabolic water. Therefore, as it is likely that D_2O is incorporated equally rapidly into the tissue fluid of insects and they may have less metabolic water available, the effects of D_2O will be even more rapid. The results obtained from the present experiments seem to be in general agreement with this hypothesis.

Brinkmann (1976) working with the flagellate *Euglena gracilis* found that ethanol probably produced period lengthening by a metabolic rather than an unspecified physical action. Furthermore, it was clear that dose-dependent effects of ethanol were not seen, but rather temperature changes altered the ability of ethanol to produce lengthened periods. This also indicated a metabolic function for the action of ethanol. Crucially, he also discovered that only those alcohols which could be metabolized via the oxidative pathways were able to lengthen the period. As insects are generally more complex organisms than either *Euglena* or the other species which have shown effects of alcohols (mainly plants), the lack of effectiveness of ethanol in inducing changes to the rhythmic pattern of the blowfly may arise from its effects being concealed by the sugar which the flies are constantly consuming, and which also feeds into the oxidative pathways. Therefore, in hindsight it may have been necessary to alter the temperature before any substantial changes to the period could have been induced by ethanol.

The effects of Li^+ on insect rhythms have in general been far smaller than

those observed with D_2O. However, Reinhard (1983, cited in Engelmann, 1987) suggested that some effects of Li^+ might be explained by one of several effects:

1. an increase in the sensitivity of the photoreceptors or the oscillators to the Zeitgeber (light in this case).
2. a weakening of the strength of the oscillators.
3. a change in the mutual coupling strength of the oscillators.

Subsequent experiments have partially ruled out the first of these, as it was found that the sensitivity of the photoreceptors actually decreased with Li^+ treatment in cockroaches (Rauch, Reinhard and Engelmann, unpublished, cited in Engelmann, 1987).

In the present experiments no detectable effects of LiCl were found on the period, active-phase length or the amplitude of the activity patterns. However, this was not surprising considering that previous researchers have only found small effects on period. Furthermore, the periodogram used for this study had a lower resolution of 0.17 hours (10 mins), which roughly equated to the size of many of the increases which had been found with Li^+. Thus, even if slight increases were present in the data it may have been almost impossible for them to be detected. It is, however, equally possible that there were no effects, for Hofmann et al. (1978) found that, while some cockroaches did show lengthened periods, many were unaffected by the treatment.

Li^+ did however have marked effects on the stability of the activity pattern, especially when present in the larval medium, and frequently resulted in a high degree of rhythm-splitting. This suggested that LiCl was profoundly affecting the overall coupling of the components of the system, perhaps by disrupting the normal development of the adult circadian system, thereby implying that some aspects of the circadian system only become fully determined post-oviposition. Although circadian oscillations have been found to influence other developmental events, little work has been carried out on their own ontogeny (for reviews, see Davis, 1981; Konopka, 1981): although beyond the scope of the present discussion this subject will be presented in greater detail in the following chapter. Therefore, the disruption to the circadian system brought about by LiCl clearly concurred with the third of Reinhard's proposals, adding weight to the assumption that this may be the area of influence of the Li^+ atom.

In conclusion, it was found that some chemical treatments could produce dramatic effects on the free-running locomotor activity rhythm of the blowfly. In many cases these results were found to be in agreement with previous concepts on the action of chemicals on circadian rhythms. Although some of the chemicals used were able to produced a lengthening of the period (if they had any effect at all) the details of their actions were not found to be the same. Furthermore, the degree to which they disrupted the coupling of the system was also seen to vary.

CHAPTER 6

Effects of Photoperiod on Circadian Development

Introduction

Although research into physiological processes has necessarily involved many differing areas of science, much recent attention has focused on attempts to understand the ways in which these processes emerge as organisms develop and mature. It is now clear that such ontogeny involves the entire life history of individuals, from the fertilized egg, through the functional maturation of their behavioural and reproductive systems, to their subsequent decline with age. As many of these functions also show some degree of circadian rhythmicity, any ontogenetic investigation should include studies on the appearance and expression of these rhythmic systems.

One of the first questions which arises in considering circadian development is how much information is transmitted from the parent to the offspring. Research into the genetics of circadian systems has found many heritable mutations affecting the free-running period (see Konopka, 1981 for a review). However, other than these studies on the encoding of specific periods in the DNA, little work has been done on whether other aspects of the circadian patterns are genetically transmissible. One of the few possible examples comes from an early study by Bateman (1955) in which he claimed to find that the phase of the pupal ecdysis rhythm could be transmitted from mother to offspring in the Queensland fruitfly *Dacus*. Subsequently, in an apparently non-circadian system, Lees (1960) found an interval timer in the aphid *Megoura* (controlling the ability to respond to short photoperiods) which was able to persist through several generations. Thus, independent of the number of generations, a non-temperature-compensated timing mechanism determined when the switch from production of parthenogenetic offspring to the production of male and female offspring would occur.

Another important area of developmental research concerns the manner in which differing functions reach maturity at different times. In terms of the developmental emergence of circadian rhythms, the bulk of such work has been confined to descriptive accounts of the process. Although these have not

provided many insights into the physiological changes underlying such development, they have been useful in displaying interesting phenomena and exposing many of the central problems in the field. The mammalian examples of this work (reviewed by Davis, 1981) have shown that much of the development of overt circadian rhythmicity occurs post-natally. Although this probably reflects the true situation, it should be recognized that the absence of evidence for pre-natal rhythms could be partially due to the difficulty associated with the analysis of these physiological processes. However, it is evident that insects present even greater problems, as the duration of their embryonic stages are very short, being measured in terms of days, rather than the weeks or months for many mammals.

The presence of significant amounts of post-natal development in the expression of rhythmicity must raise the possibility that environmental factors may influence the ultimate development of the circadian system. Most of the work which has been concerned with this question has tended to focus on whether or not individuals need to be exposed to periodic light/dark cycles during their development before they can express any rhythmicity. In general, all of these researches have found that prior exposure to LD cycles was not necessary for the development of rhythmicity. The organisms investigated in this respect have included cockroaches (Page and Block, 1980), fruitflies (Konopka, 1981), lizards (Hoffmann, 1957), rodents (Aschoff, 1955) and humans (Miles et al., 1977). The present study has shown that *C. vicina* also does not require previous exposure to light to be able to display rhythmic behaviour (see Chapter 3).

Although such studies give support to the view that circadian oscillations are innate, genetically programmed, features of the physiological system, it has not been convincingly shown that light/dark cycles have no role in the development of circadian systems (Davis, 1981). Furthermore, considering that the circadian organization in multicellular animals is derived from a complex and widely anatomically distributed neural system (Page, 1981a, b, 1984) the environment may be expected to have some effects, as it does in other aspects of central nervous system development (Page, unpublished). Thus, one could hypothesize that the mature, fully functioning, circadian system involves a basic genetic programme which is modified during development to one which more accurately reflects the local environmental conditions. This may equate to the environmental 'fine-tuning' of the circadian system envisaged by Pittendrigh

and Daan (1976c).

The features of the environment which have been shown to influence circadian development come into two main groupings, Zeitgeber and temperature effects.

Breeding animals in non-24 hour environmental LD cycles and assaying the effects on the overt rhythm is one way of investigating the influence of Zeitgebers. Thus, Hoffmann (1959) used T18 and T36 cycles during the development of a lizard, while Davis and Menaker (1981) utilized T20 and T28 LD cycles on the mouse *Mus musculus*. Although Hoffmann's study could find no effects on the adult activity rhythm from the differing T-cycles used, mice were found to show an initially lengthened period of about 23.7 hours (after a T28 cycle) which subsequently shortened to 23.2 hours. T20 cycles appeared to have no effect on the locomotor pattern, with the period remaining at about 23.2 hours throughout. Thus, these two vertebrate studies both indicated that the environment had no permanent effects on the period of the free-running circadian system. However, the effectiveness of these LD cycles in producing persisting alterations (especially in the lizard) may depend on the rhythm being entrained to such cycles during a critical developmental period (Page and Block, 1980; Davis, 1981). Thus, it is possible that the extreme photoperiods used in the lizard example may have been outside the range of entrainment of the animals' circadian system, thereby preventing the environment from influencing the rhythmic pattern.

A similar study on the cockroach *Leucophaea maderae* (Page and Block, 1980) found that exposing the animals to T22 or T26 LD cycles during post-embryonic development profoundly affected the period of the free-running rhythm, with T22 shortening the periods to about 22.7 hours and T26 producing lengthened periods of about 24.2 hours. Furthermore, these changes appeared to persist for many months, and in many cases throughout the lifetime of the individual. The permanence of these effects implied that they were probably not after-effects of entrainment, as such phenomena tend to decay relatively rapidly (Pittendrigh and Daan, 1976a). Moreover, it appeared that the light cycle may exert its influence by fixing the period of the oscillation to a limited range of values near to the period of the dominant environmental LD cycle.

Even fewer examples exist for temperature effects. However, Truman (1973) found that exposing developing silkmoths (*Antheraea pernyi*) to temperatures of 12°C phase-advanced the adult flight activity relative to moths bred at 25°C. This difference in phase-angle between the two temperature groups was found to be stable throughout the lifetime of the moths.

It has also been suggested that there may be maternal effects on circadian ontogeny, apart from the obvious genetic transmission of information. The few studies on this have suggested that the phase of the offsprings' activity may be maternally set in rodents (Reppert and Schwartz, 1983; Davis and Gorski, 1985), although this appears not to be the case in cockroaches (Page and Block, unpublished).

Another important problem in any consideration of the development of circadian organization is the timing of the emergence of a fully functioning system. The existing studies suggest that this may be long before the overt expression of the rhythm. For example, *Drosophila* and mosquito (*Anopheles gambiae*) adult activity rhythms can be phased by the light as early as the first or second larval instars (Zimmerman and Ives, 1971, and Jones and Reiter, 1975 respectively). Similarly, Minis and Pittendrigh (1968) found that the egg hatching rhythm in the moth *Pectinophora gossypiella* was differentiated by the sixth day of embryogenesis. More recently, the development of rhythmicity in the suprachiasmatic nuclei (SCN) of rats has been investigated using radio carbon labelling (Reppert and Schwartz, 1983, 1984). They were able to show that a metabolic circadian oscillation was present in the foetal SCN by the 19th day of gestation.

Although the available information on the ontogeny of circadian systems is limited, much of what exists suggests that an oscillation differentiates early in development. While specific features of this oscillation (i.e. its period) are genetic in their origin, it appears that a variety of environmental factors experienced during development can modify the overt rhythmic patterns of the circadian system.

The following experiments attempted to investigate the effects of various light cycles on the adult locomotor activity, when experienced during larval and adult stages of the life-cycle.

Materials and Methods

In all of the previous experiments the adult stock cages, and the larvae which they gave rise to, were kept in LL. However, in the present experiments this arrangement was altered to study the effects of the light Zeitgeber on the development of the circadian system. As blowflies are holometabolous insects the larval/adult transition is very clearly delineated. This made it possible to selectively expose the larvae to different photoperiods from those experienced by the adults. Thus, while the adults were still maintained in LL, their larval progeny were placed in a variety of photoperiods, i.e. 12:12, 16:8, 11:11 and 13:13. The larvae from the T24 group (12:12 and 16:8) were kept continuously in their photoperiods, and for a further 6 days after eclosion. At this point the light cycles were stopped and they were placed in DD. The T22 (11:11) and T26 (13:13) groups were both divided into two subgroups. At each T-value, one subgroup was kept in the selected photoperiod throughout larval development and for the first 4–5 days of adult life, before being put into DD. The larvae in the other two subgroups (one for each T-cycle) were removed from their photoperiods as soon as they pupated, and placed in DD. Two days after eclosion, the flies were placed in the recording devices under dim red light, where they were maintained in DD for the remainder of their lifespan.

Results

T24 Cycles

The average values for the free-running period, active-phase length and amplitude corresponding to each photoperiod are given in Table 6.1. Analysis revealed that there were no significant differences for any of these parameters between the two photoperiods (Stage 1 τ, t=0.420 with 15 d.f.; Stage 2 τ, t =0.555 with 14 d.f.; Stage 1 α, t=0.522 with 17 d.f.; Stage 2 α, t=0.312 with 14 d.f.; Stage 1 amplitude, t=0.875 with 15 d.f.; Stage 2 amplitude, t=1.105 with 12 d.f.).

Furthermore, the patterns which were observed in both of the photoperiods appeared in all respects to be the same as previous control experiments (see Chapter 4), with significant period lengthening after 6–10 days in almost every case. Indeed, only one fly in either experimental group was found not to

Experimental protocol		*Stage 1*	*N*	*Stage 2*	*N*
Larvae bred in 12:12, adults in 12:12 before DD	*Mean free-running period (hours±s.d.)*	22.70±0.36	11	24.61±0.87	9
	Mean duration of activity per cycle (hours±s.d.)	13.41±2.42	11	13.98±2.03	9
	Mean amplitude of activity per cycle (units±s.d.)	16.39±10.28	11	17.63±12.86	9
Larvae bred in 16:8, adults in 16:8 before DD	*Mean free-running period (hours±s.d.)*	22.78±0.48	9	24.39±0.73	8
	Mean duration of activity per cycle (hours±s.d.)	14.02±2.73	9	13.67±2.11	8
	Mean amplitude of activity per cycle (units±s.d.)	13.14±6.18	9	12.15±7.06	8

Table 6.1. The three parameters of the free-running rhythm in flies bred in two T24 cycles (12:12 and 16:8) and exposed to the same photoperiods before being placed in DD. These values did not differ significantly either between the two photoperiods shown here, or from the more extensive controls in Chapter 4.

conform to this pattern, having a split rhythm from the start of the recording. Comparative analysis of the present results with these controls confirmed this similarity, with the period of the rhythm (12:12, Stage 1 t=0.081 with 35 d.f., Stage 2 t=1.888 with 24 d.f.; 16:8, Stage 1 t=0.378 with 24 d.f., Stage 2 t=1.355 with 26 d.f.), the active-phase length (12:12, Stage 1 t=0.044 with 27 d.f., Stage 2 t=0.331 with 32 d.f.; 16:8, Stage 1 t=0.567 with 19 d.f., Stage 2 t=0.653 with 29 d.f.) and the amplitude (12:12, Stage 1 t=0.537 with 18 d.f., Stage 2 t=1.025 with 12 d.f.; 16:8, Stage 1 t=0.613 with 25 d.f., Stage 2 t=0.325 with 20 d.f.) showing no significant differences.

One final comparison was also made between these results and the period results from flies which had been reared in DD (from Chapter 3). This analysis showed that the periods were significantly longer after breeding the flies in DD for both photoperiods (12:12, t=3.548 with 76 d.f.; 16:8, t=2.114 with 43 d.f.). Unfortunately, it was only possible to compare the first stage of the free-run because DD reared flies show no after-effects, and thus had no period changes.

Therefore, breeding the larvae in various T24 cycles had no effect on the eventual adult activity pattern, in comparison to LL reared flies. For this reason, it was considered that the pooled T24 data would be a suitable control for the subsequent non-T24 cycles.

T22 Cycles

The group of flies which had been kept in DD from the time of their pupation (hereafter called T22.A) were seen to produce quite disturbed activity patterns. In many cases there was a clear initial rhythm with a period of less than 24 hours (on average 22.31±0.35 hours: see Table 6.2). However, 8-12 days after eclosion the patterns began to break-up, with extensive rhythm-splitting (Fig. 6.1). Of the 11 flies in this group, 6 showed this pattern while 2 had splitting from the outset of the recording, and a further 3 displayed a lengthening of the period. However, the patterns seen in this latter group were clearly different from the usual lengthening after-effects of entrainment, being relatively late changes in period (see Fig. 6.2). Comparative analyses between these results and T24 controls, showed that the initial free-running periods were shorter after T22 (t=2.841 with 25 d.f.). Coupled with this, the first stage of the free-run had significantly longer active-phases and higher levels

Experimental protocol		*Stage 1*	*N*	*Stage 2*	*N*
Larvae bred in 11:11, *adults in DD*	*Mean free-running period (hours±s.d.)*	22.31±0.35	9	24.00±0.67	3
	Mean duration of activity per cycle (hours±s.d.)	17.54±1.65	9	18.50±2.77	3
	Mean amplitude of activity per cycle (units±s.d.)	34.77±10.14	9	18.74±8.70	3
Larvae bred in 11:11, *adults in* 11:11 *before DD*	*Mean free-running period (hours±s.d.)*	22.55±0.40	13	24.35±1.18	10
	Mean duration of activity per cycle (hours±s.d.)	12.85±1.88	13	15.80±2.68	10
	Mean amplitude of activity per cycle (units±s.d.)	22.31±12.81	13	12.88±6.60	10

Table 6.2. The results of two experiments on the effects of photoperiod on development. Although all the insects were bred in 11:11, in one they were placed in DD as newly formed pupae, while in the second they were briefly exposed to the same cycle as adults. In the former many of the patterns had disintegrated by Stage 2, with the result that the sample sizes were severely reduced. The latter experiment produced patterns which were indistinguishable from the previous T24 results (see Table 6.1).

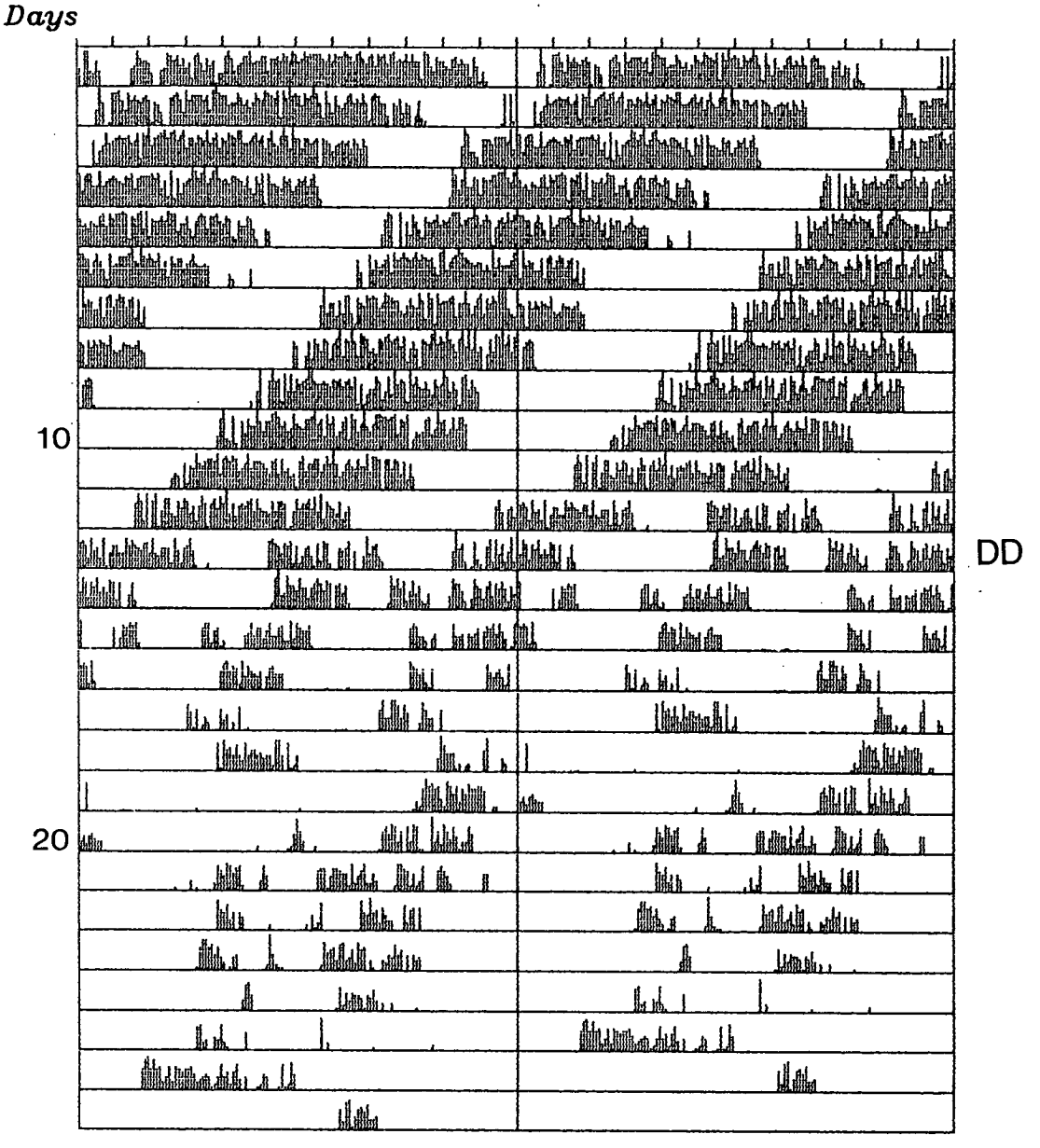

Figure 6.1. An example of rhythm-splitting seen in the activity of a fly which had been bred in T22 (11:11) but had been in DD since the time of its pupation. Note that the separate elements appeared to be free-running with quite different periods, with the result that they eventually remerged.

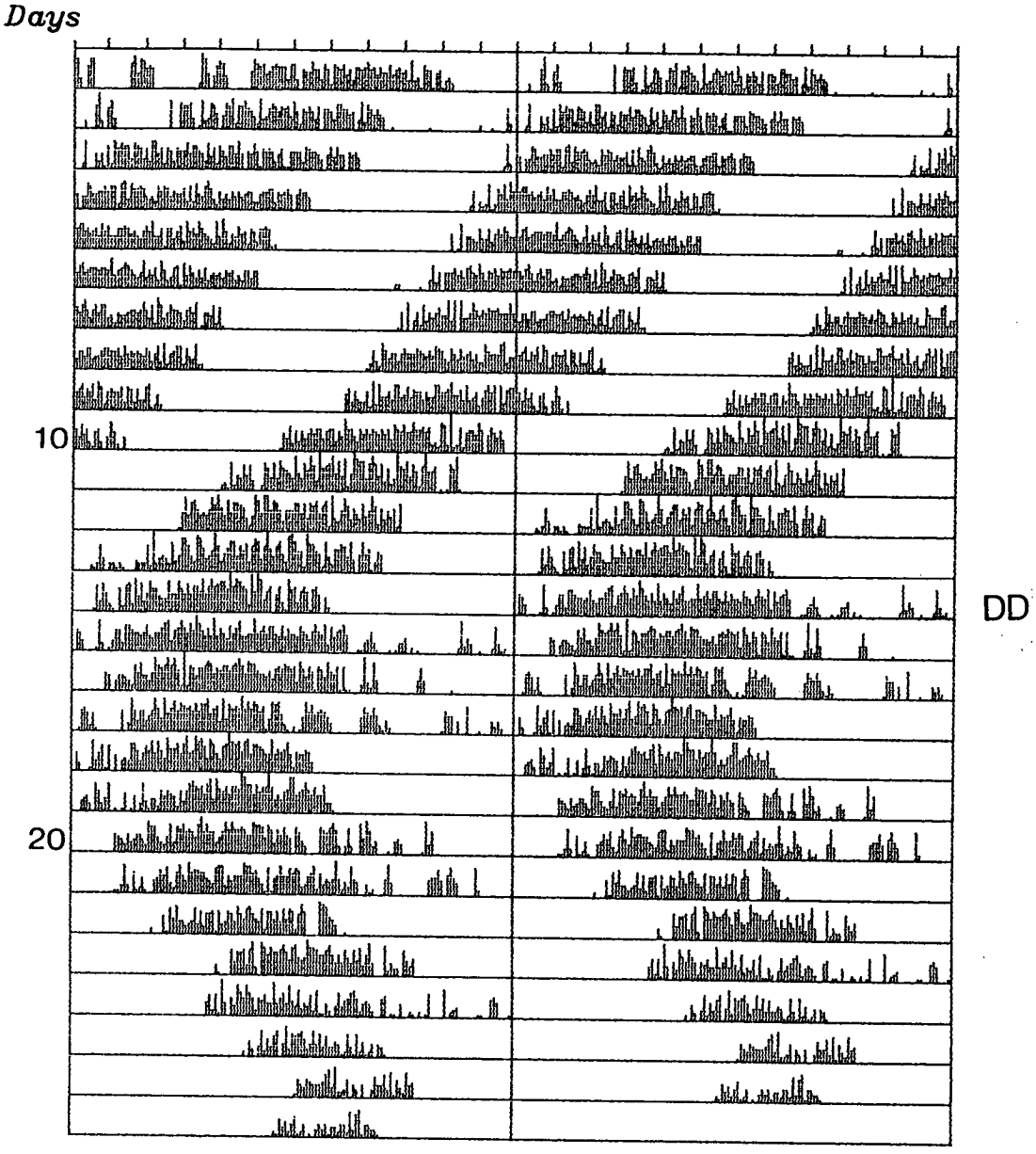

Figure 6.2. A further example of a fly bred in 11:11 but placed in DD at the time of its pupation. In this case the rhythm showed a marked lengthening of its period. This could be distinguished from the normal lengthening after-effect because of its occurrence at a much later point in the free-run.

of activity in T22 (t=4.895 with 26 d.f. and t=5.097 with 19 d.f. respectively). However, the second stage periods and amplitudes were found to be the same as the controls (t=1.240 with 8 d.f. and t=0.654 with 9 d.f.), although the active-phase lengths were again found to be significantly longer at this part of the free-run (t=2.787 with 4 d.f.). Table 6.2 contains the average values for these three parameters of the free-run.

While the Stage 1 results are interesting the second stage data must be interpreted with caution, as the sample size was so small (numbering only three flies due to the breakdown in the pattern of the remaining flies in the group).

Exposing the flies to the T22 cycle (11:11) for a short time during early adult life (hereafter called T22.B), resulted in very different adult activity patterns from those seen in T22.A above. Although the patterns were slightly disrupted, they did not appear to be very different from the control free-running rhythms, with many showing the usual lengthening of τ (see Fig. 6.3). The distribution of the activity types was as follows:

Clear and constant period:	16.7%	(N=2)
Clear but changing period:	75.0%	(N=9)
Complex patterns:	8.3%	(N=1)

Analysis confirmed that these results were very similar to the controls, with no significant differences being evident in any part of the free-runs (Stage 1 τ, t=1.272 with 29 d.f.; Stage 2 τ, t=0.430 with 16 d.f.; Stage 1 α, t=1.091 with 30 d.f.; Stage 2 α, t=2.013 with 18 d.f.; Stage 1 amplitude, t=1.825 with 22 d.f.; Stage 2 amplitude, t=0.656 with 24 d.f.). The average values for each of these features of the free-run are presented in Table 6.2.

The similarity to the controls was further emphasized by comparisons between T22.A and the present T22.B data, where many of the same differences appeared. Thus, the Stage 1 active-phase lengths and amplitudes were greater in T22.A (t=6.183 with 19 d.f. and t=2.540 with 19 d.f.). However, the first stage periods were not found to differ (t=1.475 with 19 d.f.), suggesting that the initial τ in T22.B may also have been shortened by the pre-treatment, although not enough to be significant when compared to the T24 controls. The second

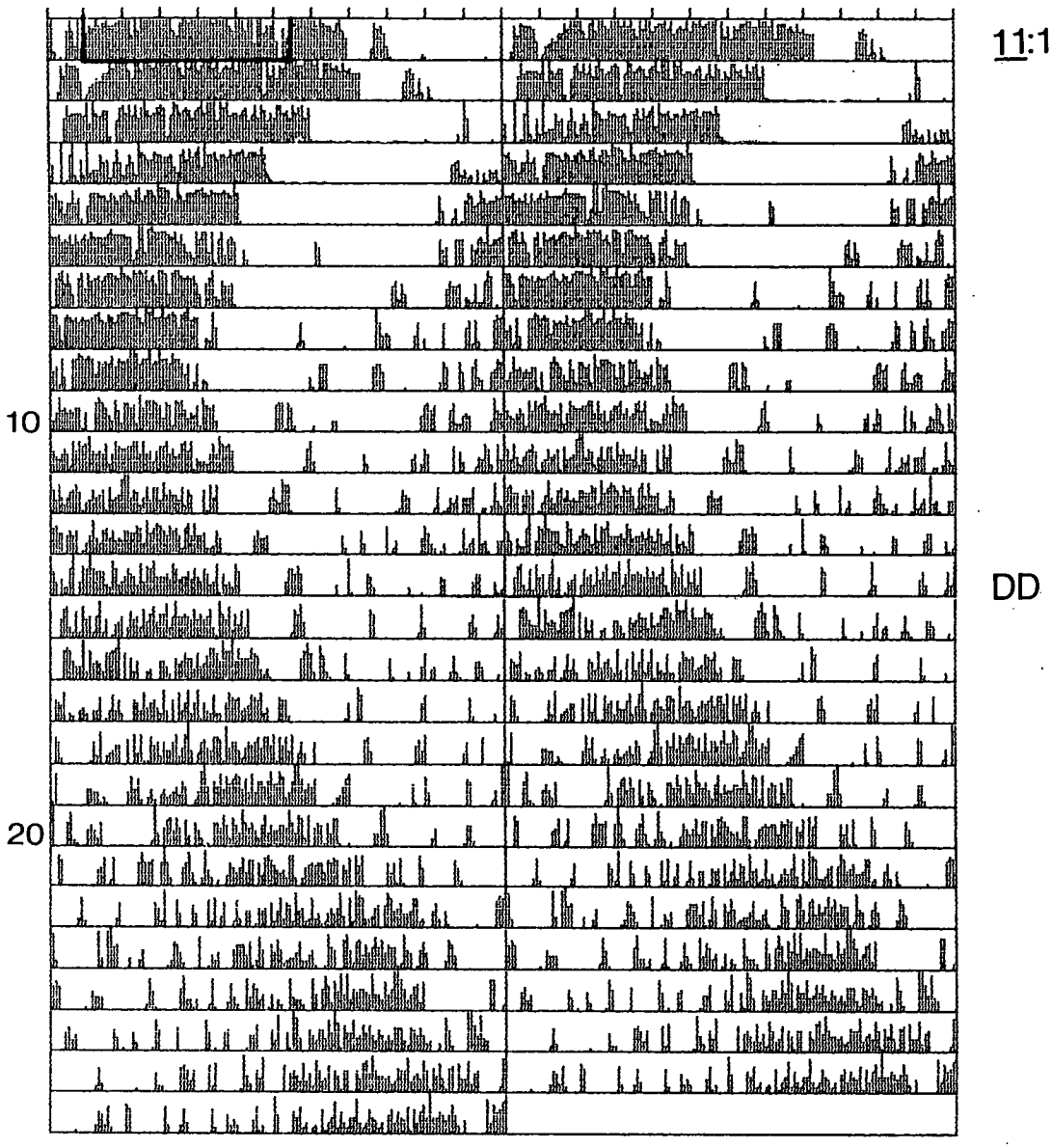

Figure 6.3. The blowfly in this record was bred in 11:11 and also experienced several cycles of the photoperiod as an adult. The resulting activity was very similar to the control free-runs, although there was a tendency for the rhythm to disintegrate during the period lengthening.

stage data were also largely in agreement with the controls, finding the periods and activity levels to be the same in T22.A and T22.B (t=0.652 with 10 d.f. and t =1.078 with 4 d.f. respectively). However, the active-phase lengths were found to be the same in the two T22 groups (t=1.491 with 6 d.f.), perhaps implying that T22.B also had a slightly increased amplitude to the activity pattern.

The results presented here imply that exposing adult flies to a small number of LD cycles was sufficient to reverse many of the disruptive effects induced by larval experience of T22 cycles.

T26 Cycles

When the larvae alone experienced these light cycles (hereafter termed T26.A), a disrupted pattern of activity was seen which closely resembled the results of the protocol used in T22.A. Thus, of the individuals which produced any worthwhile recordings, 2 showed lengthened periods, 4 had rhythmic patterns which split after several days, and 3 displayed rhythm-splitting from the beginning of the observation period. Furthermore, the records which had lengthened periods were again different from the usual situation, having relatively later lengthening.

Table 6.3 contains the average data for this experimental protocol. As the sample size for Stage 2 was so low (again due to the large number of split rhythms), it was not considered feasible to conduct statistical comparisons with this data. However, analysis of the first stage data found that the three parameters of the free-run investigated were indistinguishable from the T24 controls (τ, t=0.107 with 6 d.f.; α, t=0.320 with 10 d.f.; amplitude, t=1.460 with 6 d.f.). Therefore, although the T26 cycle produced the same type of disturbed activity pattern as had been seen with T22, the main features of the first stage of the rhythm remained largely unaffected.

As in T22, brief exposure of the adults to T26 resulted in patterns which were very different from larval exposure alone. Furthermore, these patterns were very different from the control runs, in that they had substantial numbers of flies with constant periods throughout the recording (see Fig. 6.4):

Experimental protocol		*Stage 1*	*N*	*Stage 2*	*N*
Larvae bred in 13:13, adults in DD	*Mean free-running period (hours±s.d.)*	22.78±0.99	6	24.58±0.35	2
	Mean duration of activity per cycle (hours±s.d.)	14.17±3.44	6	10.08±1.53	2
	Mean amplitude of activity per cycle (units±s.d.)	29.85±24.58	6	5.70±5.50	2
Larvae bred in 13:13, adults in 13:13 before DD	*Mean free-running period (hours±s.d.)*	22.28±0.43	10	23.00±0.69	8
	Mean duration of activity per cycle (hours±s.d.)	11.23±2.13	10	11.54±2.16	8
	Mean amplitude of activity per cycle (units±s.d.)	14.02±12.66	10	9.94±7.14	8

Table 6.3. The same type of experiment as described in Table 6.2, except that the breeding photoperiod was 13:13. As seen in 11:11, free-running the adult flies without exposure to light produced disrupted patterns (hence the reduced sample sizes at Stage 2). However, the effects of exposing the adults to the LD cycle did not compare to those seen with 11:11 as a high proportion of the flies showed little change in their period at Stage 2 and their active phase lengths were markedly reduced.

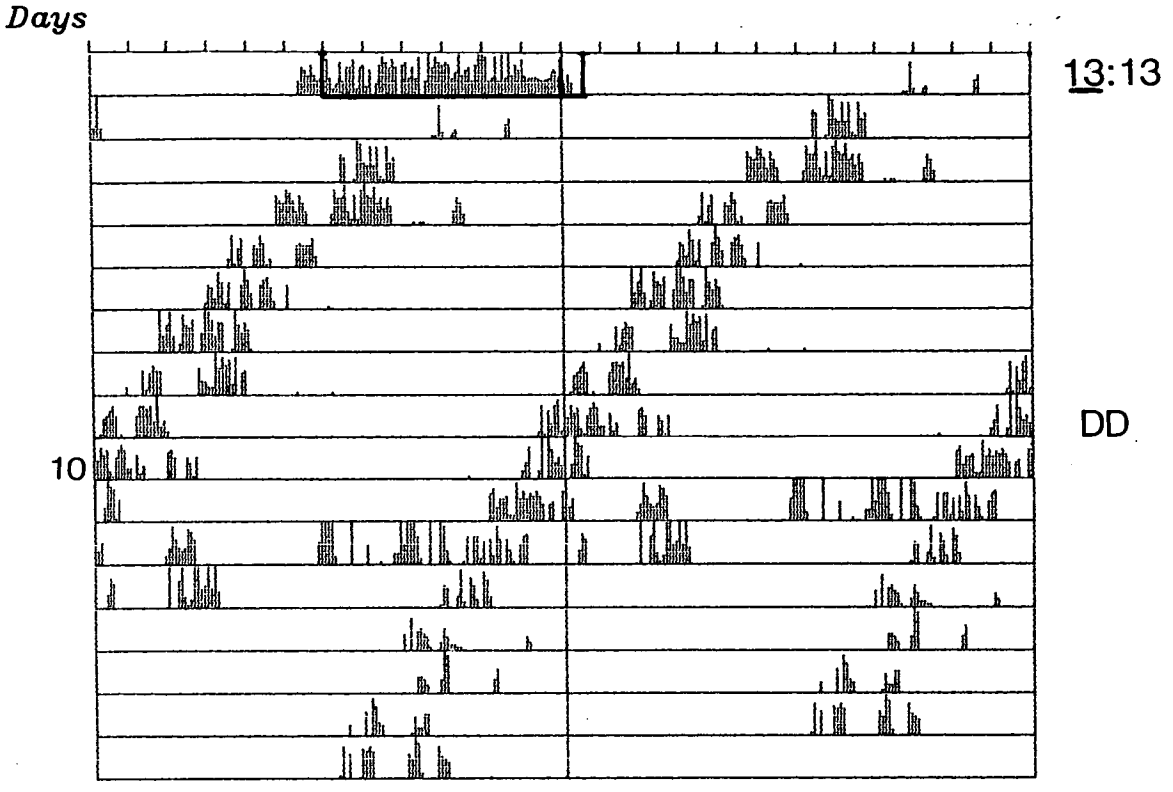

Figure 6.4. An example of a fly bred in T26 (13:13) and briefly exposed to the same cycle as an adult. Note that the free-running rhythm has a period consistently shorter than 24 hours and a very reduced active-phase length.

Clear and constant periods:	60%	(N=6)
Clear but changing periods:	30%	(N=3)
Complex patterns:	10%	(N=1)

As a result of this, significant differences were found between the present results (termed T26.B) and the controls. Thus, it appeared that both stages of the free-run had shorter periods than the T24 controls (Stage 1, t=2.749 with 23 d.f.; Stage 2, t=4.997 with 20 d.f.). The active-phase lengths were also found to be shorter at both stages after T26 (Stage 1, t=2.792 with 26 d.f.; Stage 2, t =2.532 with 18 d.f.), although the amplitude of activity was not found to differ (Stage 1, t=0.204 with 17 d.f.; Stage 2, t=1.417 with 22 d.f.).

Interestingly, comparisons made between T26.A and T26.B showed no significant differences between the Stage 1 data (τ, t=1.157 with 6 d.f.; α, t =1.884 with 8 d.f.; amplitude, t=1.465 with 7 d.f.). This was unexpected, as the T26.A data had been shown to be the same as the controls. However, this discrepancy may have resulted from the relatively larger standard deviations and low sample sizes found in T26.B.

Therefore, in comparison to T22, T26 cycles were found to alter the adult activity pattern, even when the adults themselves had been exposed to the light cycle. Despite this, the changes were less extreme than when the larvae alone experienced 13:13.

Summary

1. The adult locomotor patterns were indistinguishable when the larvae were bred in various T24 LD cycles or in LL, although the periods were significantly longer after breeding the flies in DD.
2. Exposing the larvae alone to T22 (11:11) produced highly disrupted activity patterns, with the initial periods being shorter than the T24 controls. There were also greater levels of activity and longer active-phases in this first part of the free-run.
3. Similar disrupted patterns were found when the larvae experienced T26 (13:13), although in this case the parameters of the free-run could not be distinguished from the T24 controls.
4. If the insects were exposed to either T-cycle during the adult stage, the free-running pattern was radically altered from the patterns produced after larval exposure. In 11:11 the pattern appeared in every respect to be the same as the control, while 13:13 produced records with a high proportion of unchanging periods. Furthermore, the period and active-phase lengths were clearly shorter after T26 than the T24 controls.

Discussion

While it is generally agreed that the circadian system in insects has a substantial genetic basis, the environment has also been implicated in shaping the development of this system. The results of the present experiments support the case for such involvement, especially when experienced during the larval stages.

It was of interest to note that T24 cycles had little effect in comparison to LL bred flies, but still induced shorter periods than were seen with DD bred blowflies. This is in direct contradiction with the only comparable insect study (with the cockroach *L. maderae*; Page and Block, 1980), where T24 (12:12) produced longer periods than DD bred cockroaches. The only obvious difference between flies used here that might explain this discrepancy, was that the DD bred flies had originated from a stock population which had been maintained in complete darkness for several generations. If this is subsequently found to be the source of the variation, one might conclude that these results are an example of the environment exerting its influence *via* the maternal generation. As it has been known for some time that *C. vicina* has a maternally induced photoperiodic response (Vinogradova and Zinovjeva, 1972; Saunders et al., 1986), it would not be surprising if the ambient light conditions could also produce changes to other circadian functions.

Exposing the larvae to non-24 hour cycles produced dramatic effects on the free-running locomotor pattern. These manifested themselves in a variety of forms. Firstly, T22 (11:11) induced significantly shorter initial periods than T24 cycles, while the periods appeared to be unaltered after T26 (13:13). The previously cited cockroach study (Page and Block, 1980), using the same photoperiods, found similar shortened periods with 11:11 but also showed that the initial period of the free-run was significantly lengthened by 13:13. Furthermore, while *C. vicina* displayed considerable disruption and rhythm-splitting after about 12 days in free-run, there was no indication of a similar breakdown in the cockroach activity patterns.

While it is difficult to find any particular cause for these differences, it is tempting to ascribe some of the variation to the very different life histories of these insects; cockroaches are hemimetabolous insects, while blowflies are holometabolous. These two types of life-cycle differ in that hemimetabolous

insects have immature instars which resemble, and directly develop into, the final adult form. Thus, each succeeding developmental stage incorporates large parts of the biomass of the preceding stage. Blowflies, on the other hand, have two distinct parts to the life history with the adult form being completely different from the larval stages in both appearance and lifestyle. The change between these two forms occurs during the pupal instar with little of the larval form incorporated into the adult. These contrasting life-cycles must have implications for the development of circadian systems, as well as the relationship between the functioning systems in the immature and adult forms.

When adults were allowed to emerge into the photoperiods which they had experienced as larvae, the activity patterns were once more altered. Firstly, the rhythm-splitting, which had been evident in the previous experiments, almost completely disappeared. At T22 the disrupted patterns were largely replaced by runs which resembled the free-run for LL bred flies. T26 patterns, on the other hand, were found to display a high percentage of unchanging periods.

Although the cockroach study did not employ the same type of experiment, it was found that adult and nymphal stages produced differing activity patterns in response to the same photoperiods (Page and Block, 1980). Thus, in both insects, the immature and adult forms respond in very different ways to the same photoperiodic information. However, this response cannot be considered to be comparable for each stage in the life history, for the larval effects are produced by altering the development of the circadian system, while the adult patterns arise from changes to a fully differentiated system. Despite this, there is some evidence that a separate clock may be present in the larval stages of *C. vicina*, as stepwise transfers of larvae and pupae from DD to 12:12 were found to produce very different eclosion patterns (Zinovjeva and Polyakova, 1987).

The overall implications from the present results are that the developmental processes which occur in the larval stages are very important in building up the system which controls the activity patterns of the adults. The large number of split rhythms seen after both T-cycles suggests that exposing the larval stages to exotic photoperiods was disrupting the normal development of the coupling between the constituent oscillators. Thus, the larval stages must be the point at which such interconnections attain their final form. The experiments which were presented in Chapter 5 on the effects of lithium

chloride treatment can also be related to this hypothesis, for larval exposure to this compound induced a higher proportion of split rhythms than adult exposure. As it is well known that the larval stages are negatively phototactic, with the final instars being subterranean, one would expect that these immature forms would not normally be exposed to much photoperiodic information. Despite this, it is clear that they are sensitive to LD cycles, a fact which has been noted in the induction of the diapausing state (Saunders et al., 1986).

Although larval exposure to exotic T-cycles did affect the subsequent adult activity, it was evident that adult exposure to the same photoperiods induced strong enough effects to be able to overcome many of these influences. This was especially true for 11:11, suggesting that the brief entrainment to this light/dark cycle was sufficient to reorganize the oscillators into something resembling the normal coupled system. The responses to 13:13 were, on the other hand, more difficult to explain. However, if one assumes that the coupling between the constituent oscillators was altered by the impinging light, the development of a normal phase response curve (PRC) for the adult's locomotor rhythm would also be affected, as it presumably relies on the combined properties of these oscillators for its expression. This would, in turn, affect the ability of the system to entrain in a normal fashion. Thus, while entrainment to T22 may be achieved, entrainment to T26 might be more difficult. The existence of a large proportion of patterns showing no period changes after T26 (closely mimicking the results from flies which were bred without any experience of light; see Chapter 3) implied that this cycle was ineffective in providing a strong enough entraining stimulus. This situation could come about if the phase-delaying part of the PRC was reduced in effectiveness relative to the phase-advance portion of the curve. Despite this, T26 must have exerted some normalizing influences as much of the split rhythmicity disappeared from the adult activity patterns, indicating that T26 was also able to partially reorganize the circadian system.

In some ways these proposals are counter-intuitive, for one might have expected a T26 environmental cycle to predispose the system to entrainment to such a cycle, as was suggested for the cockroach (Page and Block, 1980). However, the holometabolous nature of the blowfly's lifestyle indicates that one should not necessarily expect the two species to respond in similar ways. Furthermore, the extensive degradation of the larval tissue at the larval/pupal

transition suggests that the integration of the circadian systems in the immature and adult forms may not be as straightforward as it would appear to be in the cockroach (where many parts of the larval structures survive to the adult stage).

The changes which were seen in the active-phase length and amplitude of the activity are rather more difficult to assess. It has been noted in previous chapters that the breakdown of the rhythmic patterns was frequently associated with increased active-phases. Thus, the increases seen in this after T22 may represent another facet of the disruptive process brought about by larval exposure to the T-cycle. However, the lack of change in α after larval exposure to T26 and its decrease when the adults were also illuminated suggests, firstly, that the system may have been more tightly coupled than usual after adult entrainment and, secondly, that T26 cycles have quite different effects on the developing circadian system. Although an increase in the strength of coupling between the oscillators may be responsible for the unusual after-effects of T26, it is difficult to visualize the means by which it could occur. However, if it was assumed that the system was constructed from two populations of oscillators, phase-set by dawn and dusk respectively, one could arrive at a suitable explanation for these phenomena. Thus, under normal circumstances entrainment would rely on the light/dark transitions being able to 'separate' the oscillating populations enough to allow them to adopt their correct phase relationships. An increase in coupling between the oscillators could, therefore, impair the effectiveness of the entraining cycle, and thereby reduce the consequent after-effects.

In conclusion, one should view the development of the circadian system as a continuous interaction of genetic and environmental forces, the former providing a 'template' for the latter to manipulate. This will ensure that the most suitable system emerges for the prevailing environmental conditions. Furthermore, it should be recognized that the ability to respond to environmental forces may, in its own right, be of adaptive use to the organism, allowing the fully developed circadian system to rapidly adjust to short-term environmental changes. Thus, such developmental lability would actually be selected for. Similar types of malleability have been noted in many areas of developmental biology, and it is now recognized that the maturation of many organisms (especially *Drosophila*) involves a hierarchical loss of developmental flexibility (Lawrence, 1981; Morata and Lawrence, 1977; Struhl, 1981). This is

also reflected in the development of the circadian system, where exotic photoperiods produced different effects on the adult activity, depending on the developmental stage experiencing the LD cycle.

Although this chapter is the last containing experimental results of the locomotor rhythm in *C. vicina* no overall discussion of activity will be attempted here. It was considered more appropriate to have this overview in the final discussion, juxtaposing a similar discussion on the photoperiodic responses of the blowfly. This arrangement was found to facilitate the discussion on the relationship between these two aspects of the circadian system. As a consequence of this, the next chapter will deal with the results of the photoperiodic responses of *C. vicina*.

CHAPTER 7

Photoperiodic Responses in the Blowfly

Introduction

This last chapter involves one of the best known of all the circadian phenomena, photoperiodism. In general terms, this phenomenon comprises the range of processes by which organisms distinguish the long days (=short nights) of summer from the short days (=long nights) of winter. Although it had been known for some time that daylength affected the developmental fate of many plants, photoperiodism was only given its first detailed description by Garner and Allard in the 1920s. Subsequently, many studies have revealed its involvement in the control of seasonal activities of animals as diverse as insects, crustacea, acarina, birds, mammals and reptiles (see Saunders, 1982a). In terms of insects, the most common aspects of this phenomenon, and the most intensively studied, concerned the induction and termination of dormancy, and the control of the appearance of seasonal morphs.

Forms of dormancy are found in many insects from northern and southern latitudes, where they enable insects to avoid unfavourable environmental seasons. In tropical areas this dormant period frequently coincides with the dry season, while insects in temperate regions usually commence dormancy as winter approaches. Although ecologically there are two forms of dormancy, hibernation (winter dormancy) and aestivation (summer dormancy), in physiological terms the two most important mechanisms are quiescence and diapause. In the former the dormant state is directly imposed on the organism by the adverse conditions, and although this is relatively uncommon in temperate regions, in tropical parts of the World quiescence is used by many animals and plants as a survival mechanism over the long dry seasons. For example, Hinton (1951) found that larvae of the Chironomid *Polypedilum vanderplanki*, which breeds in exposed pools of water, become almost completely dehydrated when their pools evaporated during the dry season. In this state they were able to remain viable for many years.

Diapause differs from quiescence in two distinct respects. Firstly, rather than being a passive process, it involves the active cessation or alteration of

the neuro-endocrine system at specific stages in the life-cycle of the insect, with the dormant stage being very much dependent on the actual species involved. Secondly, this active process is initiated by environmental factors which are not, in themselves, adverse. Rather they signal the approach of future unfavourable conditions. The most important environmental factor for bringing this about is the photoperiod.

There are thought to be three main physiological types of such diapause (from Müller, 1970). The first of these is Parapause, which is an obligatory diapause usually seen in univoltine species, with the dormant stage occurring in the species-specific instar in every generation. This diapause is genetically determined and appears to be independent of the environment. The second form is Eudiapause, which differs from the former in that the dormancy is induced by photoperiod. Thus, in favourable conditions development proceeds unchecked, with diapause intervening when the unfavourable conditions approach. Although induction of this diapause is photoperiodically controlled, its termination is frequently brought about by a period of chilling. The final type of dormancy, Oligopause, differs from the previous forms in having both the induction and termination of diapause under control of the photoperiod.

Among insects species there is a great variety in the stage of the life cycle which expresses the diapause, some insects (especially those which are long-lived) having more than one diapausing stage, e.g. the dragonfly *Tetragoneuria cynosura* (Lutz and Jenner, 1964) can diapause as early nymphs in one winter and as mature nymphs in the next. The majority of insects, however, only diapause at a single stage in their life history. For example, the mosquito *Aedes togoi* (Vinogradova, 1960) and the green vetch apid *Megoura viciae* (Lees, 1959) diapause as eggs, while the bollworm *Pectinophora gossypiella* (Adkisson et al., 1963), *Ostrinia nubilalis* (Beck and Hanec, 1960), *Nasonia vitripennis* (Saunders, 1965a, b) and the blowflies *Lucilia caesar* and *Calliphora vicina* (Ring, 1967; Vinogradova and Zinovjeva, 1972) all enter diapause as larvae. Pupal diapause is also common, especially in the Lepidoptera, e.g. *Acronycta rumicis* (Danilevskii, 1965) and *Pieris rapae* (Barker et al., 1963), and the Diptera, e.g. *Sarcophaga bullata* and *S. argyrostoma* (Fraenkel and Hsiao, 1968). Adult, or reproductive, diapause has also been found to exist in several Dipteran species, such as *Musca autumnalis* (Stoffolano and Matthysse, 1967), *Phormia regina* (Stoffolano et al., 1974), *Drosophila melanogaster* (Saunders et al., 1989). There is also some evidence that

Calliphora vicina may also express an adult diapause, although it is uncertain whether this is truly physiological (Vinogradova, 1984, 1986). A more extensive review of the various insect examples can be found in Saunders (1982a).

Although a discussion of the physiological mechanisms behind these different diapausing forms is not within the scope of this introduction it is possible to make several generalizations. Thus, while it appears that larval, pupal and nymphal diapauses are brought about by an inactivation of the brain-prothoracic gland system (Saunders, 1982a) (with a subsequent low titre of ecdysteroids in the haemolymph stopping both growth and development), adult (or reproductive) diapause results from an inactivation of the brain-corpus allatum system, preventing the release of juvenile hormones and suppressing ovarian maturation.

While photoperiodic phenomena have been found in many forms, they all have one thing in common: the principal trigger for their control is nightlength (or daylength). For this reason, many workers employed investigative techniques which manipulated the photoperiodic environment of the insect. Most of these studies of the formal responses to LD cycles have involved stationary photoperiods, in which groups of insects are exposed to fixed LD cycles throughout development (or over the photosensitive phase) of T=24 hours. As the ratio of light to dark was altered the proportion of the population entering diapause was plotted as a function of the length of the light phase. The resulting fluctuation in diapause incidence is the photoperiodic response curve (PPRC). As the majority of insects are active in the summer and dormant in winter, the most common type of response from this protocol is the 'long-day response'. This term comes from the early work on photoperiodism in plants, and simply refers to the time in the year when the organism is growing and developing. Thus, short-day responses are found in those insects which aestivate, or are winter-active.

Irrespective of shape of the particular PPRC, the most important feature of these curves is the transition zone between the responses to long days and short days: this is termed the critical daylength (or critical nightlength). In long-day species, long photoperiods result in non-diapause development, while short photoperiods ultimately lead to the dormant state. In many insect species the critical photoperiod is very abrupt, sometimes requiring as little as 10-15 minutes change in lightlength for there to be a perceptible alteration in the

proportion of diapause in the population. However, for most species a 1 hour change in the length of the day is usually sufficient to convert all the individuals in a population from one developmental pathway to another. It should be stressed, however, that the PPRC represents the response of a population of insects, and although individuals may differ slightly in the threshold for their response, once that threshold is passed the insect responds in an all or nothing fashion. For comparative purposes, the critical daylength (CDL) has been defined as that point on the PPRC transition between long- and short-day responses at which 50% of the population express diapause.

Clearly, photoperiodic regimes which cover the whole T24 range from DD to LL, will include photoperiods which would not normally be experienced by insects. Danilevskii (1965) pointed out that only those photoperiods around the critical photoperiod would normally be encountered by insects in nature, thus the shape of the PPRC in this region must be of adaptive significance, and must be under strong selective pressures. The photoperiods beyond this region either do not occur in the natural environment or only do so when the insects are unresponsive to photoperiod (see Fig. 7.1). Thus, one might assume that the selective pressures are greatly reduced, or absent, in this portion of the PPRC. The fact that there is a great variability in the shape of the curve at these extreme photoperiods does imply that this may be the case. Thus many insects show a falling off of diapause in ultra-short daylengths, while others have the same level of diapause in DD as they do in the strongly diapause-inductive short days, e.g. *Leptinotarsa decemlineata* (de Wilde, 1962). *Pectinophora gossypiella*, on the other hand, had a greater proportion of the population entering diapause at DD than 2:22 or 6:18 (Pittendrigh and Minis, 1971). Furthermore, long photoperiods and LL may induce slightly higher levels of diapause than natural long photoperiods (Williams and Adkisson, 1964; Pittendrigh and Minis, 1971).

The opposite, short-day, response is found in a smaller number of species, especially those which are spring-, autumn- or winter-active, e.g. the Lepidopteran *Abraxas miranda* (Masaki, 1959) spends the summer in a pupal diapause, the adults only emerging in September/October to produce larvae which grow and feed during the winter.

Irrespective of the type of response, all species restrict their sensitivity to photoperiod to certain parts of the developmental cycle. In some the sensitive

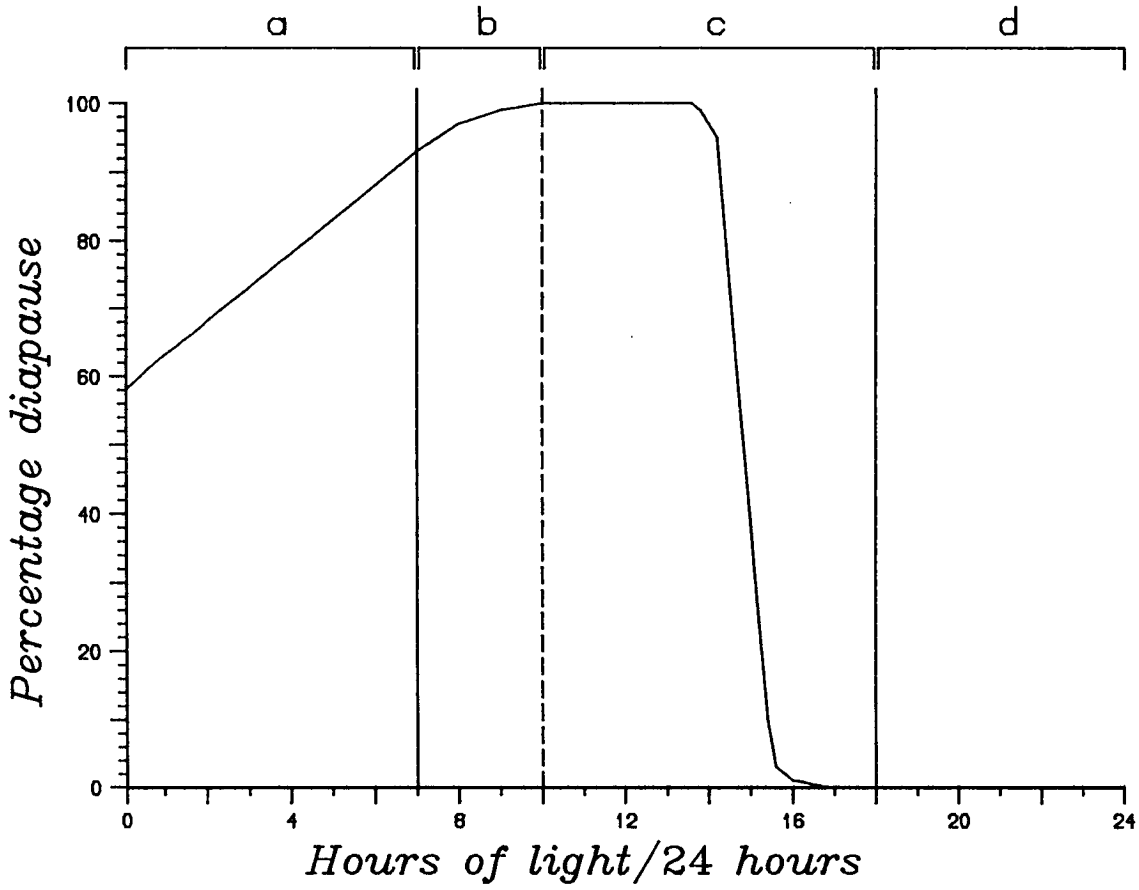

Figure 7.1. A schematic form of a long day photoperiodic response curve. The solid vertical lines represent the range of natural daylengths at 55°N. Regions a and d are therefore never experienced in nature, while b only occurs during winter when the temperature is probably below the minimum for development (and thus the insect will be in diapause). Only region c is of ecological importance (after Saunders, 1982a).

period occurs in the same instar as the resulting diapause, although in most this period is terminated before the diapausing instar. For example, in many fleshflies (Sarcophagidae) the larvae are sensitive to photoperiod from early embryogenesis, while still in the mothers' uterus (Denlinger, 1972; Roberts and Warren, 1975; Vinogradova, 1976; Saunders, 1980). In the case of *S. argyrostoma* this sensitivity continues right through development, only eventually ceasing at the time of the puparium formation (Saunders, 1971). In this species diapause is expressed in the pupal stage.

The most extreme examples of a delay between the sensitive period and the ultimate response are when both processes are in different generations. Maternal influences of this type have been found in a large number of insect species, and it seems likely that many more exist, e.g. *Nasonia vitripennis* (Saunders, 1965a, 1966a). Similar types of control have been found in several blowflies; *Lucilia caesar, L. sericata* (Ring, 1967), *Sarcophaga bullata* (Henrich and Denlinger, 1982) and, especially important in the present context, *Calliphora vicina* (Vinogradova and Zinovjeva, 1972; Saunders et al., 1986).

Despite the obvious importance of the photoperiodic response to the insect, it has been found that it can be modified by a number of environmental factors. The most important of these is temperature, which is known to be able to alter the degree of the response and the position of the CDL. Temperature pulses can also completely reverse the photoperiodic response, depending on the time in the LD cycle at which they are applied, e.g. *Acronycta rumicis* (Danilevskii, 1965) and *Nasonia vitripennis* (Saunders, 1967, 1968, 1969). Furthermore, many experiments with long-day species have shown that constant high temperatures and long daylength act together to avert diapause, while low temperatures and short days promote its induction (see Saunders, 1982a). As a result of these observations, Saunders (1971, 1981b) proposed an explanation for the interaction of photoperiod and temperature, based on the realization that many insects required a number of inductive photoperiodic cycles before diapause induction was complete. For example, *Sarcophaga argyrostoma* required 13–14 long nights to raise diapause incidence to 50% (Saunders, 1971), while *N. vitripennis* only required about 6–7 (Saunders, 1966a). This 'counter' model was envisaged as the quantitative accumulation of successive long nights until a threshold was reached, at which point induction was complete. This system has also been shown to be temperature-compensated in a number of species (Saunders, 1981b). Thus, the

effects of temperature were interpreted as an interaction of this temperature-compensated counter and a temperature-dependent developmental process. This would operate, in long-day species, by higher temperatures inducing increased growth rates, which would in turn reduce the length of the photosensitive developmental stage. Such a reduction would result in fewer long nights being 'seen' by the insect, and a concomitant decline in the level of diapause. The converse would occur at lower temperatures, with the sensitive phase increasing in length, more long nights being seen, and diapause increasing as a result. More recently this proposal has been successfully incorporated into a photoperiodic oscillatory model (Saunders and Lewis, 1987a, b; Lewis and Saunders, 1987).

As indicated above, temperature can also alter the CDL of the photoperiodic response. This is a particularly important problem as any changes to this feature of the response must inevitably affect the timing of the on-set of the diapause condition. Although it is not clear how this may function at the physiological level, one proposal (Saunders, 1982a) suggested that an overall reduction in the diapause response in increased temperatures could result in shortened CDLs without affecting the time-measuring mechanism. In essence, this reflected a general shift in the position of the PPRC, up or down, depending on whether the temperature decreased or increased respectively. As a consequence of this, those curves which have very steep transitions between the long- and short-day responses would have a largely unaltered CDL, while increasingly shallower slopes would produce larger effects on the CDL. This, therefore, may explain the observed variability in the effects of temperature on the CDL. In *Pieris brassicae* (Danilevskii, 1965) temperatures of up to 26°C have little effect on CDL, while the aphid *Megoura viciae* shortens the CDL by about 15 minutes with every 5°C rise up to 23°C, at which point the short-day induced oviparae fails (Lees, 1963). The European corn borer, *Ostrinia nubilalis* (Beck and Hanec, 1960), and the fleshfly *Sarcophaga argyrostoma* (Saunders, 1971) both show only slight changes in CDL with changing temperature.

As well as these superficial temperature effects on seasonal responses, there is substantial evidence that some species also use this environmental variable as their primary diapause inducing stimulus. In temperate areas, the number of species involved are relatively few, all tending to be soil-inhabiting (Tauber et al., 1986). However, in equatorial regions, where seasonal changes in daylength are rather small, many insects use temperature as the signal to

prepare themselves for approaching seasonal changes, e.g. the tropical fleshfly *Poecilometopa punctipennis* (Denlinger, 1974) was found to have an increasing incidence of diapause as the temperature decreased below 20°C (at which temperature no diapause was observed). Further examples of this phenomenon are discussed in Tauber et al. (1986). Even among those insects which use photoperiod as their main timing mechanism, examples can be found of successful diapause induction using thermoperiods. For example, when *N. vitripennis* adults, which had been bred in complete darkness, were exposed to a short-day (6–10 hours) thermoperiod of 13 to 23°C, high diapause percentages were observed among the larvae (Saunders, 1973a). Extending the length of the thermoperiods to over 14 hours completely averted diapause. Similarly, Skopik et al. (1986) found that exposing *Ostrinia nubilalis* to a thermoperiod of 25 to 4°C (12 hours: 12 hours) induced 100% diapause in DD, but 0% in LL. However, the situation in many insects is further complicated by the interactive properties of photoperiods and thermoperiods, such that thermoperiods can augment or overcome inductive photoperiods (see Skopik et al., 1986). In general, it appears that for most species the temperatures at night are more important in determining the developmental pathway than the temperatures during the light (Pittendrigh and Minis, 1971; Saunders, 1982a).

The preceding discussion clearly indicates that temperature and photoperiod interact in a highly complex manner throughout the induction and termination of the diapause response in many insects. As a consequence of this many of the existing studies have tended to concentrate on the influences of these two environmental factors. Despite this, it is increasingly evident that many other environmental parameters affect the seasonal responses. Chief among these are diet, population density and moisture. While all of these can influence growth, development and reproduction in much the same way as temperature, they are also known to occur in more or less regular seasonal cycles. As a result, they have the potential to act as stimuli signalling changes in the environment, and thus may be able to affect the seasonal responses of some insects. Reviews of these effects can be found in Saunders (1982a) and Tauber et al. (1986).

The dynamics of the seasonal responses are not only limited to short-term changes in form, they are also changeable over evolutionary time. This is an inevitable consequence of insects attempting to synchronize their seasonal cycles to the local conditions. Furthermore, any widespread species will

encounter a great diversity of climatic conditions in its different localities, with the result that there will be a correspondingly large variation in the form of the seasonal cycles between geographical populations.

For most insects, the greatest variations between geographical strains are usually the result of a wide latitudinal distribution. This is largely due to the fact that the daylength, and its rate of change, in any one locality are functions of the latitude. Furthermore, as climates in higher latitudes are generally colder, with the on-set of winter occurring sooner, the breeding seasons in these regions are considerably restricted in comparison to more southerly populations. The resultant behavioural modifications which have been necessary for these populations are evident in their PPRCs. For example, Danilevskii (1965), investigating the photoperiodic responses of a number of insect species from 40° to 60°N, found a general lengthening of the CDL as the latitude increased, e.g. *Acronycta rumicis* increased its CDL by about 1.5 hours for every 5° increase in latitude. The same trend was observed in *Nasonia vitripennis* (0.9 hours/5°, Saunders, 1966), *Mamestra brassicae* (1 hour/5°, Danilevskii, 1965) and *Drosophila littoralis* (1.3 hours/5°, Lankinen and Lumme, 1984). A particularly detailed study of 42 strains of *D. littoralis*, from 40°N to 70°N, found the same overall value of 1.3 hour increase in CDL for every 5° increase in latitude (Lankinen, 1986). However, it should be noted that some species have remarkably stable CDLs. Thus, *Pieris brassicae* showed little alteration in its CDL over a latitudinal range of 44° to 60°N (Danilevskii, 1965; Danilevskii et al., 1970). Similarly, Ankersmit and Adkisson (1967) found a largely invariable CDL between six populations of *Pectinophora gossypiella*, coming from latitudes ranging from Texas (32°N) to Argentina (27°S). Interestingly, the degree of the response was also found to correlate with the latitude. Thus, the PPRC was observed to be quite pronounced and very similar at the two extremes, 32°N and 27°S, while the tropical strains had weak responses which almost completely disappeared at high temperatures. Therefore, it would appear that this species had adapted to the tropical climate by reducing its diapause response, rather than altering its CDL. It has also been observed that many species show changes in their voltinism associated with these clines in CDL, with southern populations producing two or more generations in every season, while more northerly populations were univoltine.

Latitudinal clines have also been seen for other aspects of dormancy, e.g. depth (or duration) of diapause, duration of the sensitive phase, body size and

the thermal regulation of the rate of development (Tauber et al., 1986). Although most of these features are outwith the present discussion, the depth of diapause was considered of interest. The few studies which have been carried out suggest that many insects display more intense diapause the further north the locality of the population. For example, the lacewing *Chrysopa carnea* (Tauber and Tauber, 1972) showed twice the length of diapause at 42°N than at 33°N in a range of photoperiods. Further examples of this trend are given by Danilevskii et al. (1970). Although this cline presumably reflects an adaptation to the more severe northern winters, some insects display an opposite trend, whereby the southern populations have a longer diapause, and usually require a longer period of chilling, than those further north, e.g. *Teleogryllus* sp., *Lymantria dispar* and *Anopheles maculipennis messeae* (Danilevskii et al., 1970). This opposing cline may also be of adaptive advantage to the insect, for a stable diapause in southern climates would prevent the resumption of development under a warm and changeable winter.

It is generally believed that these geographical variations in the photoperiodic response have a complex genetic basis, almost certainly involving polygenic, as well as Mendelian, inheritance (Tauber et al., 1986; Saunders, 1982a). Much of the existing evidence suggests that the polygenic form may be the more common in controlling the photoperiodic response. This type of inheritance is characterized by the hybrids of a cross showing intermediate responses in certain aspects of their photoperiodism to the two parental generations. A particularly clear example of this was seen in a cross between two populations of the long-day species *Acronycta rumicis* which differed in their CDL by about 4 hours (Danilevskii, 1965), with the F1 and F2 hybrids all showing intermediate responses from the parental CDLs. It also appears that diapause-depth may be under the influence of polygenes (Tauber et al., 1986). Furthermore, Tauber et al. (1986) suggested that the existence of polygenic inheritance was supported by the observations that many of these photoperiodic clines occur as a continuum over the geographical range of most insects.

The family to which *Calliphora vicina* belongs generally express their diapause as larvae, adults or pupae; in the specific case of *C. vicina* this is as a post-fed larvae, just prior to pupariation. They may, however, also diapause as adults, or as both larvae and adults (Vinogradova, 1984, 1986). Recent work has revealed that larval diapause in *C. vicina* occurs as an interruption of endocrine

activity in the brain-ring gland axis, resulting in lower titres of ecdysteroids in the haemolymph (Richard and Saunders, 1987). As was found for *Manduca sexta* (Bowen et al., 1984), this was probably due to a failure of the brain to release prothoracicotropic hormone (PTTH), although *in vitro* studies suggest there is also a refractoriness on the part of the ring glands to PTTH (Richard and Saunders, 1987). A more detailed picture of the physiology of *C. vicina* can be found in Vinogradova (1984).

Although the eggs and larvae of *C. vicina* may show a slight sensitivity to daylength (Saunders et al., 1986), the photoperiodic regulation of diapause is largely maternal. Thus, in both Russian (56°N) and Scottish (55°N) strains, larvae bred in DD expressed levels of diapause entirely dependent on the photoperiods to which the adults had been exposed (Vinogradova and Zinovjeva, 1972; Saunders et al., 1986; Vaz Nunes and Saunders, 1989). As was expected for a long-day species, short days were found to induce diapause, provided the larval rearing temperature was below 15°C, while long days (or rearing the larvae at temperatures above 15°C) resulted in non-diapause development, and hence rapid pupariation. Furthermore, the diapause-averting effect of high temperatures did not appear to operate before the late larval wandering stage (Vaz Nunes and Saunders, 1989). Thus, transferring diapause-destined larvae from 11 to 23°C at at various times before the wandering stage produced high diapause incidence, while the same transfer 1-2 days after the onset of wandering induced far lower levels of diapause. It has been suggested that this diapause-avertion effect of temperature may act at the level of the brain-ring gland complex, affecting the brain's decision whether, or not, to release PTTH (Richard and Saunders, 1987).

The maternal photoperiod determines not only the proportion of the progeny entering diapause, but also the characteristics of the diapause, a more intense form typically occurring in the progeny of short-day-treated females (Vinogradova, 1974). Saunders (1987) showed a tight correlation of the average duration of diapause to the percentage incidence of diapause. Furthermore, it was found that 9-10 days of short photoperiods had to be experienced by the fly before significant levels of diapause were induced. This number of days was found to be temperature-compensated between 18 and 24°C. Saunders (1987) was also able to show that the diapause history of adults had no detectable effects on the diapause expression of their larvae. Thus, adults from a diapausing population showed levels of larval diapause equal to those seen

from adults of a non-diapausing group. Despite this, there is some evidence that long term exposure of the puparia to cold temperatures reduces the diapause incidence in the progeny of the resulting females (Bogdanova and Zaslavskii, 1985).

Although the duration of diapause in larvae from short-day mothers, reared at 10°C and held at 4°C, was found to be about 2-3 months, recovery from this state could occur within 48 hours if the larvae were transferred to 25°C (Saunders et al., 1986). Although it is possible to reactivate larvae using photoperiod, it seems likely that temperature is the most important factor determining the termination of diapause in nature (Vinogradova, 1974; Saunders et al., 1986).

As with many other insect species, geographical variability in the photoperiodic response is known to exist in the Calliphoridae. Although little work has been done with *C. vicina*, there is the suggestion of a cline in the CDL. For example, while a Scottish strain (55°N) had a CDL of 14.5 hours (Saunders et al., 1986), in Gorky (U.S.S.R., 56°N) it was in the region of 15-16 hours (Vinogradova and Zinovjeva, 1972), and a further population in Belomorsk, U.S.S.R. (64°N) was found to have a CDL of between 16 and 17 hours (Vinogradova, 1974). Another feature of the photoperiodic response of *C. vicina* which has been found to systematically vary with latitude is the depth or duration of diapause. In strains ranging from 38°N to 60°N, it was found that the more southerly populations had little or no diapause expression (even at low temperatures), while the northern groups all showed diapause, albeit to varying degrees (Vinogradova, 1986).

The existence of the critical daylength, as a sharp discontinuity between long- and short-day responses, has always inferred that insects must possess some kind of intrinsic clock which enables them to measure the length of the day or the night. As a result a great deal of study, over many years, has focused on attempts to understand how this time-measurement is achieved. Thus, although the physiological processes behind this phenomenon are still largely unknown, the system has been thoroughly described in terms of its formal properties, and a number of models have been derived to explain these features (see the General Introduction).

Unfortunately, despite these indications of the existence of an internal

'clock', photoperiodic responses themselves provide little direct evidence for the involvement of circadian oscillations; there is no overt rhythm which can be easily observed, as there was for locomotor activity. Furthermore, as photoperiodic phenomena tend to be once in a lifetime events, with the sensitive phase usually occurring well before the output of the system, the only parameter available for experimental manipulation is the proportion of the population entering or leaving diapause. For these reasons some early workers suggested that a simultaneous analysis of photoperiodic induction and an overt rhythm was the best way of approaching the subject. This, obviously, was based on the assumption that both systems were either linked to the same pacemaker, or had some similarities in their properties (Pittendrigh and Minis, 1964). As this is an important concept, especially with regard to the present study on photoperiodism and locomotor activity in *C. vicina*, it will be dealt with in greater detail in the Final Discussion.

Despite these obvious experimental difficulties, it has been shown that circadian oscillations are involved in the control of photoperiodism in a large number of animals and plants (see Saunders, 1982a). The most decisive evidence for such involvement comes from experimental protocols which utilized abnormal LD cycle-lengths, in particular night-interruption experiments (or Bünsow protocols) and Nanda-Hamner (or resonance) protocols (Nanda and Hamner, 1958). Both of these experimental techniques involve coupling a short day, or photophase (about 12 hours), with a variable night, or scotophase (8-72 hours). In the former (night-interruption) regime the length of the light and dark phases are selected and held constant while the scotophase is scanned with brief light pulses. Resonance experiments, on the other hand, have a fixed photophase coupled to successively longer scotophases, such that a series of extended LD cycles are produced. These usually vary over a wide range of T-values, from T18 to T90, and thus are expected to include several multiples of the period of the internal oscillator, τ. If peaks and troughs of the product of induction (in this case diapause) are seen with either protocol, this is taken to imply that the mechanism of time-measurement is a function of a circadian oscillation. By this means, many insects and other arthropods have been found to display a circadian basis to their photoperiodism, e.g. *Sarcophaga argyrostoma* (Saunders, 1973a, 1976), *Pieris brassicae* (Claret et al., 1981), *Nasonia vitripennis* (Saunders, 1970, 1974), *Tetranychus urticae* (Veerman and Vaz Nunes, 1980) and *Calliphora vicina* (this study, published in Vaz Nunes et

al., 1989). Some species, however, do not produce such fluctuations of induction. Rather it was found that beyond a critical night length all T-cycles were equally inductive, e.g. *Megoura viciae* (Lees, 1973), *Plodia interpunctella* (Takeda and Masaki, 1976), and *Pectinophora gossypiella* (Pittendrigh and Minis, 1971). These insects are therefore said to have hourglass time-measuring systems. However, it should be stressed that even in some of these species there is evidence for the involvement of some sort of circadian rhythmicity (see Saunders, 1981b).

Although these peaks of induction appear to represent a circadian system underlying photoperiodism, finding suitable explanations for their existence is rather more difficult. In terms of resonance experiments, it is thought that when the period of the driving LD cycle is close, or equal to, a multiple of the true period of the oscillator (τ) the two oscillating systems resonate sympathetically, resulting in a high inductive effect. If, on the other hand, the LD cycle is not near a multiple of τ there will be no resonance, and thus no inductive effect. This was confirmed in a study carried out with the fleshfly *Sarcophaga argyrostoma* (Saunders, 1982b), in which the ranges of entrainment of the pacemaker were calculated (based on the External Coincidence Model) for LD cycles from 12:8 (T18) to 12:72 (T84) using the phase response curve of the pupal eclosion rhythm. These computations indicated that there were three main ranges of T-cycles to which the oscillator could entrain; T20-T29, T44-T53 and T68-T77. Within each of these ranges the photoinducible phase (ϕ_i) was calculated to be in the light at the lower end of the range, but passed into the dark at the longer T-values. A comparison of the theoretical positions of this transition point for each T-range, with the results of a Nanda-Hamner, revealed that the transition coincided almost exactly with the ascending slope of each diapause peak. At the T-values between these ranges no induction was possible as the oscillator could not entrain to the LD cycles.

Explanations of the night-interruption experiments are more difficult, but it is generally agreed that the troughs of low diapause are the result of a coincidence of the photoinducible phase with the light pulse (Saunders, 1982a). This explanation was derived from the results of T24 night-interruption experiments, in which two diapause-aversion peaks were found, one corresponding to a coincidence of ϕ_i with the short scanning light pulse and the other due to ϕ_i being phase-delayed into the beginning of the main light period (Pittendrigh and Minis, 1964; Pittendrigh, 1966). As the ECM predicted

that diapause incidence will decrease whenever ϕ_i comes into the light, these coincidences will result in two points of diapause-aversion.

Although both of these protocols appear to give clear indications of the involvement of a circadian or an hourglass response in any particular species, recent experiments have indicated that these two apparently disparate time-measuring systems may be very similar in their basic properties. The most suggestive of these came from temperature manipulations of Nanda-Hamner responses, where lowering the ambient temperature for a normally periodic response resulted in a gradual filling up of the non-diapause troughs, until the shape of the curve became indistinguishable from the typical hourglass response (Saunders, 1973b, 1982a; Pittendrigh, 1981; Takeda and Skopik, 1985). Raising the temperature level, on the other hand, caused a universal decline in the overall expression of diapause. Although such effects are difficult to explain using the conventional ECM, recent models incorporating damped circadian oscillators have provided possible explanations for such phenomena (Lewis and Saunders, 1987; Saunders and Lewis, 1987a, b).

The aim of the present chapter was to investigate some aspects of the photoperiodic response of *C. vicina*. To this end the flies were exposed to Nanda-Hamner protocols and various environmental disturbances. The results of these experiments were then considered in the light of recent photoperiodic models.

Materials and Methods

The experimental groups of flies were all kept in light-tight boxes in constant temperature rooms, as described in the General Materials and Methods. Meat was presented to the flies on the third day after emergence and every day thereafter, with egg-collections every day from day 11 to day 14. The larvae from all experiments were maintained at 11°C in DD, thus ensuring that the only variable environmental factors affecting diapause were those experienced by the adults. The newly pupated larvae were collected from the sawdust around these cultures every two days. Non-diapausing individuals usually begin pupariation about day 16 post-oviposition at 11°C, with all having formed pupae by day 24. Diapausing larvae, on the other hand, obviously pupariated much more slowly, as the diapausing stage in this species is at the end of the last larval instar. The convention adopted for defining

diapause percentages within a population, was to assume that larvae which had not pupariated by day 30 were in diapause. This choice was somewhat serendipitous, as recent work has shown that the ring glands in *C. vicina* become refractory to PTTH stimulation 26 days after hatching at 11°C (Richard and Saunders, 1987). Thus, by day 30 it is almost certain that any non-pupariated larvae are in diapause (each plotted point using at least 200 larvae).

In order to study the effects of the experimental protocols on the depth of diapause some larval cultures were maintained in the sawdust at 11°C until they spontaneously broke their diapause and formed puparia.

Some of the regimes employed in this study exposed the adults to various chemical treatments. These treatments were given to the flies as solutions in their drinking water from the moment they eclosed. Furthermore, as the meat used inevitably contained a high proportion of water, it was first necessary to dehydrate it, by chopping it into very small pieces and storing at 4°C for 24 hours, and then rehydrate by soaking for 1 hour in the same solution given in the adult drinking water. This method was preferred over attempts to dehydrate using H_2SO_4 (as outlined in Solomon, 1951), firstly due to its practicability, and secondly, because it appeared to be more effective in terms of the overall weight loss (data not given).

The meat was given to the flies from day 3 onwards, but eggs were only collected on alternate days from day 12 to day 16. This later collection time was necessary due to the slightly slower growth-rate of the follicles, although they appeared to be normal when they reached their mature size.

Results

Photoperiodic Response Curves

The experimental flies came from the two previously described strains of *C. vicina*, i.e. Scottish (55°N) and U.S. (36°N). Groups of flies from both strains were maintained at 20°C in a variety of photoperiods from 4:20 to 20:4, as well as DD and LL. The diapause expressed by larvae hatching from eggs laid on day 12 was plotted against the prevailing photoperiod to give the phase response curves (PPRCs). The results for the Scottish and U.S. strains are presented in Fig. 7.2a and Fig. 7.3a respectively. Clearly, both strains showed

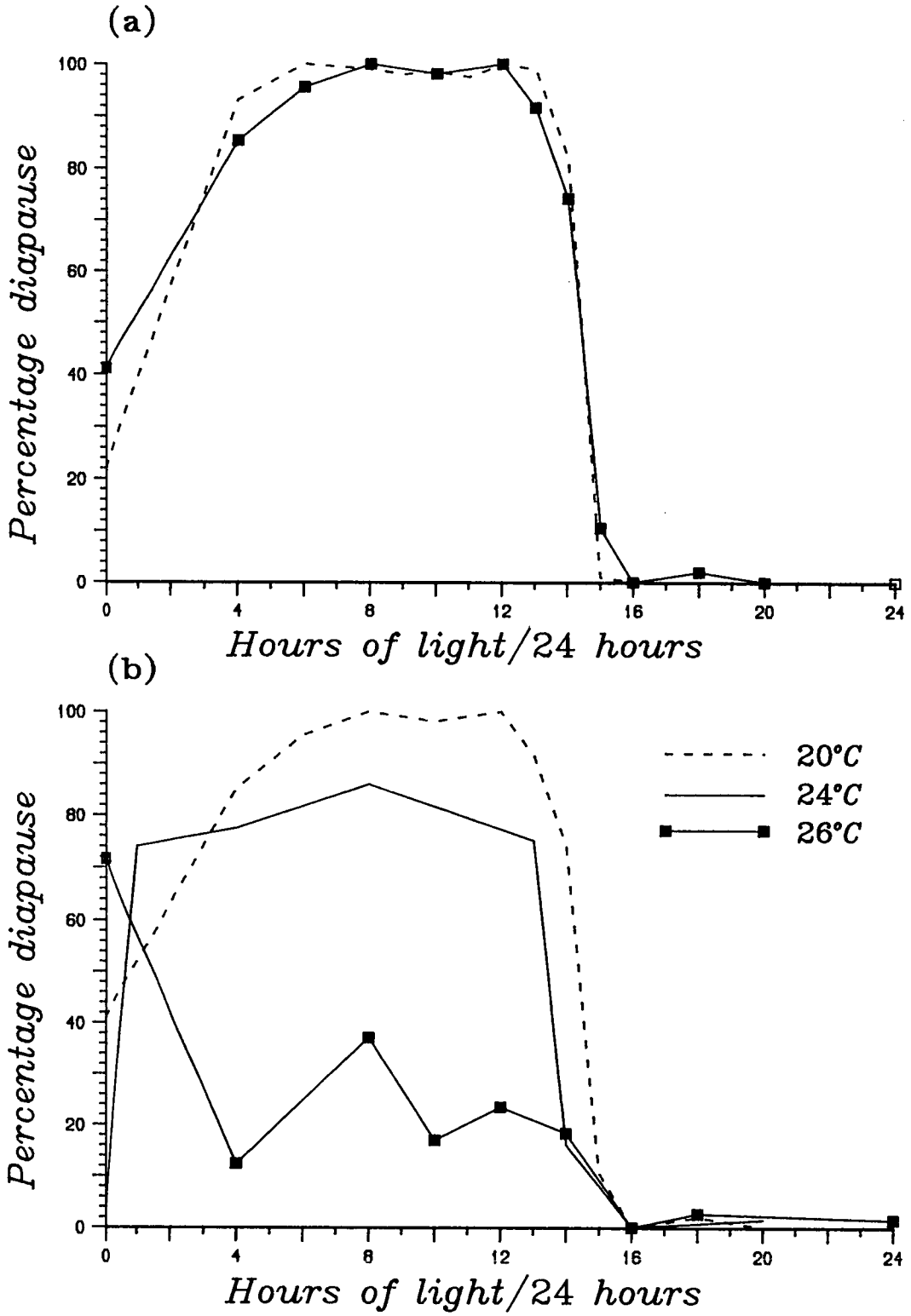

Figure 7.2. Photoperiodic response curves (PPRCs) for the Scottish *C. vicina* strain (55°N). (a) Both of these curves represent PPRCs at 20°C. The solid line is taken from the present study, the dashed line indicates the results of a previous PPRC for the same strain (Saunders, 1987). Clearly there is very little difference between the two examples. (b) This graph consists of three PPRCs for the Scottish strain at 26°C, 20°C (both from the present study) and 24°C (Vaz Nunes, unpublished). The degree of diapause incidence was evidently highly dependent on the temperature experienced by the adult blowflies.

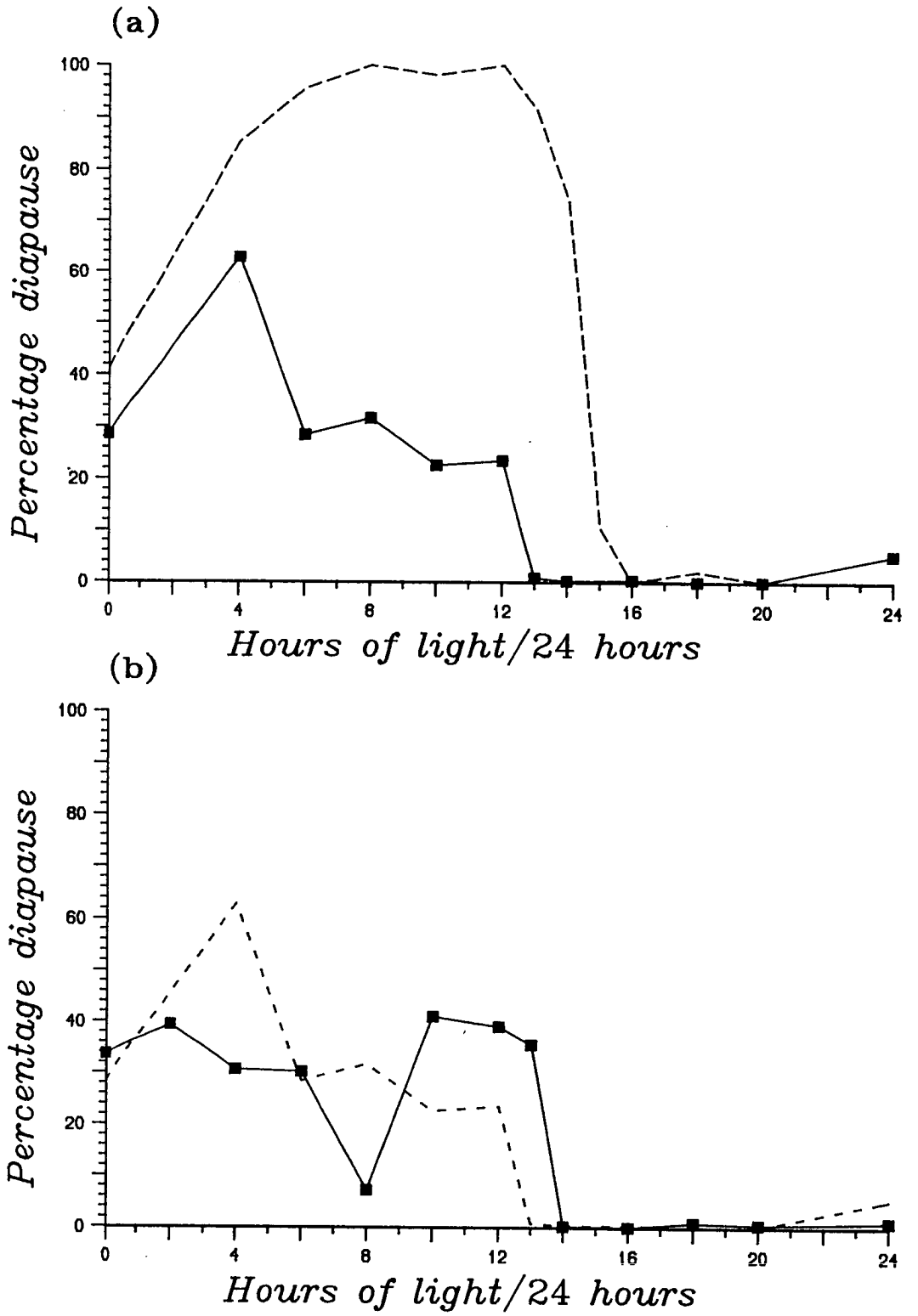

Figure 7.3. Photoperiodic response curves for the U.S. strain of *C. vicina* (36°N). (a) The solid line is the response from this strain at 20°C; for comparison the response of the Scottish strain at the same temperature is represented by a dashed line. (b) The solid line indicates the results from the U.S. strain at 23°C, the dashed line the response of the same strain at 20°C (from the PPRC above). It is apparent that different strains of *C. vicina* have very different responses to the prevailing photoperiod.

PPRCs characteristic of long-day species, with maximum diapause induction in short days. The pattern produced by the Scottish strain was identical to a previously published PPRC for this strain at 20°C (Saunders, 1987, see Fig. 7.2a). Furthermore the critical daylength (CDL) was found to be 14.3 hours in the present experiments, almost indistinguishable from the previously reported value of 14.5 hours (Saunders, 1987).

The U.S. strain, on the other hand, differed in several important respects from the Scottish (see Fig. 7.3a). Firstly, the level of diapause induction in short days was found to be far lower in the U.S., being 33.7% (averaged over the photoperiods from 4:20 to 12:12). The Scottish strain expressed an average diapause of 95.8% over the same photoperiods. Secondly, the CDL was found to be between 12 and 13 hours, a much shorter value than the 14.3 hours described above. There also appeared to be a slight increase in diapause induction at LL in the U.S., a feature not seen in the Scottish strain. As well as these differences, the percentage diapause in DD was found to be slightly lower (being 28.4% for U.S. compared to 41% for Scottish).

In general, the variations in PPRC accorded well with those expected from strains originating from two separate latitudinal localities.

As it had previously been found that temperature changes could induce alterations in insects' PPRCs, it was decided to expose adults from both strains to temperatures other than 20°C. Thus, the Scottish adults were kept at 26°C, while the U.S. were maintained at 23°C. Although a previous PPRC for the Scottish strain at 24°C (Vaz Nunes, unpublished) had shown decreased diapause (about 63.4%, averaged from 4:20 to 12:12), the present results showed an even more marked decline when the temperature was further increased to 26°C: the new level being 22.4%, for 4:20 to 12:12 (see Fig. 7.2b). Interestingly, the diapause present in DD responded in the opposite direction to increased temperature, showing an increase to 71.5% (although Vaz Nunes' results showed a greatly decreased diapause in DD at 24°C). Included in these temperature effects were shorter CDLs. Although the low level of diapause in the present 26°C experiment did not allow this to be clearly seen, the previous 24°C results indicated that the CDL had shortened to about 13.5 hours.

The U.S. strain, on the other hand, did not display substantial diapause reduction with increasing temperature, for while 23°C produced 31.1% diapause

(between 2:22 and 12:12), at 20°C it was found to be 33.7%. Furthermore, the diapause in DD only showed a slight increase over this temperature rise, going from 28.4% at 20°C to 33.5% at 23°C (Fig. 7.3b). The CDL, however, clearly lengthened to 13–14 hours with the temperature increase.

While the temperature effects in the Scottish strain were similar to the findings from previous studies, i.e. lower diapause and shorter CDL with increasing temperature, the U.S. strain behaved in quite the opposite manner having a largely unchanging diapause incidence and a lengthened CDL.

Nanda-Hamner Protocols

The resonance experiments were performed with the Scottish strain at two temperatures, 20°C and 23°C. At each temperature, the adults were exposed to a 12 hour photophase (L=12) coupled to scotophases from 6 to 68 hours. The incidence of diapause was recorded for larvae derived from eggs laid on day 12 post-emergence. At 20°C (Fig. 7.4a) the results from these T-cycles resembled an hourglass type of response, with all LD cycles from T28–T72 having 80 to 100% diapause. Despite this, there was some indication of 24 hour periodicity, with slight reductions in diapause being evident at T36 and T60. T-cycles beyond 72 hours showed a sharp decline in diapause level, until at T80 (12:68) there was almost complete non-diapause.

Raising the temperature to 23°C, on the other hand, produced quite a different response. In this case, there was clear 24 hour periodicity (Fig. 7.4b), with peaks of diapause induction at T24, T48 and T72. In conjunction with this, it was noted that each peak comprised two apices, and that there was a gradual fall in the maximum diapause with successive peaks. Subsidiary peaks were also observed at T38 and T62, coinciding with the lowest points of diapause induction. Unexpectedly, there was also found to be a difference in the critical nightlengths (CNL) for the two temperature responses, with 20°C having a value of 10–12 hours (approx. 11.5 hours) and 23°C being between 6 and 8 hours (approx. 7.5 hours).

Although PPRCs provide information on the type of photoperiodic response exhibited by insects, they cannot determine whether the clock is measuring the length of the day or the night, or perhaps a combination of the two. The Nanda-Hamner protocol, however, can provide such information when the length of the photophase is manipulated. Thus, if the photophase is increased,

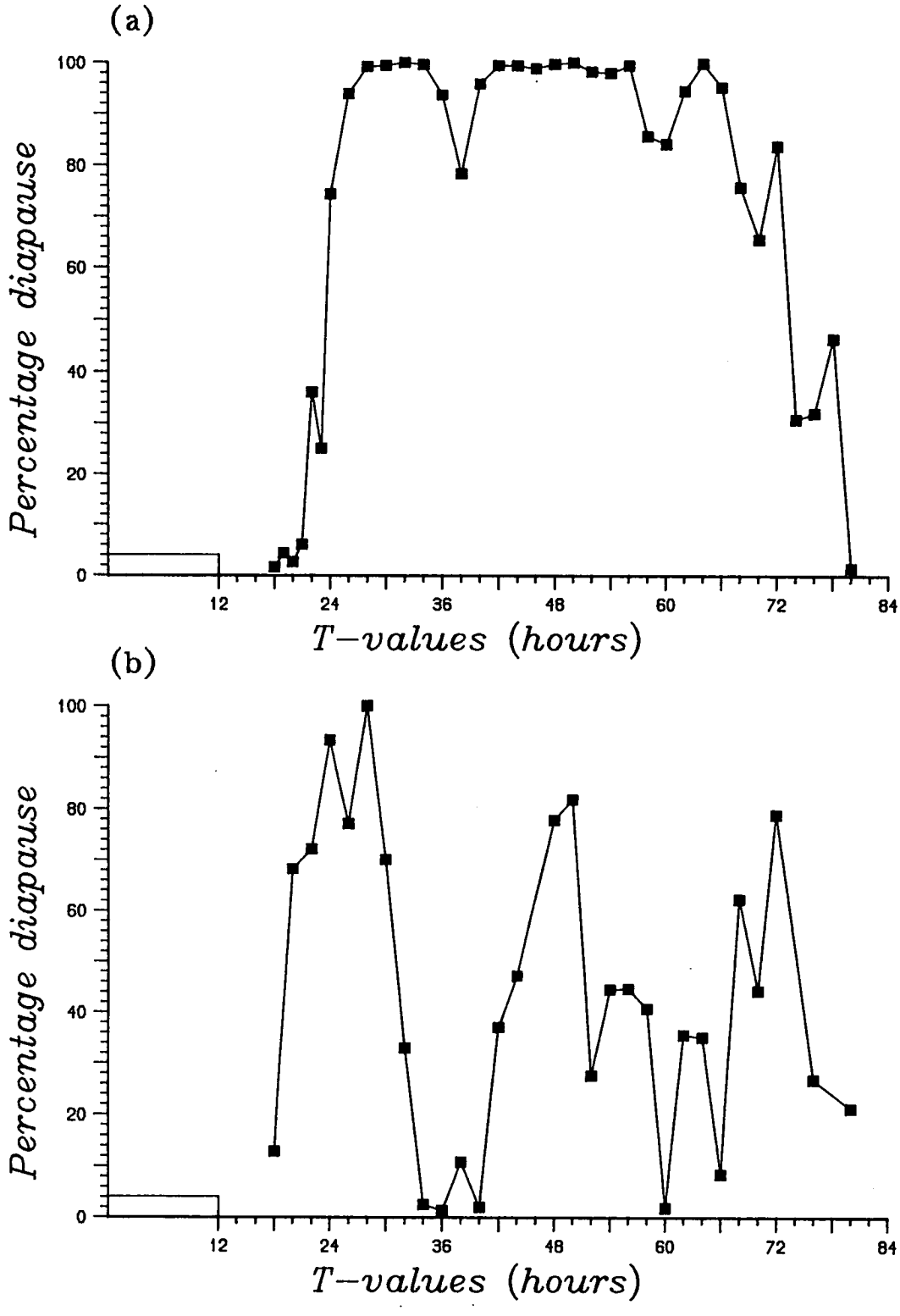

Figure 7.4. Two Nanda-Hamner responses for the Scottish strain, (a) 20°C and (b) 23°C. In both examples a photophase of 12 hours was used (L=12). Note that the former resembles an hourglass type response, while the latter exhibited periodic diapause induction at 24 hourly intervals. More detailed descriptions of these will be found in the text.

and the clock is measuring daylength, the response should not be altered in its position on the x-axis. If, on the other hand, nightlength is the important factor, one would expect the first peak of diapause induction to occur at successively later T-values, the movement along the x-axis equalling the increase in photophase length. In the present experiments, when the photophase was increased to 16 hours (at 23°C), the resulting response was unequivocal; the circadian system was measuring the length of the night (Fig. 7.5). All of the peaks of induction had been shifted roughly 4 hours to the right (except the first one which was about 6 hours later), while maintaining all of the features of the response. Thus, there was a general decline of maximum diapause in successive peaks and small subsidiary peaks in the non-diapause troughs. It was noticeable, however, that the CNL for this L=16 resonance response was between 10 and 12 hours (approx. 10.8 hours), in comparison to the 6-8 hours for the L=12 response. It was considered possible that this very short CNL found in L=12 (at 23°C) may be an erroneous result, as the L=16 value above was similar to that obtained for L=12 at 20°C. This would also partially explain why the first diapause peak at L=16 appeared to show a greater delay than the subsequent ones.

The U.S. strain was also tested for its response to a Nanda-Hamner. At 20°C, and with a photophase of 12 hours, larvae from day 12 eggs produced a clear periodic pattern of diapause induction (Fig. 7.6). Although the first inter-peak interval appeared to be in the region of 22 hours, the subsequent peaks occurred 24 and 48 hours later. Although the maximum diapause induction appeared to be slightly lower in all of the peaks for the U.S. strain, the same gradual decline was observed with increasing T-values. The rate of the decline was, however, greater than had been seen in the Scottish strain. Furthermore, the CNL of this response was found to be longer than any of the Scottish results, at 12-16 hours (approx. 14.5 hours). This longer CNL concurred with the shorter CDL found in the PPRC for this strain. One further feature was also present which had not previously been observed in the Scottish responses to this protocol, namely an extra peak at T88.

In conclusion, these Nanda-Hamner results confirm that both strains of *C. vicina* probably have a circadian oscillator (or oscillators) controlling their photoperiodic responses, which uses nightlength to indicate the passage of time. They also generally support the finding of the PPRC study, that the more southerly U.S. strain has a shorter CDL.

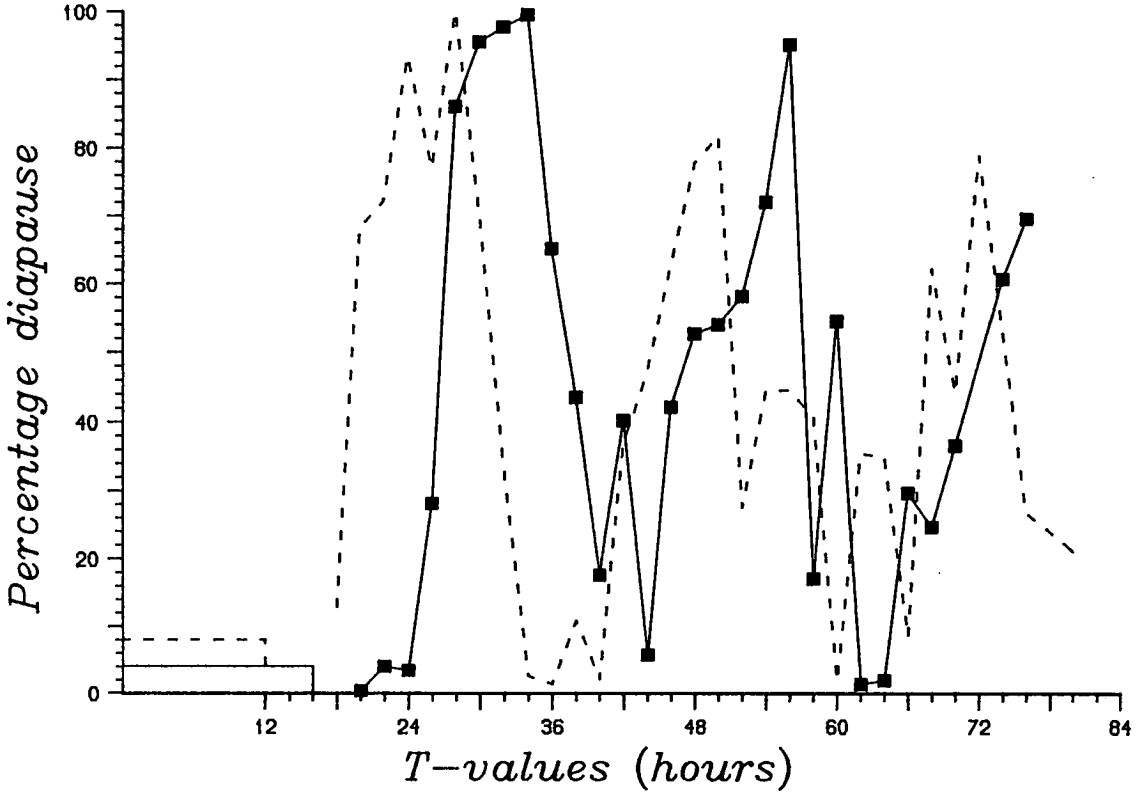

Figure 7.5. The solid line indicates the results of a Nanda-Hamner with a 16 hour photophase (L=16) at 23°C. For comparative purposes the response for a 12 hour photophase (from Fig. 7.4b) is given as a dashed line. It would appear that the two responses are very similar, except that the curve for L=16 is shifted roughly 4 hours to the right. This suggests that it is nightlength which is being measured by the photoperiodic system.

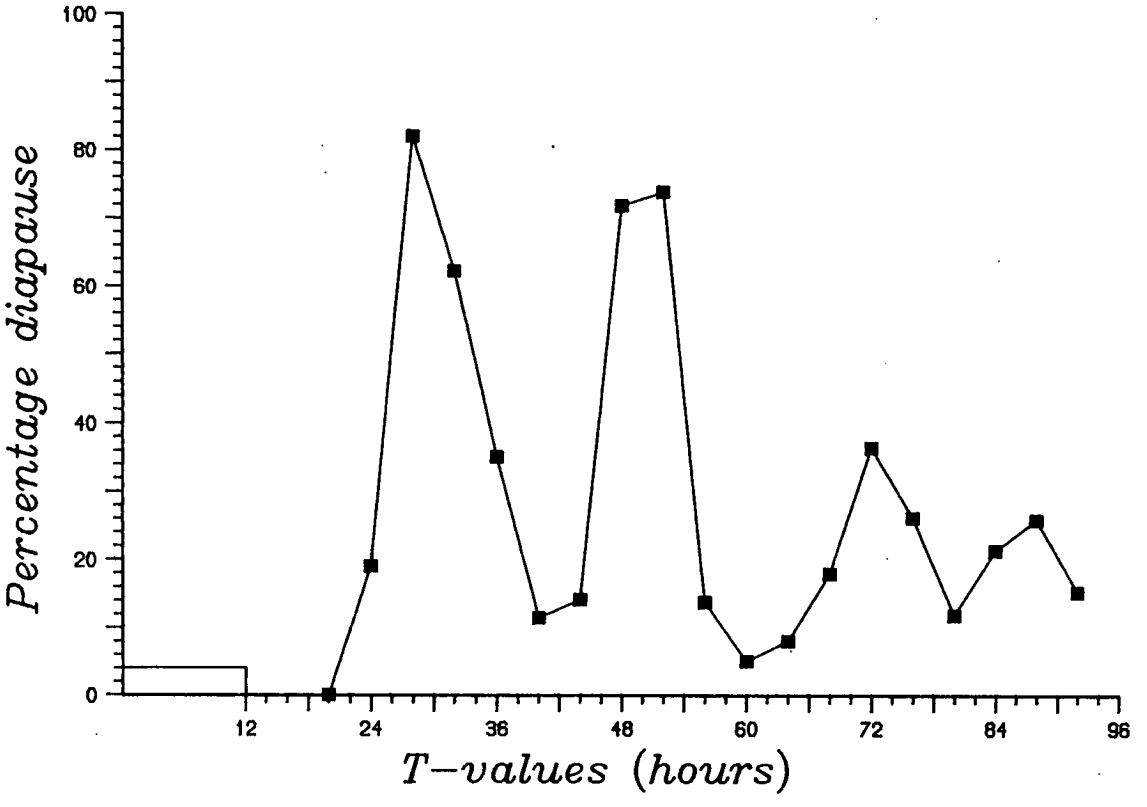

Figure 7.6. A Nanda-Hamner response for the U.S. strain at 20°C, using a 12 hour photophase. Note that an oscillating response is also seen in this strain with a 24 periodicity, although the diapause levels are slightly lower than the Scottish strain. Furthermore, the response appears to damp out more rapidly.

Manipulations of the Photoperiodic Response

Deuterium Oxide

In the previous chapters on adult locomotion, it had been noted that exposure to various chemical treatments induced alterations to the observed rhythmic patterns. Furthermore, such changes had proved to be valuable aids towards an understanding of the system controlling locomotor behaviour. Thus, it was considered appropriate to investigate the responses of the photoperiodic system under similar treatments.

The initial experiment was a control for the technique used to provide the adults with their chemical solutions. This control resembled the following experiments in every detail, except that the dried meat was rehydrated using pure water. The PPRCs were calculated using larvae from day 12 eggs, with the adults maintained at 23°C. As can be seen from Fig. 7.7a this control very closely matched the previous PPRC at 20°C, although it was slightly lower in terms of its overall diapause. This slight lowering of the response may have been the result of the higher temperature used. It also appeared that the CDL may have been slightly shorter as a result of the technique, although this was not thought to be significant.

The first chemical treatment used 5% D_2O solutions, given to the adult flies in their meat and drinking water. Photoperiodic response curves were constructed for both strains using the larvae from day 12 eggs at 23°C. Under this treatment the Scottish strain showed a distinctive response. At all photoperiods from DD to 12:12 D_2O induced lower levels of diapause (see Fig. 7.7b): this was approximately 51.2% between 2:22 and 12:12. However, when the photoperiod increased to 13:11, the D_2O lost its ability to alter the response, and the subsequent parts of the curve resembled the water experiments in most respects, although there was an indication that the CDL was slightly longer after D_2O treatment (see Fig. 7.7a).

Repeating the experiment with the U.S. strain, at 20°C, gave the result in Fig. 7.8. A control experiment was not carried out for this strain, as it was considered that the previous control had shown the regimen to have only small effects on the response. The results indicated that far from there being a decline in the overall level of the response in the U.S. strain, there was a slight increase to 42.2% between 4:20 and 12:12. The diapause in DD was, however,

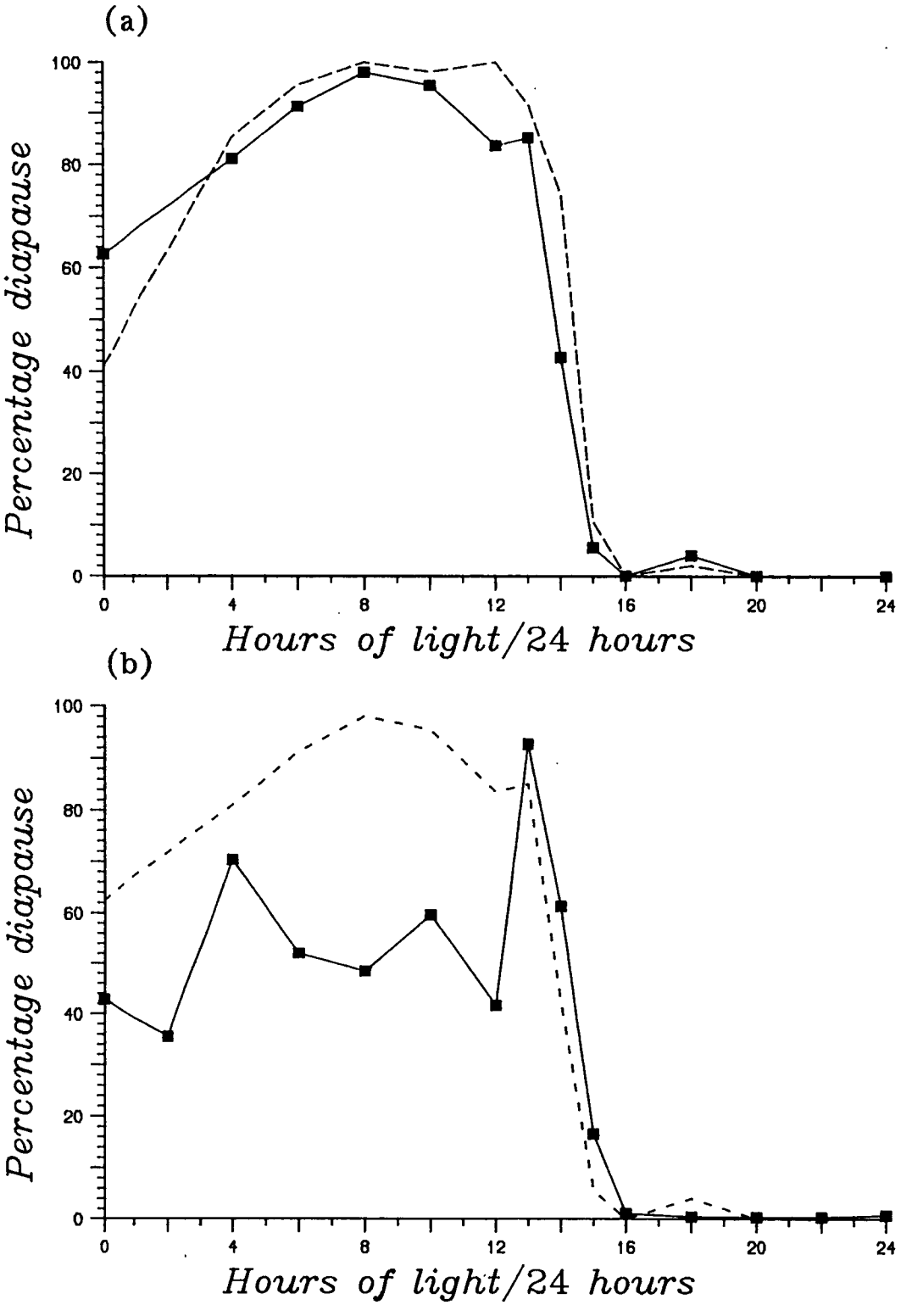

Figure 7.7. The effects of deuterium oxide (D_2O) on the photoperiodic response of the Scottish strain at 23°C. (a) The solid line is the water control for the protocol used to provide the flies with D_2O. Comparison with the response seen in normal culturing (dashed line, from Fig. 7.2a) indicated the technique had little effect on the response. (b) In this instance the solid line shows the effects of 5% D_2O, while the dashed represents the control from (a) above. The diapause was seen to be much reduced in all short photoperiods, although the critical daylength (CDL) was largely unaffected by the treatment.

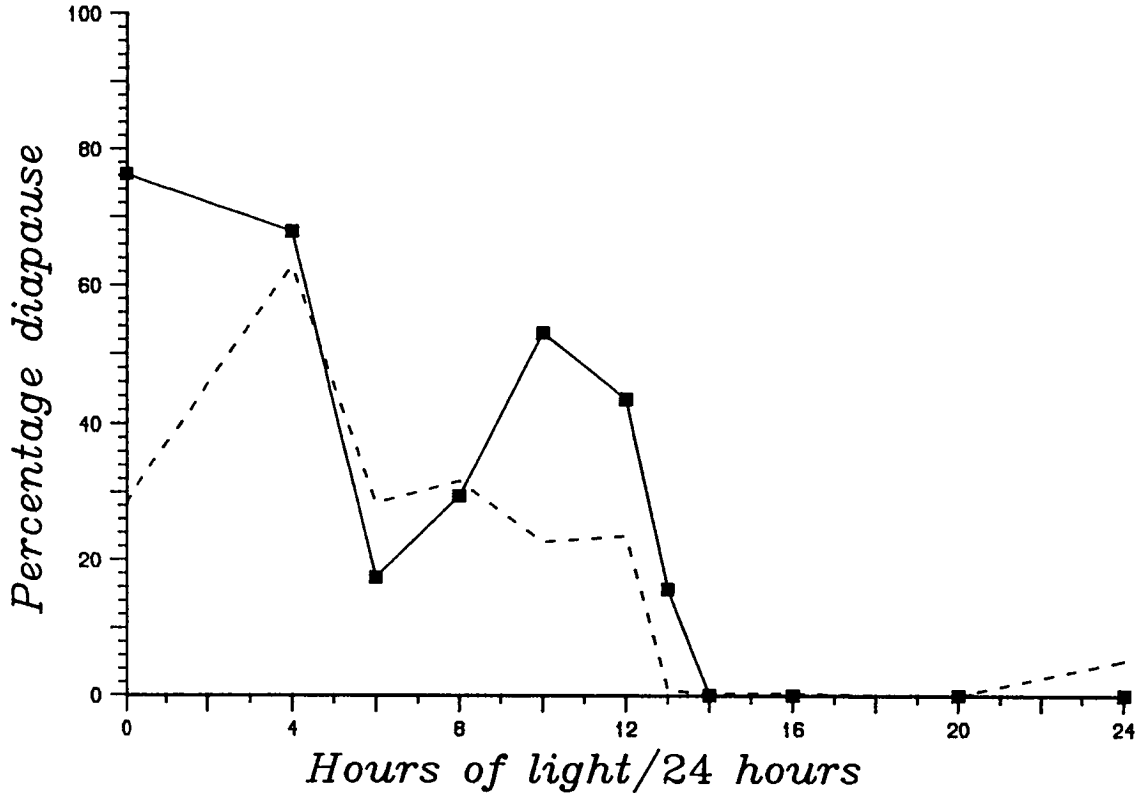

Figure 7.8. The effects of D_2O on the U.S. strain at 20°C. The dashed line shows the response of this strain at the same temperature, when only exposed to water (taken from Fig. 7.3a). Note that the diapause level appears to be unaffected by the treatment, while the CDL was clearly lengthened by an hour.

seen to increase markedly with D_2O treatment, becoming 76.2% from the previous 28.4%. However, the most noticeable alteration to the response was in the CDL, which was seen to increase to 13–14 hours, from the previous 12–13 hours.

The same 5% D_2O treatment was applied to Scottish flies in a Nanda–Hamner protocol. As before, the temperature for the adult cultures was set to 23°C, and larvae from day 12 eggs were used to provide the diapause results. In the PPRC with D_2O it had been observed that there was a great disparity in the diapause response between 13:11 and 12:12. For this reason, it was decided to conduct two resonance experiments using photophases of 12 and 13 hours respectively. The results were intriguing. With L=12, although the response was less clear than previous Nanda–Hamner experiments, peaks of induction were still visible at T24 and T48 (Fig. 7.9a). The third peak (expected at T72) was obscured by an early rise in diapause induction. Despite this, the most peculiar feature of the response was the extremely low diapause found at T24, not reaching above 45%. The CNL appeared to have remained unchanged at 10–12 hours, although the substantial diapause percentage at T20 (approx. 25%) suggested that the range of CNL values may have been extended if the experiment had examined T-values below this. Repeating the experiment for L=13, provided a much clearer response, with three diapause peaks at T24, T48 and T72 (Fig. 7.9b). Again the CNL was found to lie between 10 and 12 hours. However, the single most distinguishing feature of this result, when compared to L=12, was the high diapause present at T24. Indeed, this response closely resembled that seen with water, with the exception of sharper peaks at the resonant T-values.

Although the effects of D_2O were rather difficult to interpret, it did appear that the CDL (in the Scottish strain) may have been largely protected from its influence, presumably by some homoeostatic mechanism. This was indicated by the relatively slight lengthening of the CDL in the PPRC, and the disparate responses seen for the two photophases in the resonance experiments. Although the U.S. strain was less susceptible to gross changes in the level of response, its CDL was altered by the treatment to a far greater extent. Interestingly this mirrors the differing temperature effects on the PPRCs of the two strains. Although the circadian system in *C. vicina* has been shown to measure the length of the night, the present Nanda–Hamner results with the Scottish strain seem to indicate that the system is also sensitive to the length

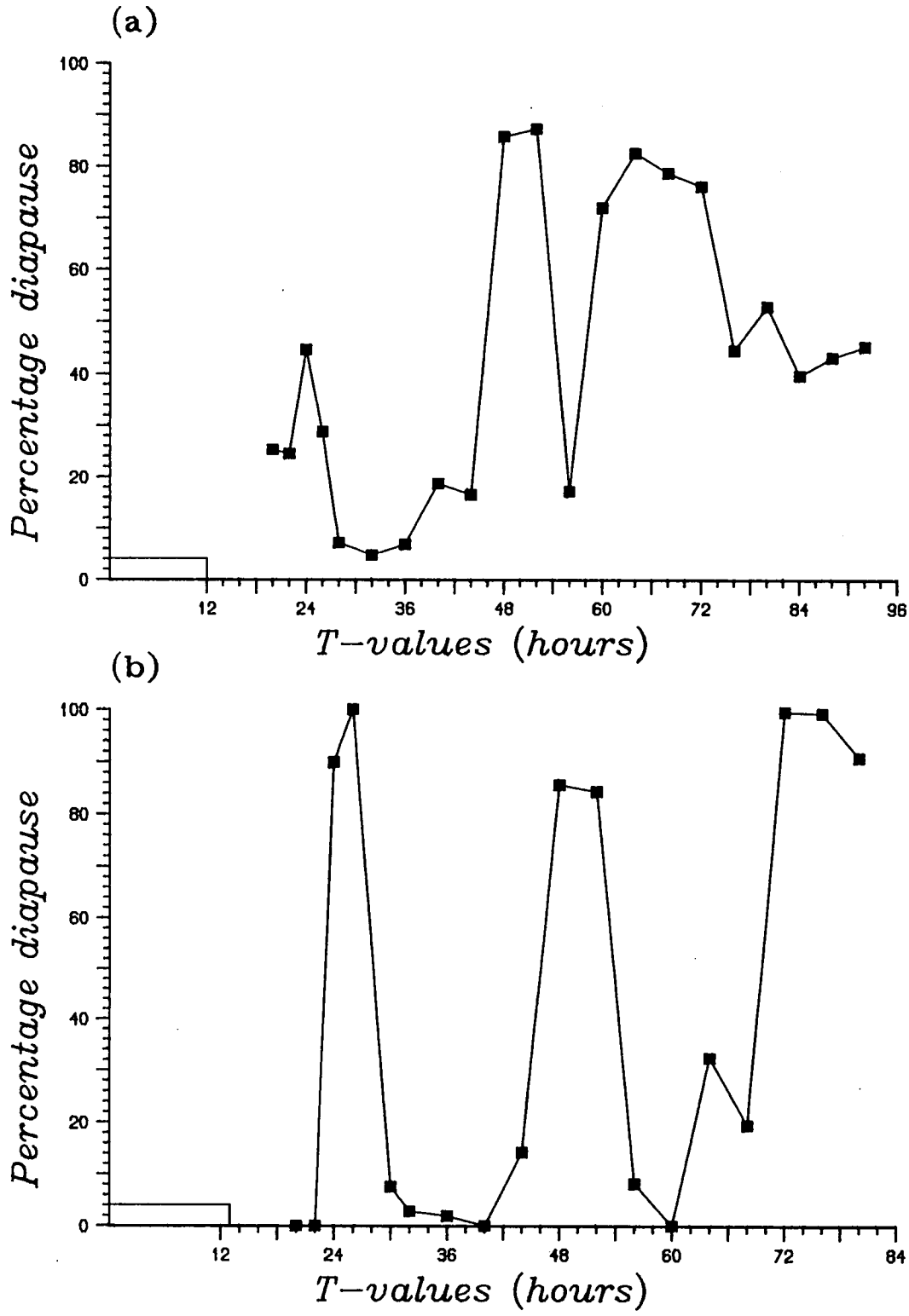

Figure 7.9. The Nanda-Hamner responses of the Scottish strain at 23°C in the presence of 5% D_2O, with (a) a 12 hour photophase and (b) a 13 hour photophase. It was notable that the while both produced oscillating responses, that seen in L=12 was more disrupted. This was emphasized by the reduced height of the first peak and the excessive width to the third. These results bear comparison to the PPRC seen in Fig. 7.7b, where a 12:12 photoperiod showed a reduced response to 13:11.

of the photophase. In a normally functioning system the night responses may completely obscure the effects of the light, but with the introduction of D_2O the system is destabilized to the benefit of the light responses. Alternatively, D_2O may be affecting the counter mechanism which accumulates successive long nights. As this system has been shown to be temperature-compensated (Saunders, 1981b), one may expect that the accumulation process for those photoperiods closest to the CDL would be under the greatest homoeostatic control. Clearly in hindsight it would have been instructive to repeat the D_2O Nanda-Hamner protocol for a variety of photophases, in both the Scottish and the U.S. strains.

Other Treatment Regimes

Only two other chemicals were applied to the photoperiodic response, both with the Scottish stock at 23°C. As for all previous photoperiodic experiments, larvae from day 12 eggs were used as indicators of the response. The first regime involved the application of 0.16% acetone, in precisely the same manner as had been used for D_2O, in a PPRC. There appeared to be almost no effect of the treatment on either the overall level of induction or on the CDL (Fig. 7.10a). The final treatment, using 0.005M lithium chloride (LiCl), was given during a resonance experiment. The results, although only covering a short range of T-values, were found to be very unusual (Fig. 7.10b), for as well as the expected peaks of diapause induction at T24 and T48 there were two further peaks at T32 and T38. The CNL was also observed to be slightly shorter, at 8-10 hours. There appeared to be no obvious explanation for these peculiar results, but it did appear as if the pattern had broken down under the influence of the LiCl. This conclusion is supported by the similar results seen with locomotor activity, in which this chemical produced an unusually high percentage of split rhythms.

Larval Development

It has been shown previously that the duration of larval development in *C. vicina* was positively correlated with the incidence of diapause (Saunders, 1987). This, therefore, indicates that the length of larval development must be a reflection of the duration or depth of diapause. As previous studies have shown that this feature of the photoperiodic response can vary between geographical strains, it was decided to study this effect in the two available populations of

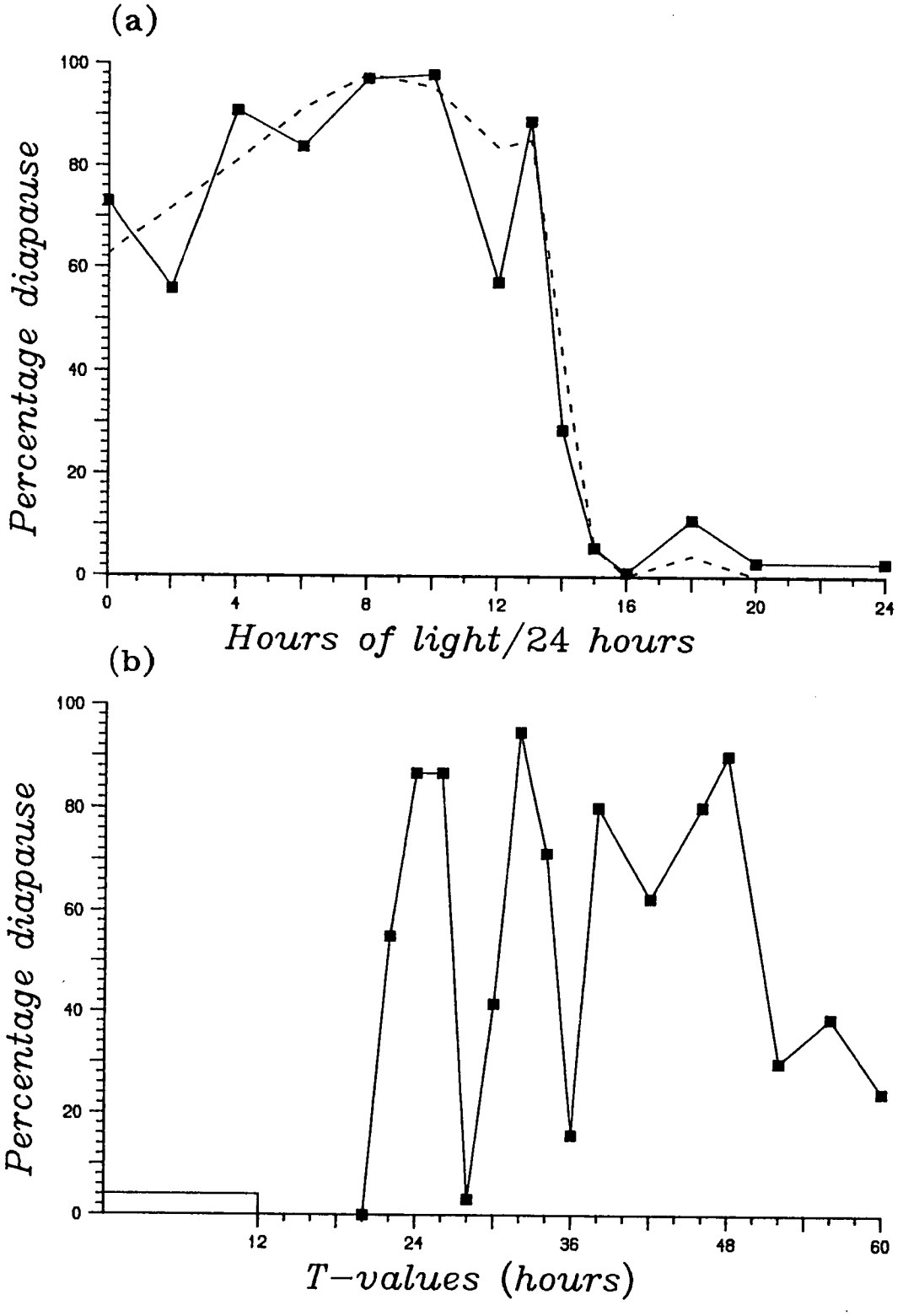

Figure 7.10. (a) The effects of 0.16% acetone at 23°C on the photoperiodic response of the Scottish strain. There appeared to be no effect of this treatment on the PPRC. (b) A Nanda-Hamner protocol at 23°C with the Scottish strain. In this instance the adult flies were exposed to 0.005M lithium chloride (LiCl). The normal oscillatory pattern seemed to have disintegrated, and as such the effects of this treatment resembled those observed with LiCl on the locomotor rhythms.

C. vicina. For the purposes of these experiments, the duration of larval development was defined as the time from hatching, to the point at which 95% of each experimental group had pupariated. It was decided not to use 100% pupariation, as the values obtained seemed to be artificially protracted by a very small number of late-pupariating individuals. Furthermore, 95% values proved to give a tighter correlation to the incidence of diapause.

Figure 7.11 shows the resulting regression for both strains. Superficially the two populations seemed to have similar relationships between diapause incidence and length of larval development. Comparative analyses of the regression coefficients confirmed that the correlations were not significantly different between the two strains (variance of difference in regression coefficients, d=0.847; not significant). Data were also available for the effects of D_2O on the development time in the U.S. strain (Fig. 7.12). When the D_2O treatment was compared with the previous regression for the same strain, it was found that D_2O had slightly reduced the regression coefficient (see Fig. 7.12), although this alteration proved to be non-significant (d=1.259). Unfortunately there were insufficient data to repeat the comparison for the effects of D_2O on the Scottish strain.

Thus, it was clear that depth of diapause did not differ between these two latitudinal populations of the blowfly, nor did D_2O effect any significant alterations to this relationship in the U.S. strain.

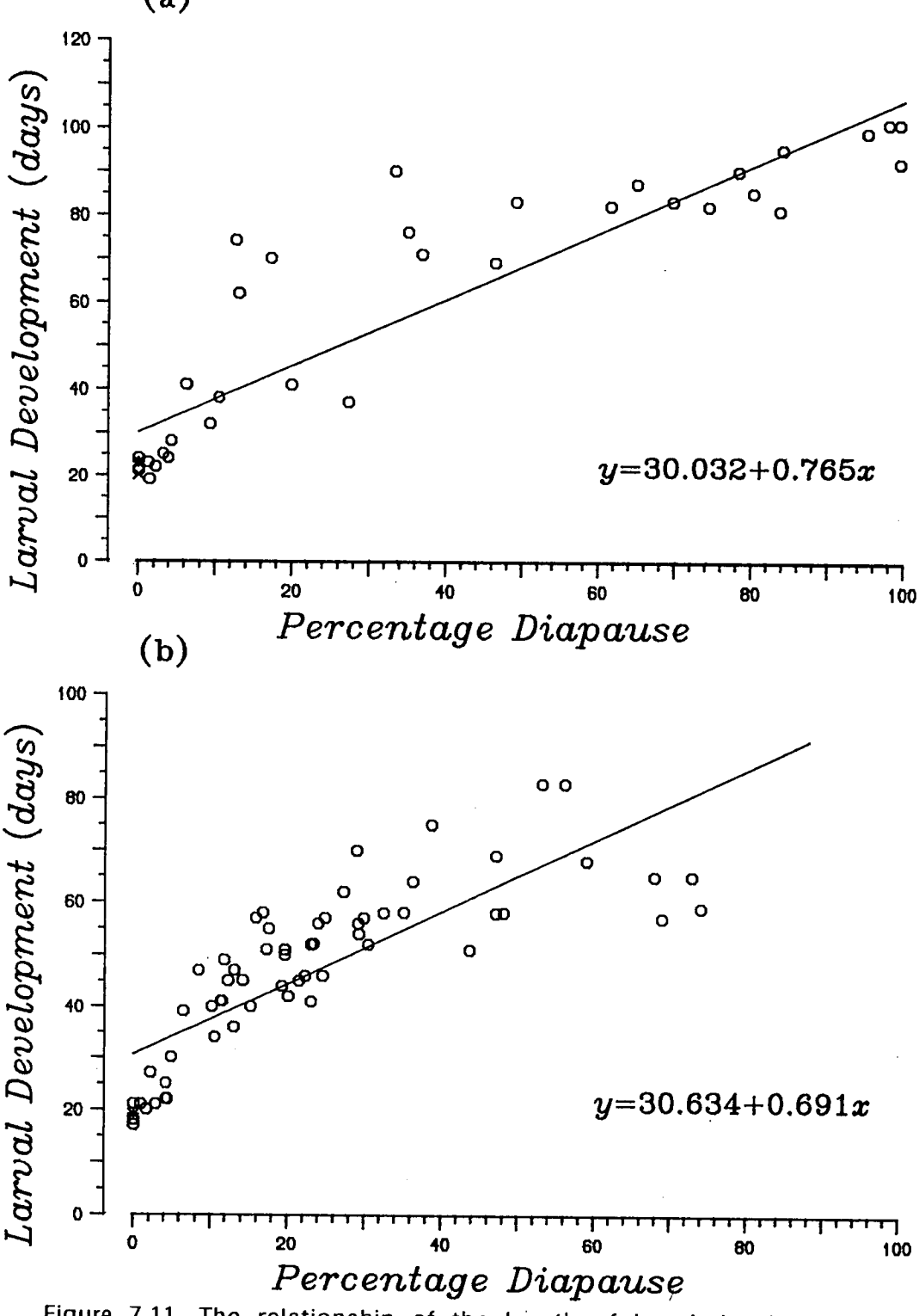

Figure 7.11. The relationship of the length of larval development to the incidence of diapause in various groups of blowflies, in (a) the Scottish and (b) the U.S. strain. The data for both strains came from larvae produced by adults which had been exposed to 20°C, with single datum points represented by circles, double points by crosses and the coincidence of three or more points by asterisks. Both of the strains showed a tight correlation between the two parameters (Scottish, r=0.907 with 37 d.f., goodness-of-fit, F=170.700 with 1 and 37 d.f.; U.S., r=0.807 with 61 d.f., goodness-of-fit, F=113.724 with 1 and 61 d.f.). However, comparison of the regression coefficients found that the two strains did not differ significantly in their relationship of diapause incidence and larval development time (d=0.847, not significant).

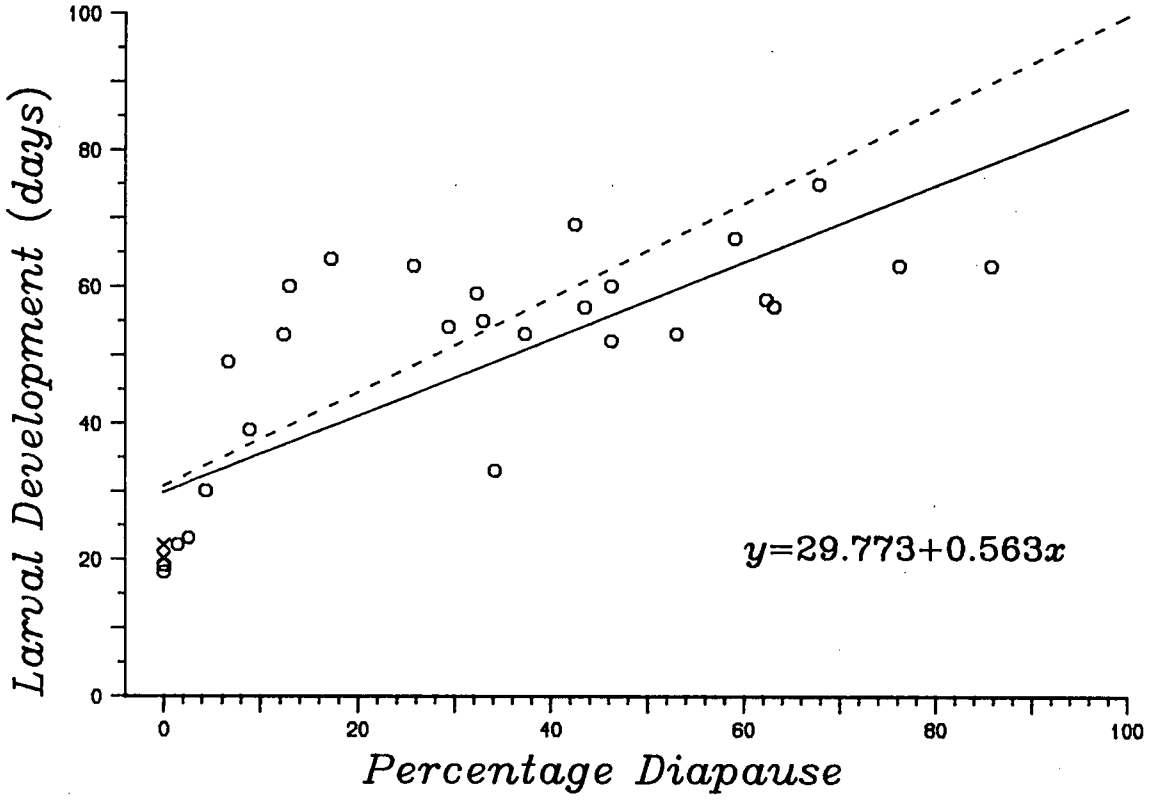

Figure 7.12. A study of the effects of 5% D_2O on the relationship between diapause incidence and length of larval development for the U.S. strain at 20°C. The solid line is the regression for the data present on the graph, while the dashed line indicates the regression from Fig. 7.11b. There was a clear positive correlation (t=7.184 with 31 d.f.; r=0.790 with 31 d.f.; goodness-of-fit, F=51.610 with 1 and 31 d.f.), although this was not found to differ from the previous regression (d=1.259, not significant). As for the previous figure the circles represent one, and the crosses two, data points.

1. Although both strains displayed long-day responses to photoperiod, these were found to differ in CDL and levels of diapause induction, the southern strain having a lower diapause incidence at short photoperiods and a shorter CDL.
2. The PPRCs of the strains also differed in their responses to environmental disturbance, with the U.S. lengthening its CDL with increased temperatures or D_2O treatment, while the Scottish strain showed a shortened CDL and a decline in the level of diapause with increased temperature. Although deuterium oxide produced a reduction in the diapause in the Scottish strain there was no evidence of any effect on the CDL.
3. The incidence of diapause in DD increased after a temperature increase for both strains, although D_2O treatment resulted in differing DD responses in each strain, with the U.S. increasing its diapause while the Scottish showed a reduction. Acetone was found to have no effect on the response in the Scottish strain.
4. Nanda-Hamner protocols revealed the involvement of a circadian oscillator in the photoperiodic responses, with nightlength appearing to be the crucial time cue. Temperature effects were also induced in the response, such that hourglass-like patterns were observed at lower temperatures.
5. Deuterium oxide application to these Nanda-Hamner experiments resulted in unusual patterns, depending on the length of the photophase. Lithium chloride produced a highly disrupted pattern, indicative of a breakdown in the circadian organization.
6. No variation was found in the depth of diapause between the two strains, and D_2O was found to have no significant effects on this response in the U.S. strain.

Discussion

The results from these experiments have clearly shown that the photoperiodic responses of *C. vicina* exhibited the characteristics of a long-day insect, with diapausing progeny resulting from exposure to short days. The similarity between the two strains did not, however, extend much beyond this, for the U.S. had consistently lower levels of diapause and a shorter CDL. Despite this, it was assumed that the two strains must have the same basic 'clock' controlling their photoperiodic phenomena. Furthermore, the Nanda-Hamner protocols clearly revealed that for both populations this clock was circadian in nature, and had a period of about 24 hours. If resonance experiments are revealing the presence of an oscillator underlying photoperiodism, the rapidity with which the response decays may reflect the degree of damping of that oscillator. Thus, from the present results the U.S. strain might be expected to have the more damped pacemaker controlling its photoperiodic responses. Is this, therefore, the basis of the observed differences between the strains?

A recent model, devised to explain the features of the photoperiodic responses in *Sarcophaga argyrostoma*, may hold the key to answering this question (Lewis and Saunders, 1987; Saunders and Lewis, 1987a, b). Although the model has already been described in the General Introduction, it would be beneficial to reiterate its main points again. In essence, the model involved a damped circadian oscillator which functioned according to the principles of the External Coincidence Model (ECM). The performance of this oscillator was controlled by the interaction of a temperature-dependent synthesis of an oscillating chemical and its temperature-independent loss. Both synthesis and loss were incorporated into a negative feedback system, in which a time-delay factor governed the formation of the oscillation. Alterations in the synthesis rate of the chemical resulted in greater or lesser degrees of damping in the oscillator, with lower rates of synthesis increasing the damping. The time-delay factor was not only essential for the formation of the oscillation, but also controlled its period. Thus, increases to the time-delay increased the period of the oscillation.

The induction of diapause was dependent upon the production of a 'diapause titre' (in the model this was denoted as a diapause-inducing chemical

INDSUM), with the degree of the response determined by the concentration of this substance at the end of the photosensitive phase. This INDSUM was synthesized, in a temperature-dependent fashion, when the oscillation fell below a threshold during the subjective night. This point in the cycle was considered to be equivalent to the photoinducible phase, ϕ_i, in the ECM. Although, INDSUM was synthesized when ϕ_i coincided with the dark, illumination of this point in the cycle leads to its destruction. Thus, in successive long nights INDSUM was seen to accumulate to a point at which diapause was induced. A more recent, updated, version of this model (Saunders and Lewis, 1988) considered the total accumulation of INDSUM to be more accurately reflected in an integration of the whole area of the oscillation above the threshold.

The real strengths of this model lie in its ability to accurately simulate the photoperiodic responses of a large number of insects. Furthermore, these simulations can be achieved by the manipulation of relatively few components of the system. For example, the degree of damping of the oscillator can be increased by reducing the synthesis rate, which, in turn, is reflected in the observed PPRCs as a shortening of the CDL. Therefore, the greater damping apparent in the Nanda-Hamner responses of the southern strain of the blowfly, may explain the shorter CDLs seen in this population.

Although, it was noted that both strains expressed the same diapause incidence in DD, for the Scottish strain this level was considerably below that found in the other short photoperiods. These features can also be explained using the Lewis and Saunders' model. Thus, if an oscillator is self-sustaining, one would expect it to continue to oscillate in DD, with the result that the ϕ_i would pass through the threshold many times during the photosensitive phase, and diapause incidence would be high. If, on the other hand, the oscillator was damped the number of cycles in which ϕ_i did this would be limited by the degree of damping. Thus one would expect to find lower levels of diapause in DD when the oscillator was damped, with the incidence of diapause giving some indication of the amount of damping. Following this reasoning, however, the present results would suggest that the two strains of *C. vicina* did not have very different degrees of damping, although the lower diapause levels for the U.S. suggest that it may have slightly more than the northern strain. Therefore, alternative explanations must be found to explain the shorter CDL and lower diapause induction in the U.S. strain.

The theoretical PPRCs produced by this model were also shown to be affected by changes in the time-delay (TD) of the feedback loop, such that relatively small increases in TD (equivalent to lengthening the period) produced substantial shortening of the CDL. In the present experiments, the only available measure of changes to the period of the photoperiodic oscillator was contained in the Nanda-Hamner regimes. These indicated that both strains appeared to have similar periods for their oscillator, around 24 hours. However, it should be recognized that the estimations of period using this technique are fairly imprecise, and, as such, small changes in τ may not be detected.

In an attempt to approach the problem from another angle, it was noted that a number of previous workers had advocated the use of parallel studies of circadian phenomena, combining photoperiodic experiments with those on other more overtly rhythmic elements of the system. Although, it is uncertain how closely one can relate two different circadian outputs in any species, one may expect correlations between certain aspects of these various outputs. The most obvious candidate for such a correlation is probably the period of the oscillation. Thus, a recent application of this principle with *Drosophila littoralis* (Lankinen, 1986) found a north-south cline of shortening CDL was associated with lengthening period in the free-running eclosion rhythm. It was therefore concluded that the clock behind photoperiodism may also have shown a similar cline in its period, and that the alterations in CDL might have arisen from these changes. Using the same principle, comparisons were made between the locomotor activity rhythms and photoperiodic responses of *C. vicina*.

It was clear from the discussion in Chapter 4 that there was some evidence that the oscillating elements in the locomotor behaviour of the southern strain did display periods which were longer than those seen in the Scottish strain. Furthermore, the difference between the strains was found to be in the region of 1 hour: in the Lewis and Saunders' model theoretical increases of this magnitude were capable of shortening the CDL by several hours. Therefore, it was assumed that these long-period elements in the locomotor activity reflected similar components in the photoperiodic responses of the blowfly. Thus, some of the variation between the two strains may be due to slightly different periods to the oscillation.

This cannot, however, explain the general lowering of diapause induction which was seen in the U.S. strain. There are, however, several further factors

which could provide suitable explanations. For example, in the Lewis and Saunders' model this situation could occur if the southern population was producing less INDSUM for every coincidence of ϕ_i and the threshold. Alternatively, the U.S. strain may be less sensitive to the light stimulus. This could mean that the oscillation may not be fully 'boosted' at the beginning of every dark phase, and the resulting decay of the oscillation after only a few LD cycles would lower the levels of INDSUM present at the end of the photosensitive phase. Although the similarity of the DD diapause levels in both strains tended to discount the southern strains simply producing less INDSUM, the second proposal cannot be discarded until the U.S. strain is tested for its photoperiodic responses at differing light intensities. Similar differences in light sensitivity (or 'subjective light intensity') have been proposed for different geographical strains of *Drosophila littoralis* (Lankinen, 1986) and *D. auraria* (Pittendrigh and Takamura, 1989).

However, the results from the resonance experiments suggested that the alteration in light sensitivity may not act in this manner. Rather it appeared that the efficiency of light in destroying the INDSUM product may have been increased in the U.S. strain. This proposal comes from the observation that Nanda-Hamner responses were higher in this strain than those seen in PPRCs. Interestingly, in these resonance experiments the diapause percentage at T24 was found to be the same as that seen for the same photoperiod in the PPRC. In the resonance profile, however, the diapause continued to rise over the next few T-cycles up to the first peak at T28, and remained high for the next inductive peak. This implied that the inductive ability of short T-cycles was reduced in the southern strain, a situation which could only be overcome when the nights were extended. One possible explanation for this may be that light during the photophase reduced the diapause titre accumulated during the scotophase. Thus, maximum expression of induction was only possible in cycles with very long nights, as only under these conditions were substantial quantities of INDSUM built up. Furthermore, the natural damping of the oscillator only allowed two large peaks of inductive response, with the peak at T72 falling below 40%.

It is, therefore, probable that the differences in the features of the photoperiodic response of the two strains were due to the interaction of a number of variable factors, e.g. damping, period length and light sensitivity.

The responses of the two strains to temperature were also found to differ, with the Scottish strain showing a marked reduction in diapause incidence as temperature increased, while the U.S. was largely unaffected (albeit over a smaller temperature range: however, the stability of the diapause response was supported by preliminary experiments at 12°C which also found no increase in diapause percentage in the U.S. strain). In conjunction with these effects, the CDL was seen to be lengthened in the southern strain and shortened in the northern strain.

The ability of temperature to alter photoperiodic responses has been reported for a number of insect species. In general, it has been supposed that increased temperatures affected a temperature-dependent development by shortening the photosensitive phase (Saunders, 1982a). This, in turn, would result in fewer inductive cycles being experienced by the insect, and therefore lower levels of diapause expressed. However, this explanation may not be relevant to the present study, as all the larvae used in these experiments came from eggs laid on day 12, and thus (assuming the photosensitive phase occurs throughout the adult females' lifetime) all of the cultures must have experienced the same number of inductive cycles. Alternatively, Lewis and Saunders' model indicated that such temperature increases can also reduce the degree of damping of the oscillator (by increasing the synthesis rate of the oscillating chemical). A change of this nature could lead to a lengthening of the CDL. Although, this might explain the change in CDL seen in the U.S. strain, this supposition is not supported by the results in DD. As a more persistent rhythm should, theoretically, result in higher diapause in DD, one would expect the U.S. strain to show just such an increase if its lengthened CDL was the result of a less damped oscillator. Unfortunately this is not what is observed, the U.S. strain having no change in its diapause expression in DD. Although, these problems seem to be irreconcilable, the effects of temperature on the various factors controlling diapause induction are so far-reaching, that increasing temperatures could potentially result in either increasing or decreasing incidences of diapause (Vaz Nunes et al., 1989).

The same principles could be assumed to apply to the temperature effects on the Nanda-Hamner protocols. Thus, at lower temperatures more inductive cycles would be experienced, due to a lengthening of the photosensitive phase, and the diapause incidence would be expected to be higher. With this temperature decrease there may also be an increase in the damping of the

oscillation. Although such increased damping could explain the rapid decline in the inductive response after T72 at the lower temperature, the fact that all of the cultures were likely to have experienced the same number of inductive photoperiodic cycles goes against interpreting the hourglass-like response in terms of a reduced photosensitive phase.

An alternative explanation for some of these changes has been proposed by Pittendrigh (1981), based on a hypothetical system of a pacemaker controlling nine slave oscillators. If one assumes that in a tiered circadian mechanism the overt expression of the system is controlled by the temperature-dependent interaction of these slaves (and that their mutual phase positioning is an important element in photoinduction) then temperature could have any number of effects on photoperiodic expression depending on the periods, coupling factors and damping coefficients of such slaves.

However, in order to fully understand the disparities in the responses of the two strains, it must be assumed that they are a reflection of differing selection pressures acting on the photoperiodic system. These selection forces, in turn, stem from the different climatic conditions in the habitats of each strain. For example, in the more extreme northern climates one may expect diapause expression to be in the larval rather than the adult stage. Furthermore, the severity of the winters will not only increase the significance of the CDL, but it may also lead the insect to use temperature increases as a means to terminate its diapause. Therefore, in the Scottish strain, one might expect the CDL to be homoeostatically protected from many environmental influences, while the diapause induction at short days may be more affected by such factors, e.g. temperature increases. On the other hand, the less rigorous winters experienced by the southern strain might allow many individuals to diapause (or quiesce) as adults. Larval expression of diapause would therefore be less important, and as such the CDL may not have developed an effective homoeostatic mechanism. However, once diapause is induced one might expect it to be impervious to temperature effects. Otherwise diapause would be broken by short-term temperature increases. A study of a central Asiatic population of *C. vicina* found that significantly lower temperatures were necessary for diapause induction in extreme southern populations, further implying that blowflies in such localities are highly dependent on temperature for the initiation of diapause induction (Kudryavtseva, 1986).

It is highly probable, that in order for such different requirements to be expressed in the photoperiodic responses, selection pressures have needed to alter a variety of factors in the system controlling photoperiodism. For this reason, many of the present results seemed to indicate modifications in a number of the elements controlling the expression of the oscillator.

The severity of northern winters would also lead one to expect that the populations in these regions would have a deeper diapause. Although this has been found in a number of species there was no evidence from the present experiments that, for any particular diapause incidence, northern populations had longer larval development (and therefore longer diapause duration). Despite this, it has been found that extreme southern populations of *C. vicina* have very long larval development times (Kudryavtseva, 1986). This may not, however, be significant, as it has been shown in the present study and Saunders (1987) that the length of larval development is correlated to diapause incidence. Therefore, comparisons must be made between groups which have a similar diapause incidence. In hindsight, a better protocol for studying diapause-depth would have been to expose diapausing larvae to a set temperature increase on successive days after induction. This would then reveal any differences in the form of the induction. The rationale behind this protocol comes from the realization that the depth of diapause is not an absolute quantity, but a feature which can change as diapause progresses (Bodnaryk, 1977).

Strain differences were also apparent in the responses to D_2O treatment. There are few examples of the effects of this molecule on photoperiodism, but those which do exist suggest that D_2O reduces the inductive effect of short days, e.g. the fleshfly *Sarcophaga crassipalpis* (Rockey and Denlinger, 1983) and the hamster (Eskes and Zucker, 1978). The Scottish strain was found to show a similar effect in the present experiments. Rockey and Denlinger attempted to explain their results on the basis of the period lengthening effects of D_2O. Theoretically, this would produce a constant phase readjustment of the oscillator to the LD cycle, with the result that ϕ_i would be gradually moved into the light. Thus, even in short days, this would result in less diapause induction. Following this argument, it would be expected that D_2O would have no effect in DD and, in the context of the recent Lewis and Saunders model, the increase in τ would be expected to shorten the CDL. Unfortunately, neither of these effects were observed in the Scottish strain, where the CDL was unaltered and

diapause levels in DD actually declined slightly. Although the U.S. strain did show greater diapause in DD, the overall level of diapause in short days actually showed a slight increase with D_2O application. Furthermore, the CDL was clearly lengthened by the treatment. These results imply that more complex answers may be required to fully explain these effects of D_2O.

Saunders and Lewis (1987a) also found that increases in τ could decrease the damping of the oscillator slightly. This creates even further problems as decreased damping would lengthen CDL, while increased τ would be expected to shorten it. Thus, some of the effects of D_2O must reflect a balance between these two influences. Interestingly, some of the responses to D_2O seem to mirror those found with increased temperature, e.g. the diapause incidence at short days in the U.S. strain were unaffected by the treatment. By the same token, the inductive effect of short days was reduced and the CDL was lengthened in the Scottish and U.S. strains respectively. It appears, therefore, that those aspects of the response which are of greatest importance to each strain are homoeostatically protected from excessive environmental disruption.

The results of the Nanda-Hamner protocols present further problems to interpretation. Firstly, there was no evidence that the peaks of induction were further apart, i.e. there was no increase in period. Secondly, each peak seemed more sharply defined than had been found with the previous water experiments. However, the most obvious effect of the treatment was the difference in the size of the first peak in L=12, compared to L=13. It is difficult to imagine the causes for these effects, but it is possible that the new phase positions of ϕ_i following D_2O treatment may be largely responsible. Thus, in short T-cycles (T24, 12:12) ϕ_i may coincide with the photophase, causing a reduced diapause titre (as proposed by Rockey and Denlinger, 1983). Extending the night will, on the other hand, allow the ϕ_i to coincide more often with the dark, and therefore the inductive effect will be increased. Clearly, only a detailed analysis of entrainment of the system, through the development of phase response curves, can begin to validate this hypothesis. However, it is tempting to assume that the obvious differences between L=12 and L=13 may result from homoeostatic protection of the CDL. Thus, as the lengths of the photophases in the Nanda-Hamner approach the CDL, one might expect D_2O to be less influential. Therefore, repeating these resonance experiments over a wide range of photophases may help to clarify this problem. The lack of evidence in the Nanda-Hamner for lengthening of the period may again be due

to the inaccuracy in determining the exact position of the inductive peaks. The finding that D_2O treated locomotor rhythms rarely showed periods lengthened by more than 1 hour (in both Stage 1 and Stage 2) would mean that even the third inductive peak would only lag the water experiments by 2 hours. This tends to confirm that small period lengthening of the photoperiodic oscillatory system cannot be detected by the Nanda-Hamner protocol,

Acetone had no effects on the photoperiodic response. This finding is in agreement with the results of similar treatments on the locomotor activity rhythm. Equally the effects of LiCl could be related to the locomotor activity experiments. Thus, it had been found that LiCl caused a destabilization between the various oscillating elements making up the clock controlling activity. The pattern seen in the Nanda-Hamner may also be explained by a similar disruption to the oscillating system. This would support the proposition that Nanda-Hamner protocols are revealing the presence of oscillating systems in photoperiodic phenomena, and furthermore these systems may comprise more than one oscillator. This suggests that lithium chloride may be a useful tool for further photoperiodic research.

In conclusion, it has been established that the photoperiodic system in *C. vicina* is probably controlled by a circadian oscillator, which measures the length of the scotophase. However, as the characteristic features of the response were found to vary greatly between the two geographical strains used, one must assume that local selective forces have had a strong influence in shaping the observed photoperiodic patterns.

CHAPTER 8

General Discussion

Throughout many of the preceding chapters continued reference has been made to the similarities which may or may not exist between the circadian systems underlying locomotor behaviour and photoperiodism. As was stated in the General Introduction they were initially considered to be two aspects of the same oscillatory system, both being termed 'hands of the clock'. The aim of this discussion is to present an overview of both locomotion and photoperiodism, and to consider whether this was a justifiable assumption.

An appropriate starting point for any critical comparisons for these systems would be to summarize the existing knowledge on their physical positioning within the insect body. In general terms any circadian mechanism can be reduced to three stages.

1. Photoreceptor(s), mediating the mechanism of entrainment of the oscillating system by the light/dark cycles.
2. Oscillator(s), comprising several pacemakers or one pacemaker driving several slave oscillations.
3. Observed, overt rhythm.

Clearly it is important in any study of circadian phenomena to ascertain the precise anatomical position of both photoreceptor and oscillator(s), as this is relevant to the functioning of the clock.

In terms of photoreception there are few general rules. While it is evident that the compound eyes of many cockroaches and crickets are essential for the entrainment of the locomotor rhythms, as their occlusion or surgical destruction causes the rhythm to free-run in LD cycles as though the insects were in DD (Roberts, 1965; Nishiitsutsuji-Uwo and Pittendrigh, 1968a; Loher, 1972), the ocelli may also be necessary for entrainment of locomotor activity in some species, e.g. *Acheta domesticus* (Nowosielski and Patton, 1963). Although the precise role of these 'primitive' photoreceptors probably varies between different insect species (Wilson, 1978), some recent research with the cricket *Teleogryllus commodus* (Rence et al., 1988) suggested that these ocelli may interact with the compound eyes to modulate the rhythmic response. This was inferred from experiments in which the period of the stridulation rhythm

was significantly lengthened by occlusion of the ocelli or severance of the three ocellar nerves. In many ways these photoreceptors are suited to this task as they have been found to be several log units more sensitive to light than the compound eyes and to have a very large visual field (Wilson, 1978).

Despite this it is equally evident that many species, including the examples from the higher flies, utilize extraretinal receptors for entrainment of their activity and have little recourse to their compound eyes or ocelli, e.g. the grasshopper *Chorthippus curtipennis* (Loher and Chandrashekaran, 1970), the cricket *Ephippiger* (Dumortier, 1972), the silkmoths *Antheraea pernyi, Hyalophora cecropia* and *Samia cynthia* (Truman, 1974), the house fly *Musca domestica* (Helfrich et al., 1985) and *Drosophila melanogaster* (Dushay et al., 1989). Similarly the existing evidence for the position of the photoperiodic light sensor has also indicated that organized photoreceptors (i.e. the compound eyes and ocelli) are not involved (de Wilde et al., 1959; Lees, 1964). Rather it appears that the photoreceptor controlling activity and photoperiodism is brain-centred in all of these species (Lees, 1964; Williams and Adkisson, 1964; Claret, 1966a, b).

The investigations into the anatomical location of the 'clock' itself have also left little doubt that it resides in the brain (or associated structures) of the majority of insect species. In the specific case of the locomotor behaviour in cockroaches (Nishiitsutsuji-Uwo and Pittendrigh, 1968b; Roberts, 1974; Page, 1981a) and crickets (Cymborowski, 1981; Loher, 1972) the optic lobes have been implicated as the probable site for the oscillator(s). The regions within these organs which function as the clock appear to be the lobula in cockroaches (Page, 1981a) and the lamina/medulla in the case of *Acheta* (*=Gryllus*) *bimaculatus* (Tomioka, 1985), although this latter study did suggest that there was some evidence for an oscillatory structure outside the optic lobes. Furthermore, the optic lobes need to be neurally connected to the brain before the activity rhythm can be established in both cockroaches and crickets (Brady, 1969; Page, 1984; Tomioka, 1985).

In other insect orders the optic lobes appear to be dispensable for the expression of rhythmic locomotor behaviour, e.g. silkmoths (Truman, 1974), housefly *Musca domestica* (Helfrich et al., 1985) and *Drosophila melanogaster* (Helfrich and Engelmann, 1983). Although the Lepidopteran examples required intact neural pathways for normal expression of activity (Truman, 1974), it has

been suggested that the Dipteran output from the clock may be humoral (Handler and Konopka, 1979).

With the photoreceptor for photoperiodic phenomena positioned in the brain it is more difficult to confirm that this is also the site of the pacemaker(s). However, selective destruction of minute areas of brain tissue using microcautery techniques has shown that in the aphid *Megoura viciae* and the silkmoth *Antheraea pernyi* the clock is also brain-centred, probably in (or close to) the median neurosecretory cells (Steel and Lees, 1977, and Williams, 1969 respectively). The output from this clock is probably humoral, and involves the brains' decision whether or not to release neurohormones (Saunders, 1982a).

Although the lower insects display differences in the anatomical position of certain elements of the circadian system controlling locomotion and photoperiodism, e.g. the photoreceptors, these distinctions become blurred in the Diptera, where the entire process appears to reside in the brain. Furthermore, while the output of the clock controlling cockroach and cricket locomotor activity is neurally mediated and that of photoperiodism is humoral, the Diptera may have a humoral output for both types of response. In an attempt to order this diversity Truman (1971) proposed that animal clocks be divided into two groups, the first (Type 1) being developmental rhythms including eclosion and photoperiodism, and the second (Type 2) being behavioural rhythms, e.g. locomotor activity. Type 1 clocks characteristically used extraretinal photoreception, with a clock positioned in the brain and a humoral output from the system. Type 2, on the other hand, utilized the organized photoreceptors for light sensing, with the brain being insensitive to light, and the output being nervous. While these categories may apply reasonably well to the rhythms observed in the lower insects, they do not seem to be useful in considering Dipteran rhythms. Thus, it must be concluded that, as yet, there is no anatomical evidence that locomotion and photoperiodism are controlled by different clocks in higher flies such as *Calliphora vicina*.

However, it may be possible to compare the formal properties of these two responses. The present experiments have clearly shown that the system controlling locomotor activity in *C. vicina* is circadian and probably comprises several oscillators or groups of oscillators. In unperturbed free-run the system

as a whole displays a constant rhythm of about 23.14 hours, and in this state the oscillators are assumed to be tightly coupled. After LL or LD cycles (diel and non-diel) the free-running pattern showed certain after-effects. The experiments with temperature and D_2O indicated that the major after-effects (the period lengthening 6-10 days into the free-run and rhythm-splitting) were probably due to a breakdown in this homoeostatic coupled state, with the alteration in coupling allowing these non-temperature-compensated oscillators to be more easily seen. Although it is not certain whether these oscillators are slaves to a single pacemaker or are each pacemakers in their own right, it is interesting to note that the stable system appears to exhibit some homoeostatic properties which the disrupted patterns were not able to do. Thus, these oscillators could be compared to the slaves in Pittendrigh's tiered model (Pittendrigh, 1967; Pittendrigh and Bruce, 1957, 1959; Pittendrigh et al., 1958). Further, it was hypothesized in Chapter 4 that it would perhaps even be beneficial to an organism to evolve a hierarchical structure to its circadian system, as it would allow the slave oscillators to adapt to local environmental conditions without disrupting the main pacemaker(s).

Throughout much of the literature on locomotor activities mention has been made of rhythmic systems comprising two populations of oscillators, one phase-set by the dawn and the second by the dusk (see Chapter 2). Similar ideas have also been used in the present experiments. However, it may not be necessary to group the oscillators in this fashion, for exposing a number of oscillators (each with differing periods and coupling strengths) to a diel LD cycle would result in those with periods of less than 24 hours being phase-set to the lights-on transition, while the long period oscillators ($\tau > 24$ hours) would be phased by the lights-off. Some of the short period oscillations may even include ultradian periods which are submultiples of the Zeitgeber cycle. This would therefore give the appearance of a system made up of two populations of oscillators, when in fact it would be more accurate to consider the system as a number of independent, but coupled, rhythmic elements.

Thus, the after-effects and split patterns can be explained in one of two ways. Firstly, these changes to the pattern could be the result of varying numbers of the rhythmic components splitting away from and rejoining a main mutually coupled group of oscillators, or secondly various rogue rhythmic elements (which could not be entrained by the previous LD cycle) producing after-effects by crossing and recrossing the main group. Hypothetically both of

these propositions may be correct. This, in essence, is the basis to the previously described Christensen and Lewis model (Gander and Lewis, 1979; Christensen et al., 1984).

However, the two population model cannot be discounted as it does bear direct comparison to the physical construction of the system in cockroaches (Page, 1981a) where bilaterally redundant oscillators in the optic lobes are mutually coupled to control locomotor activity. Similar coupled systems are thought to exist in rodents (Pittendrigh and Daan, 1976c) and lizards (Underwood, 1977), with the pacemakers in each case being differentially responsive to evening and morning light.

Many of the suppositions discussed above are contained in a computer model devised by Pittendrigh (1981) involving nine slave oscillators coupled to a single pacemaker. Each of these slaves can entrain separately to the prevailing photoperiod, their eventual phase relationship being dependent upon their period and coupling to the other slaves. Changes in the form of the entraining photoperiod (both in terms of its length and the ratio of light to dark) can produce marked changes in the mutual phase relationships of these slaves. Although this (in conjunction with Christensen and Lewis' model) provides convincing explanations for the observed locomotor activity patterns, it will also have important consequences for any consideration of photoperiodic phenomena, as seasonal changes in photoperiod will necessarily produce changes in the phase relationships between the slaves and the slaves to the pacemaker.

Although the Nanda-Hamner results described in Chapter 7 imply the involvement of a circadian system in diapause induction, little evidence is available on how this organization may be structured. In the many years since Bunning's original hypothesis that circadian rhythmicity was causally involved in photoperiodism, various models have been proposed to interpret the results of photoperiodic manipulative experiments. Of these the External Coincidence Model (ECM) (Pittendrigh, 1966, 1972; Pittendrigh and Minis, 1964) and the Internal Coincidence Model (ICM) (Pittendrigh, 1972) have proved to be the most resilient. Although the ECM has proved especially useful for interpreting the responses to numerous experimental protocols, in the light of the fact that many overt rhythmicities are controlled by several oscillators, one must envisage the oscillating system as being a combination of the two models. In

this sense the nine-slave oscillator paradigm described above may provide a basis for understanding not only photoperiodism, but also the relationship photoperiodic responses may have with locomotor activity.

Furthermore, this model also provides some insights into a phenomenon related to Nanda-Hamner regimes, frequently termed 'extended circadian surfaces'. In essence these 'surfaces' are three dimensional plots of diapause induction as a function of the length of the light and the dark (and thus the length of the entraining T-cycle). This is equivalent to producing a number of Nanda-Hamner protocols for a range of differing photophases, the combined results looking somewhat like an ordnance survey map (see Pittendrigh, 1972; Saunders, 1974, 1982a). The exact meaning of the complex patterns seen has never been clearly understood, although Saunders (1974) indicated that they seemed to illustrate that the ascending slopes of Nanda-Hamner responses for the parasitic wasp *Nasonia vitripennis* were phase-set by the dusk, while the descending slopes were phase-set by dawn. Thus these 'surfaces' may indicate that two populations of oscillators may be involved in the control of the inductive response in this wasp species.

However, Pittendrigh's nine-slave model can readily produce similar circadian surfaces, if contours of iso-phase position are plotted for a number of Nanda-Hamner type photoperiods (Pittendrigh, 1981). Although, in this case the phase position of only two of the slaves was considered, the results strongly implied that the level of diapause induction was dependent on the relative phase position of the various slaves. Furthermore, this model can also provide an alternative explanation of the effects of temperature on Nanda-Hamner responses, because of the temperature-dependent coupling both between the slaves, and between these and the pacemaker. Thus, temperature changes would be expected to produce radical alterations in the height of the inductive response and the critical daylength (if one assumes that induction is a function of the specific phase relationship between these slave oscillators).

Therefore, as both locomotor activity and photoperiodism appear to be explicable using this model, does it perhaps indicate functional similarities between the two systems, or indeed is the same pacemaker governing both responses? It would not be altogether surprising if the two systems did have some functional similarities, for the organism would only need to evolve one

type of clock, which could be used for many different developmental and behavioural events. However, it would be equally specious to assume that any two responses of the system would not need to have the same pacemaker governing their action.

The most obvious way to approach a comparison of pacemakers is to analyse their phase response curves (PRCs), as it is assumed that light sensitivity is a function of the pacemaker. While this is relatively easy for locomotor rhythms, it is quite difficult for photoperiodic responses. Indeed photoperiodic PRCs have been constructed for only two species, one of which was a plant species (*Chenopodium rubrum*, see Saunders, 1982a, p.220) and the other the Dipteran fleshfly *Sarcophaga argyrostoma* (Saunders, 1976). The insect PRC (using 15 minute pulses) was found to have a low amplitude, with weak phase-advances and phase-delays: this is termed a Type 1 PRC after a series of papers published by Winfree (see Saunders, 1982a for a review of this work). The opposite strong response curve, in which light produced large phase-advances and delays, was termed Type 0. The existing PRCs for locomotor rhythms have also been found to be Type 1 responses although this can vary depending on the duration and intensity of the light pulse (Jones et al., 1972a; Saunders and Thomson, 1977; Wiedenmann, 1977b). These findings emphasize the caveat for any comparison of different rhythmic outputs, i.e. it is usually necessary to obtain a series of PRCs for different intensities and lengths of light pulse. Unfortunately such families of PRCs were not attempted in *C. vicina* for either locomotion or photoperiodism as they were considered too laborious and time-consuming. Thus alternative features of the pacemaker have to be found to compare these two systems. One of the obvious candidates for this was the phase relationship of the rhythms to their entraining LD cycles.

It was clear from Chapter 3 that, irrespective of the T24 LD cycle involved, the entrained activity rhythm was positioned in the centre of the photophase. Obviously, this phase relationship only reflects that of the combined output of the slave oscillators, and cannot tell us much about the position of the pacemaker itself. However, it is evident that it is the role of the pacemaker to maintain the position of the activity in the light, *C. vicina* being a diurnal species, and depending on the period of the oscillating system (and the shape of the PRC) the pacemaker will have to change its position relative to the LD cycle in order to do this. In steady state entrainment two parts of the

oscillation will be exposed to a light/dark transition, one in the subjective day and one in the subjective night; as the action of light is non-parametric in all but long photoperiods these transitions will produce phase changes in the same manner as single light pulses, the direction and degree of the change depending on the shape of the PRC. If the period of the oscillator is 24 hours, the phase-advances will need to exactly match the phase-delays if the oscillation is to maintain its phase position. However, as the true period of the oscillator is thought to be nearer 23 hours, these transitions will have to coincide with the oscillation in such a way that the combined phase change is equal to a phase advance of 1 hour every cycle.

Pittendrigh and Daan (1976b) used this principle to calculate the phase position of the middle of the subjective day (Circadian time, Ct 6), over the whole T24 photoperiodic range, for different theoretical PRC shapes and for differing periods to the endogenous rhythm. Assuming that the locomotor PRC shows a low amplitude Type 1 response in *C. vicina* (as many insects seem to have), the calculated position of Ct 6 for an oscillator with a period of less than 24 hours is several hours before the middle of the Zeitgeber photophase. Furthermore, this relative position was found to be maintained in all photoperiods, with the result that Ct 6 eventually moved into the light in longer photoperiods. This leads one to conclude that the constancy observed in the phase position of the overt activity rhythm marks an equally stable phase relationship of the oscillator to the LD cycle. It should be pointed out, however, that the theoretical phase position of the oscillator is based on the assumption that the PRC is perfectly symmetrical. In reality, of course, this is rarely the case (see Daan and Pittendrigh, 1976a), and indeed one may expect the PRC of *C. vicina* to have a larger delay portion if its free-running period is less than 24 hours. Even so Pittendrigh's model showed that in most photoperiods the oscillation will phase-lead the Zeitgeber by a few hours in each cycle.

In terms of the photoperiodic responses the only available indicator of the position of the oscillator is the position of ϕ_i. Although the position of this part of the oscillation is likely to be different in each photoperiod, eventually it will pass into the light. At this point the induction of diapause will be curtailed and the observed response will decline rapidly. This corresponds to the critical daylength observed in PPRCs. In the case of *C. vicina* this occurs roughly 14.5 hours after lights-on. It would appear that in photoperiods longer than 12 hours the oscillator is reset to its starting point once the rhythm is released

into darkness (Pittendrigh, 1966). The position of ϕ_i is therefore revealed by the dawn tracking backwards over earlier parts of the endogenous oscillation. As the CDL is 14.5 hours, the critical nightlength (CNL) must be 9.5 hours. Therefore ϕ_i occurs 9.5 hours after dusk, and must therefore be in the late subjective night. This indicates that the circadian oscillation is more or less in synchrony with the LD cycle. The subsequent finding that the period of the photoperiodic oscillation is near 24 hours confirms that this is probably the case.

Thus it would appear that the oscillators controlling locomotor activity and photoperiodism are not the same, as they appear to adopt slightly different phase positions in 24 hour LD cycles. In many ways this finding is dependent on the most critical comparative test of their similarity, their respective periods. As has been stated in previous chapters, the Nanda-Hamner experiments and unperturbed locomotor free-runs have indicated that the photoperiodism and locomotion have periods of roughly 24 hours and 23.14 hours respectively. Although it has been stated that the period of the pacemaker is difficult to estimate from a Nanda-Hamner, if the photoperiodic response had had a period of 23 hours each of the three peaks would have been increasingly advanced, with the result that the third would be expected to occur at T69, three hours earlier than the observed peak at T72. Therefore it can be confidently stated that the photoperiodic response did not have a period of about 23 hours, but rather it was closer to 24 hours. Furthermore, if one follows the computed models Pittendrigh and Daan (1976b) devised for rodent locomotor rhythms one would expect most photoperiodic clocks to have a symmetrical Type 1 PRC and a period close to 24 hours, for under these circumstances the endogenous oscillation would closely match the environmental cycle and the position of ϕ_i would be relatively stable in most natural photoperiods.

Although the two rhythms may be controlled by different oscillators, there is a case for saying that the two systems may function in a similar way, perhaps in the manner proposed by Pittendrigh (1981).

Pittendrigh and Minis (1964, 1971), and Pittendrigh (1966), discussed the importance of using overt rhythms, such as activity and eclosion, as indicators of the phase position of the more covert photoperiodic response. In these papers they studied three overt rhythms in *Pectinophora gossypiella*, i.e. egg hatch, pupal eclosion and oviposition. Although initially they found quite a clear

association between eclosion and photoperiodism, the three overt rhythms displayed very different properties. For example, while oviposition and eclosion had periods of about 22.5 hours, egg hatch free-ran with a period close to 24 hours. In addition, while red light failed to entrain the eclosion or oviposition rhythms (although it does shorten τ perceptibly in the former, Pittendrigh and Minis, 1971), the larvae of *P. gossypiella* can distinguish long and short red light photoperiods (14 and 12 hours) to produce 2% and 100% diapause respectively. The obvious conclusion from this is that overt rhythms and diapause induction are not controlled by the same clock, and as such overt rhythms are not ideal indicators of the properties of the photoperiodic oscillator.

Another comparable study on overt rhythms and photoperiodism in the fleshfly *Sarcophaga argyrostoma* (see Saunders, 1986) came to the same conclusion. Two overt rhythms appeared to be under the control of circadian oscillators, i.e. larval wandering and pupal eclosion, but differed in several important respects, most noticeably their period and phase. The former was found to have a free-running τ of <24 hours and a phase relationship close to dusk, while the period of the latter was about 24 hours with a phase position close to dawn. Engelmann and Mack (1978) also found significant differences between the clocks controlling locomotor activity and eclosion in *Drosophila pseudoobscura*.

In conclusion, while *C. vicina* appears to have circadian oscillators underlying both locomotion and photoperiodism, the evidence suggests they are not the same pacemaker. However, this does not imply that it is ultimately fruitless to compare the functional properties of different rhythmic events, for only by such comparisons can the important variable features of circadian rhythms be identified. Indeed the basis of most modern circadian research depends upon such inter-specific variation, for it defines the limits of the models which must be adopted to explain the rhythmic behaviour in insects.

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References

ADKISSON, P.L., BELL, R.A. and WELLSO, S.G. (1963) Environmental factors controlling the induction of diapause in the pink bollworm, *Pectinophora gossypiella* (Saunders). *J. Insect Physiol.* **9**, 299-310.

ANKERSMIT, G.W. and ADKISSON, P.W. (1967) Photoperiodic responses of certain geographical strains of *Pectinophora gossypiella* (Lepidoptera). *J. Insect Physiol.* **13**, 553-564.

ASCHOFF, J. (1955) Tagesperiodik von Maus estammen unter konstanten Umgebungesbedingungen. *Pfluegers Arch. Gesamte Physiol.* **262**, 51-59.

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

ASCHOFF, J. and SAINT PAUL, U. von (1982) Circadian rhythms in the blowfly *Phormia terraenovae* : the period in constant light. *Physiol. Entomol.* **7**, 365-370.

BAILEY, S.E. and LAZARIDOU-DIMITRIADOU, M. (1986) Circadian components in the daily activity of *Helix lucorum* L. from northern Greece. *J. Molluscan Stud.* **52**, 190-192.

BARKER, R.J., MAYER, A. and COHEN, C.F. (1963) Photoperiodic effects in *Pieris rapae*. *Ann. entomol. Soc. Am.* **56**, 292-294.

BARTELL, R.J., SHOREY, H.H. and BARTON-BROWNE, L. (1969) Pheromonal stimulation of the sexual activity of the sheep blowfly *Lucilia cuprina* (Calliphoridae) by the female. *Anim. Behav.* **17**, 576-585.

BATEMAN, M.A. (1955) The effect of light and temperature on the rhythm of pupal ecdysis in the Queensland fruit-fly, *Dacus (Strumeta) tryoni* (Frogg.). *Aust. J. Zool.* **3**, 22-33.

BECK, S.D. and HANEC, W. (1960) Diapause in the European corn borer, *Pyrausta nubilalis* (Hubn.). *J. Insect Physiol.* **4**, 304-318.

BODNARYK, R.P. (1977) Stages of diapause development in the pupae of *Mamestra configurata* based on beta-ecdysone sensitivity index. *J. Insect Physiol.* **23**, 537-542.

BOGDANOVA, T.P. and ZASLAVSKII, V.A. (1985) Effect of diapause, reactivation

and long-term exposure to cold of preimaginal stages on the formation of maternal effects in *Calliphora vicina* (Diptera, Calliphoridae). *Entomol. Obozr.* **30,** 458-463.

BOWEN, M.F., SAUNDERS, D.S., BOLLENBACHER, W.E. and GILBERT, L.I. (1984) *In vitro* reprogramming of the photoperiod clock in an insect brain-retrocerebral complex. *Proc. Nat. Acad. Sci., U.S.A.* **81,** 5881-5884.

BRADY, J. (1967) Control of the circadian rhythm of activity in the cockroach. I. The role of the corpora cardiaca, brain and stress. *J. exp. Biol.* **47,** 153-163.

BRADY, J. (1969) How are insect rhythms controlled? *Nature, Lond.* **223,** 781-784.

BRADY, J. (1972) Spontaneous, circadian components of tsetse fly activity. *J. Insect Physiol.* **18,** 471-484.

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

BRADY, J. (1988) Circadian ontogeny in the tsetse fly: a permanent major phase change after the first feed. *J. Insect Physiol.* **34,** 743-749.

BRINKMANN, K. (1976) The influence of alcohols on the circadian rhythm and metabolism of *Euglena gracilis. J. Interdiscip. Cycle Res.* **7,** 149-170.

BROWN, R.H.J. and UNWIN, D.M. (1961) An activity recording system using infra-red detection. *J. Insect Physiol.* **7,** 203-209.

BRUCE, V.G. (1960) Environmental entrainment of circadian rhythms. *Cold Spring Harbor Symp. Quant. Biol.* **25,** 29-48.

BRUCE, V.G. and PITTENDRIGH, C.S. (1960) An effect of heavy water on the phase and period of the circadian rhythm in *Euglena. J. cell. comp. Physiol.* **56,** 25-31.

CALDAROLA, P.C. and PITTENDRIGH, C.S. (1974) A test of the hypothesis that D_2O affects circadian oscillations by diminishing the apparent temperature. *Proc. Nat. Acad. Sci., U.S.A.* **71,** 4386-4388.

CARPENTER, G.A. and GROSSBERG, S. (1985) A neural theory of circadian rhythms: split rhythms, after-effects and motivational interactions. *J. theor. Biol.* **113,** 163-223.

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

CHANDRASHEKARAN, M.K. (1967b) Studies on the phase-shifts in endogenous rhythms. II. The dual effects of light on the entrainment of the eclosion rhythm in *Drosophila pseudoobscura*. *Z. vgl. Physiol.* **56**, 163-170.

CHANDRASHEKARAN, M.K., JOHNSSON, A. and ENGELMANN, W. (1973) Possible 'dawn' and 'dusk' roles of light pulses shifting the phase of a circadian rhythm. *J. comp. Physiol.* **82**, 347-356.

CHRISTENSEN, N.D. and LEWIS, R.D. (1982) The circadian locomotor rhythm of *Hemideina thoracica* (Orthoptera: Stenopelmatidae): the circadian clock as a population of interacting oscillators. *Physiol. Entomol.* **7**, 1-13.

CHRISTENSEN, N.D. and LEWIS, R.D. (1983) The circadian locomotor rhythm of *Hemideina thoracica* (Orthoptera: Stenopelmatidae): a population of weakly coupled feedback oscillators as a model of the underlying pacemaker. *Biol. Cybern.* **47**, 165-172.

CHRISTENSEN, N.D., LEWIS, R.D. and GANDER, P.H. (1984) Properties of a feedback model for the circadian clock of *Hemideina thoracica*. *Biol. Cybern.* **51**, 87-92.

CLARET, J. (1966a) Recherche du centre photorecepteur lors de l'induction de la diapause chez *Pieris brassicae* L. *C. r. hebd. Seances Acad. Sci. Ser. D Sci. Nat.* **262**, 1464-1465.

CLARET, J. (1966b) Mise en evidence du role photorecepteur du cerveau dans l'induction de la diapause chez *Pieris brassicae* (Lepido). *Ann. Endocrinol.* **27**, 311-320.

CLARET, J. (1985) Two mechanisms in the biological clock of *Pieris brassicae* L.: an oscillator for diapause induction; an hour-glass for diapause termination. *Experientia* **41**, 1613-1615.

CLARET, J., DUMORTIER, B. and BRUNNARIUS, J. (1981) Mise en evidence d'une composante circadienne dans l'horloge biologique de *Pieris brassicae* (Lepidoptera), lors de l'induction photoperiodique de la diapause. *C. r. Seances Acad. Sci. Ser. III Sci. Vie,* **292**, 427-430.

CLOPTON, J.R. (1984a) Mosquito circadian and circa-bi-dian flight rhythms: a two-oscillator model. *J. comp. Physiol. A,* **155**, 1-12.

CLOPTON, J.R. (1984b) Mosquito circadian flight rhythms: differential effects of

constant light. *Am. J. Physiol.* **247,** 960-967.

CLOPTON, J.R. (1985) Circa-bi-dian rhythmicity in the flight activity of the mosquito *Culiseta incidens. Comp. Biochem. Physiol. A,* **80,** 469-476.

CRUMP, A.J. and BRADY, J. (1979) Circadian activity in three species of tsetse fly: *Glossina palpalis, austeni* and *morsitans. Physiol. Entomol.* **4,** 311-318.

CURIO, E. (1976) *The Ethology of Predation,* Zoophysiology and Ecology Vol. 7. Springer, Berlin.

CYMBOROWSKI, B. (1973) Control of the circadian rhythm of locomotor activity in the house cricket. *J. Insect Physiol.* **19,** 1423-1440.

CYMBOROWSKI, B. (1981) Transplantation of circadian pacemaker in the house cricket *Acheta domesticus* L. *J. Interdiscip. Cycle Res.* **12,** 133-140.

DAAN, S. and ASCHOFF, J. (1981) Short-term rhythms in activity. In *Handbook of Behavioural Neurobiology,* Vol. 4: Biological Rhythms (Ed. ASCHOFF), pp. 491-522. Plenum Press, New York.

DAAN, S. and PITTENDRIGH, C.S. (1976a) A functional analysis of circadian pacemakers in nocturnal rodents. II. The variability of phase response curves. *J. comp. Physiol. A,* **106,** 253-266.

DAAN, S. and PITTENDRIGH, C.S. (1976b) A functional analysis of circadian pacemakers in nocturnal rodents. III. Heavy water and constant light: homeostasis of frequency? *J. comp. Physiol. A,* **106,** 267-290.

DAAN, S. and SLOPSEMA, S. (1978) Short term rhythms in foraging behaviour of the common vole, *Microtus arvalis. J. comp. Physiol. A,* **127,** 215-228.

DANILEVSKII, A.S. (1965) *Photoperiodism and Seasonal Development of Insects,* 1st English edition. Oliver and Boyd, Edinburgh.

DANILEVSKII, A.S., GORYSHIN, N.I. and TYSHCHENKO, V.P. (1970) Biological rhythms in terrestrial arthropods. *Annu. Rev. Entomol.* **15,** 201-244.

DAVIS, F.C. (1981) Ontogeny of circadian rhythms. In *Handbook of Behavioural Neurobiology,* Vol. 4: Biological Rhythms (Ed. ASCHOFF), pp. 257-274. Plenum Press, New York.

DAVIS, F.C. and GORSKI, R.A. (1985) Development of hamster circadian rhythms.

I. Within-litter synchrony of mother and pup activity rhythms at weaning. *Biol. Reprod.* **33,** 353-362.

DAVIS, F.C. and MENAKER, M. (1981) Development of the mouse circadian pacemaker: independence from environmental cycles. *J. comp. Physiol. A,* **143,** 527-540.

DENLINGER, D.L. (1972) Induction and termination of pupal diapause in *Sarcophaga* (Diptera: Sarcophagidae). *Biol. Bull., Woods Hole,* **142,** 11-24.

DENLINGER, D.L. (1974) Diapause potential in tropical fleshflies. *Nature, Lond.* **252,** 223-224.

DOWSE, H.B., HALL, J.C. and RINGO, J.M. (1987) Circadian and ultradian rhythms in period mutants of *Drosophila melanogaster. Behav. Genet.* **17,** 19-35.

DOWSE, H.B. and PALMER, J.D. (1972) The chronomutagenic effect of deuterium oxide on the period and entrainment of a biological rhythm. *Biol. Bull., Woods Hole,* **143,** 513-524.

DOWSE, H.B. and RINGO, J.M. (1987) Further evidence that the circadian clock in *Drosophila* is a population of coupled ultradian oscillators. *J. Biol. Rhythms,* **2,** 65-76.

DOWSE, H.B. and RINGO, J.M. (1989) Rearing *Drosophila* in constant darkness produces phenocopies of period circadian mutants. *Physiol. Zool.* (in press).

DUMORTIER, B. (1972) Photoreception in the circadian rhythm of stridulatory activity in *Ephippiger* (Ins., Orthoptera). Likely existence of two photoreceptive systems. *J. comp. Physiol.* **77,** 80-112.

DUSHAY, M.S., ROSBASH, M. and HALL, J.C. (1989) The disconnected visual system mutations in *Drosophila melanogaster* drastically disrupt circadian rhythms. *J. Biol. Rhythms,* **4,** 1-27.

EARNEST, D.J. and TUREK, F.W. (1985) Neurochemical basis for the photic control of circadian rhythms and seasonal reproductive cycles: role for acetylcholine. *Proc. Nat. Acad. Sci., U.S.A.* **82,** 4277-4281.

ENGELMANN, W. (1972) Lithium slows down the *Kalanchoe* clock. *Z. Naturforsch. Teil B,* **27,** 477.

ENGELMANN, W. (1973) A slowing down of circadian rhythm by lithium ions. *Z.*

Naturforsch. Teil C, **28,** 733-736.

ENGELMANN, W. (1987) Effects of lithium salts on circadian rhythms. In *Neuropsychiatric Disorders and Disturbances in the Circadian System of Man* (Ed. HALARIS) pp. 263-289. Elsevier.

ENGELMANN, W., BOLLIG, I. and HARTMANN, R. (1976) Wirkung von Lithiumionen auf zirkadiane Rhythmen. *Arzneim. forsch. (Drug Res.),* **26,** 1085-1086.

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

ENGELMANN, W., PFLUG, B., KLEMKE, W. and JOHNSSON, A. (1982) Lithium-induced change of internal phase relationship of circadian rhythms in humans, and other observations. In *Circadian Rhythms in Psychiatry,* pp. 89-107. Boxwood Press.

ENRIGHT, J.T. (1965a) The search for rhythmicity in biological time-series. *J. theor. Biol.* **8,** 426-468.

ENRIGHT, J.T. (1965b) Accurate geophysical rhythms and frequency analysis. In *Circadian Clocks* (Ed. ASCHOFF), pp. 31-42. North-Holland Publishing Co., Amsterdam.

ENRIGHT, J.T. (1971a) Heavy water slows biological timing processes. *Z. vgl. Physiol.* **72,** 1-16.

ENRIGHT, J.T. (1971b) The internal clock of drunken isopods. *Z. vgl. Physiol.* **75,** 332-346.

ENRIGHT, J.T. (1981) Data analysis. In *Handbook of Behavioural Neurobiology,* Vol. 4: Biological Rhythms (Ed. ASCHOFF), pp. 21-39. Plenum Press, New York.

ESKES, G.A. and ZUCKER, I. (1978) Photoperiodic regulation of the hamster testis: dependence on circadian rhythms. *Proc. Nat. Acad. Sci., U.S.A.* **75,** 1034-1038.

ESKIN, A. (1971) Some properties of the system controlling the circadian activity of sparrows. In *Biochronometry* (Ed. MENAKER), pp. 55-80. National Academy of Sciences, Washington, D.C.

ESKIN, A. (1982) Differential effects of amino acids on the period of the

circadian rhythm from the *Aplysia* eye. *J. Neurobiol.* **13,** 231–239.

FELDMAN, J.F. (1967) Lengthening the period of a biological clock in *Euglena* by cycloheximide, an inhibitor of protein synthesis. *Proc. Nat. Acad. Sci., U.S.A.* **57,** 1080–1087.

FRAENKEL, G. and HSIAO, C. (1968) Manifestations of a pupal diapause in two species of flies, *Sarcophaga argyrostoma* and *S. bullata. J. Insect Physiol.* **14,** 689–705.

GANDER, P.H. and 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.

GREEN, G.W. (1964a) The control of spontaneous locomotor activity in *Phormia regina* Meigen. I. Locomotor activity patterns of intact flies. *J. Insect Physiol.* **10,** 711–726.

GREEN, G.W. (1964b) The control of spontaneous locomotor activity in *Phormia regina* Meigen. II. Experiments to determine the mechanisms involved. *J. Insect Physiol.* **10,** 727–752.

GROSSE, W–R. (1985a) The synchronization of circadian locomotor activity in *Calliphora vicina. Wiss. Z. Martin Luther Univ. Halle-Wittenberg Math-Naturwiss. Reihe,* **34(1),** 105–112.

GROSSE, W–R. (1985b) Synchronization of circadian locomotor activity of *Calliphora vicina. Wiss. Z. Martin Luther Univ. Halle-Wittenberg Math-Naturwiss. Reihe,* **34(3),** 22–29.

GROSSE, W–R. (1985c) Zur endogene Grundlage der circadianen Aktivitat bei *Calliphora vicina* R.D. *Zool. Jahrb. Abt. Allg. Zool. Physiol. Tiere,* **89,** 491-507.

GWINNER, E. (1974) Testosterone induces 'splitting' of the circadian locomotor activity in birds. *Science, Wash.* **185,** 72–74.

HALL, J.C. and ROSBASH, M. (1987) Genetic and molecular analysis of biological rhythms. *J. Biol. Rhythms,* **2,** 153–178.

HAMBLEN, M., ZEHRING, W.A., KYRIACOU, C.P., REDDY, P., YU, Q., WHEELER, D.A., ZWIEBEL, L.J., KONOPKA, R.J., ROSBASH, M. and 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* ^{O} and *per* $^{-}$ mutants. *J. Neurogenet.* **3,**

249-291.

HAN, S-Z and ENGELMANN, W. (1987) Azadirachtin affects the circadian rhythm of locomotion in *Leucophaea maderae. J. Interdiscip. Cycle Res.* **18,** 71-82.

HANDLER, A.M. and KONOPKA, R.J. (1979) Transplantation of a circadian pacemaker in *Drosophila. Nature, Lond.* **279,** 236-238.

HARDIE, J. (1987) The photoperiodic control of wing development in the black bean aphid, *Aphis fabae. J. Insect Physiol.* **33,** 543-549.

HARKER, J.E. (1954) Diurnal rhythm in *Periplaneta americana* L. *Nature, Lond.* **173,** 689-690.

HARKER, J.E. (1956) Factors controlling the diurnal rhythm of activity of *Periplaneta americana* L. *J. exp. Biol.* **33,** 224-234.

HARRIS, G.J. and MORGAN, E. (1983) Estimates of significance in periodogram analysis of damped oscillations in a biological time-series. *Behav. Anal. Lett.* **3,** 221-230.

HAUPT, A. and BUSVINE, J.R. (1968) The effect of overcrowding on the size of houseflies (*Musca domestica* L.). *Trans. R. Entomol. Soc. Lond.* **120,** 297-311.

HELFRICH, C. (1986) Role of the optic lobes in the regulation of the locomotor activity rhythm of *Drosophila melanogaster*: behavioural analysis of neural mutants. *J. Neurogenet.* **3,** 321-343.

HELFRICH, C., CYMBOROWSKI, B. and ENGELMANN, W. (1985) Circadian activity rhythm of the house fly continues after optic lobe tract severance and lobectomy. *Chronobiol. Int.* **2,** 19-32.

HELFRICH, C. and ENGELMANN, W. (1983) Circadian rhythm of the locomotor activity in *D. melanogaster* and its mutants 'sine oculis' and 'small optic lobes'. *Physiol. Entomol.* **8,** 257-272.

HENRICH, V.C. and DENLINGER, D.L. (1982) A maternal effect that eliminates pupal diapause in progeny of the fleshfly *Sarcophaga bullata. J. Insect Physiol.* **28,** 881-884.

HINTON, H.E. (1951) A new chironomid from Africa, the larvae of which can be dehydrated without injury. *Proc. zool. Soc. Lond.* **121,** 371-380.

HOFFMANN, K. (1957) Angeborene Tagesperiodik bei Eidechsen. *Naturwiss.* **44,** 359-360.

HOFFMANN, K. (1959) Die Aktivitatsperiodik von im 18-und 36-Stunden-Tag erbruteten Eidechsen. *Z. vgl. Physiol.* **42,** 422-432.

HOFFMANN, K. (1965) Overt circadian frequencies and circadian rule. In *Circadian Clocks* (Ed. ASCHOFF), pp. 87-94. North-Holland Publishing Co., Amsterdam.

HOFFMANN, K. (1971) Splitting of the circadian rhythm as a function of light intensity. In *Biochronometry* (Ed. MENAKER), pp. 134-150. National Academy of Sciences, Washington, D.C.

HOFMANN, K., GUENDEROTH-PALMOWSKI, M., WIEDENMANN, G. and ENGELMANN, W. (1978) Further evidence for period lengthening effect of Li^+ on circadian rhythms. *Z. Naturforsch. Sect. C Biosci.* **33,** 231-234.

JACKLET, J.W. (1977) Neuronal circadian rhythms: phase-shifting by a protein synthesis inhibitor. *Science, Wash.* **198,** 69-71.

JEREBZOFF, S. (1987) Are there ultradian rhythms at the molecular level? *J. Interdiscip. Cycle Res.* **18,** 9-16.

JILGE, B., FRIESS, L. and STAEHLE, H. (1986) Internal coupling of five functions of rabbits exhibiting a bimodal circadian rhythm. *J. Interdiscip. Cycle Res.* **17,** 7-28.

JILGE, B. and STAEHLE, H. (1984) The internal synchronization of 5 circadian functions of the rabbit. *Chronobiol. Int.* **1,** 195-204.

JOHNSSON, A., ENGELMANN, W., PFLUG, B. and KLEMKE, W. (1980) Influence of lithium ions on human circadian rhythms. *Z. Naturforsch. Sect C Biosci.* **35,** 503-507.

JONES, M.D.R. (1964) The automatic recording of mosquito activity. *J. Insect Physiol.* **10,** 343-351.

JONES, M.D.R. (1973) Delayed effect of light on the mosquito 'clock'. *Nature, Lond.* **245,** 384-385.

JONES, M.D.R. (1976) Persistence in continuous light of a circadian rhythm in the mosquito *Culex pipiens fatigans* Wied. *Nature, Lond.* **261,** 491-492.

JONES, M.D.R. (1982) Coupled oscillators controlling circadian flight activity in the mosquito, *Culex pipiens quinquefasciatus*. *Physiol. Entomol.* **7**, 281-289.

JONES, M.D.R., CUBBIN, C.M. and MARSH, D. (1972a) The circadian rhythm of flight activity of the mosquito *Anopheles gambiae*: the light-on response. *J. exp.Biol.* **57**, 337-346.

JONES, M.D.R., CUBBIN, C.M. and MARSH, D. (1972b) Light-on effects and the question of bimodality in the circadian flight activity of the mosquito *Anopheles gambiae*. *J. exp. Biol.* **57**, 347-357.

JONES, M.D.R. and GUBBINS, S.J. (1978) Changes in the circadian flight activity of the mosquito *Anopheles gambiae* in relation to insemination, feeding and oviposition. *Physiol. Entomol.* **3**, 213-220.

JONES, M.D.R. and GUBBINS, S.J. (1979) Modification of female circadian flight activity by a male accessory gland pheromone in the mosquito, *Culex pipiens quinquefasciatus*. *Physiol. Entomol.* **4**, 345-351.

JONES, M.D.R., HILL, M. and HOPE, A.M. (1967) The circadian flight activity of the mosquito *Anopheles gambiae* : phase setting by the light regime. *J. exp. Biol.* **47**, 503-511.

JONES, M.D.R. and REITER, R. (1975) Entrainment of the pupation and adult activity rhythms during development in the mosquito *Anopheles gambiae*. *Nature, Lond.* **254**, 242-244.

KATZ, J.J., CRESPI, H.L., CZAJKA, D.M. and FINKEL, A.J. (1962) Course of deuteration and some physiological effects of deuterium in mice. *Am. J. Physiol.* **203**, 907-913.

KONOPKA, R.J. (1981) Genetics and development of circadian rhythms in invertebrates. In *Handbook of Behavioural Neurobiology,* Vol. 4: Biological Rhythms (Ed. ASCHOFF), pp. 173-181. Plenum Press, New York.

KONOPKA, R.J. and BENZER, S. (1971) Clock mutants of *Drosophila melanogaster*. *Proc. Nat. Acad. Sci., U.S.A.* **68**, 2112-2116.

KUDRYAVTSEVA, N.M. (1986) Larval diapause in the central Asiatic population of *Calliphora vicina* (Diptera, Calliphoridae). *Entomol. Obozr.* **65**, 16-20.

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

LANKINEN, P. and 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 (Ed. PORTER & COLLINS), pp. 97–114. Pitman, London.

LAWRENCE, P.A. (1981) The cellular basis of segmentation in insects. *Cell,* **26,** 3–10.

LEE, J.D. and LEE, T.D. (1982) *Statistics and Computer Methods in Basic,* International Student Edition. Van Nostrand Reinhold Co. Ltd.

LEES, A.D. (1959) The role of photoperiod and temperature in the determination of parthenogenetic and sexual forms in the aphid *Megoura viciae* Buckton. I. The influence of these factors on apterous virginoparae and their progeny. *J. Insect Physiol.* **3,** 92–117.

LEES, A.D. (1960) The role of photoperiod and temperature in the determination of parthenogenetic and sexual forms in the aphid *Megoura viciae* Buckton. II. The operation of the 'interval timer' in young clones. *J. Insect Physiol.* **4,** 154–175.

LEES, A.D. (1963) The role of photoperiod and temperature in the determination of parthenogenetic and sexual forms in the aphid *Megoura viciae* Buckton. III. Further properties of the maternal switching mechanism in apterous aphids. *J. Insect Physiol.* **9,** 153–164.

LEES, A.D. (1964) The location of the photoperiodic receptors in the aphid *Megoura viciae. J. exp. Biol.* **41,** 119–133.

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

LEES, A.D. (1981) Action spectra for the photoperiodic control of polymorphism in the aphid *Megoura viciae. J. Insect Physiol.* **27,** 761–771.

LEES, A.D. (1986) Some effects of temperature on the hour glass photoperiod timer in the aphid *Megoura viciae. J. Insect Physiol.* **32,** 79–89.

LEUTHOLD, R. (1966) Die Bewegungsaktivitat der weiblichen Schabe *Leucophaea maderae* (F.) im Laufe des Fortpflanzungszyklus und ihre experimentalle Beeinflussung. *J. Insect Physiol.* **12,** 1303–1331.

LEWIS, R.D. (1976) The circadian rhythm of the weta, *Hemideina thoracica* (Orthoptera); free-running rhythms, circadian rule and light entrainment.

Int. J. Chronobiol. **3,** 241-254.

LEWIS, R.D. and 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. and TAYLOR, L.P. (1965) Diurnal periodicity of flight by insects. *Trans. R. Entomol. Soc. Lond.* **116,** 393-479.

LIPTON, G.R. and SUTHERLAND, D.J. (1970) Activity rhythms in the American cockroach *Periplaneta americana. J. Insect Physiol.* **16,** 1555-1566.

LOHER, W. (1972) Circadian control of stridulation in the cricket *Teleogryllus commodus* Walker. *J. comp. Physiol.* **79,** 173-190.

LOHER, W. and CHANDRASHEKARAN, M.K. (1970) Circadian rhythmicity in the oviposition of the grasshopper *Chorthippus curtipennis. J. Insect Physiol.* **16,** 1677-1688.

LOHMANN, M. (1964) Der einfluss von Beleuchtungsstarke und Temparatur auf die Tagesperiodische Laufaktivitat des Mehlkafers, *Tenebrio molitor* L. *Z. vgl. Physiol.* **49,** 341-389.

LUDLUM, C.T. and SIEBER, K-P. (1988) Effects of azadirachtin on oogenesis in *Aedes aegypti. Physiol. Entomol.* **13,** 177-184.

LUTZ, F.E. (1932) Experiments with Orthoptera concerning diurnal rhythms. *Am. Mus. Novit.* **550,** 1-24.

LUTZ, P.E. and JENNER, C.E. (1964) Life-history and photoperiodic response of nymphs of *Tetragoneuria cynosura* (Say). *Biol. Bull., Woods Hole,* **127,** 304-316.

MANNING, A. (1981) *An Introduction to Animal Behaviour,* 3rd Edition. Edward Arnold Ltd.

MARTIN, W. and BRINKMANN, K. (1976) A computer program system for the analysis of equispaced time series. *J. Interdiscip. Cycle Res.* **7,** 251-258.

MASAKI, S. (1959) Seasonal changes in the mode of diapause in the pupae of *Abraxas miranda* Butler. *Bull. fac. Agric. Hirosaki Univ.* **5,** 14-27.

McDANIEL, M., SULZMAN, F.M. and HASTINGS, J.W. (1974) Heavy water slows the

Gonyaulax clock: a test of the hypothesis that D_2O affects circadian oscillations by diminishing the apparent temperature. *Proc. Nat. Acad. Sci., U.S.A.* **71**, 4389–4391.

MENAKER, M. and ZIMMERMAN, N.H. (1976) Role of the pineal gland in the circadian system of birds. *Am. Zool.* **16**, 45–55.

MILES, L.E.M., RAYNAL, D.M. and WILSON, M.A. (1977) Blind man living in normal society has circadian rhythms of 24.9 hours. *Science, Wash.* **198**, 421–423.

MINIS, D.H. and PITTENDRIGH, C.S. (1968) Circadian oscillation controlling hatching: its ontogeny during embryogenesis of a moth. *Science, Wash.* **159**, 534–536.

MOLINA-BORJA, M., GONZALEZ, J.G., SOUTULLO, T.G. and DIAZ, C.G. (1986) 24-H entrainment and ultradian fluctuations in the activity of lizard *Gallotia galloti galloti* (Sauria, Lacertidae). *J. Interdiscip. Cycle Res.* **17**, 295–306.

MORATA, G. and LAWRENCE, P.A. (1977) Homoeotic genes, compartments and cell determination in *Drosophila*. *Nature, Lond.* **265**, 211–216.

MÜLLER, H.J. (1970) Formen der Dormanz bei Insekten. *Nova Acta Leopold.* **35**, 7–27.

NANDA, K.K. and HAMNER, K.C. (1958) Studies on the nature of the endogenous rhythm affecting photoperiodic response of biloxi soy bean. *Bot. Gaz.* **120**, 14–25.

NAYER, J.K. and SAUERMAN, D.M. (1971) The effect of light regimes on the circadian rhythm of flight activity in the mosquito *Aedes taeniorhynchus*. *J. exp. Biol.* **54**, 745–756.

NISHIITSUTSUJI-UWO, J. and PITTENDRIGH, C.S. (1968a) Central nervous system control of circadian rhythmicity in the cockroach. II. The pathway of light signals that entrain the rhythm. *Z. vgl. Physiol.* **58**, 1–13.

NISHIITSUTSUJI-UWO, J. and PITTENDRIGH, C.S. (1968b) Central nervous system control of circadian rhythmicity in the cockroach. III. The optic lobes, locus of the driving oscillation? *Z. vgl. Physiol.* **58**, 14–46.

NJUS, D., SULZMAN, F.M. and HASTINGS, J.W. (1974) Membrane model for the circadian clock. *Nature, Lond.* **248**, 116–120.

NORRIS, M.J. (1965) The influence of constant and changing photoperiod on

imaginal diapause in the red locust (*Nomodacris septemfasciata* Serv.). *J. Insect Physiol.* **11**, 1105–1119.

NOWOSIELSKI, J.W. and PATTON, R.L. (1963) Studies on circadian rhythms of the house cricket *Gryllus domesticus*. *J. Insect Physiol.* **9**, 401–410.

PAGE, T.L. (1981a) Localization of circadian pacemakers in insects. In *Biological Clocks in Seasonal Reproductive Cycles* (Ed. FOLLET & FOLLET), Colston Papers No. 32, pp. 113–124. John Wright, Bristol.

PAGE, T.L. (1981b) Neural and endocrine control of circadian rhythmicity in invertebrates. In *Handbook of Behavioural Neurobiology,* Vol. 4: Biological Rhythms (Ed. ASCHOFF), pp. 145–172. Plenum Press, New York.

PAGE, T.L. (1984) Neural organization of a circadian clock in the cockroach *Leucophaea maderae.* In *Photoperiodic Regulation of Insect and Molluscan Hormones,* Ciba Foundation Symposia 104 (Ed. PORTER & COLLINS), pp. 115–135. Pitman, London.

PAGE, T.L. and BLOCK, G.D. (1980) Circadian rhythmicity in cockroaches: effects of early post-embryonic development and ageing. *Physiol. Entomol.* **5**, 271–281.

PALMER, J.D. and DOWSE, H.B. (1969) Preliminary findings on the effect of D_2O on the period of circadian activity rhythms. *Biol. Bull., Woods Hole,* **137**, 388.

PARKER, A.H. (1962) Studies on the diurnal rhythms of the house fly *Musca domestica* L. in a dry tropical environment. *Acta Trop.* **19**, 97–119.

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

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 systems I. The driving oscillation and its assay in *Drosophila pseudoobscura. Proc. Nat. Acad. Sci., U.S.A.* **58**, 1762–1767.

PITTENDRIGH, C.S. (1972) Circadian surfaces and the diversity of possible roles of circadian organizations in photoperiodic induction. *Proc. Nat. Acad. Sci., U.S.A.* **69**, 2734–2737.

PITTENDRIGH, C.S. (1974) Circadian oscillations in cells and the circadian organization of multicellular systems. In *The Neurosciences Third Study Program* (Ed. SCHMITT & WORDEN), pp. 437-458. M.I.T. Press, Cambridge, Massachusetts.

PITTENDRIGH, C.S. (1981) Circadian organization and the photoperiodic phenomena. In *Biological Clocks in Seasonal Reproductive Cycles* (Ed. FOLLET & FOLLET), Colston Papers No. 32, pp. 1-36. John Wright, Bristol.

PITTENDRIGH, C.S. and BRUCE, V.G. (1957) An oscillator model for biological clocks. In *Rhythmic and Synthetic Processes in Growth* (Ed. RUDNICK), pp. 75-109. Princeton University Press, Princeton, New Jersey.

PITTENDRIGH, C.S. and BRUCE, V.G. (1959) Daily rhythms as coupled oscillator systems and their relation to thermoperiodism and photoperiodism. In *Photoperiodism and Related Phenomena in Plants and Animals* (Ed. WITHROW), pp. 475-505. Am. Assoc. Adv. Sci., Washington, D.C.

PITTENDRIGH, C.S., BRUCE, V.G. and KAUS, P. (1958) On the significance of transients in daily rhythms. *Proc. Nat. Acad. Sci., U.S.A.* **44,** 965-973.

PITTENDRIGH, C.S. and CALDAROLA, P.C. (1973) General homeostasis of the frequency of circadian oscillations. *Proc. Nat. Acad. Sci., U.S.A.* **70,** 2697-2701.

PITTENDRIGH, C.S., CALDAROLA, P.C. and COSBEY, E.S. (1973) A differential effect of heavy water on temperature-dependent and temperature-compensated aspects of the circadian system of *Drosophila pseudoobscura*. *Proc. Nat. Acad. Sci., U.S.A.* **70,** 2037-2041.

PITTENDRIGH, C.S. and COSBEY, E.S. (1974) On the very rapid enhancement by D_2O of the temperature-tolerance of adult *Drosophila*. *Proc. Nat. Acad. Sci., U.S.A.* **71,** 540-543.

PITTENDRIGH, C.S. and DAAN, S. (1974) Circadian oscillators in rodents: a systematic increase of their frequency with age. *Science, Wash.* **186,** 548-550.

PITTENDRIGH, C.S. and DAAN, S. (1976a) A functional analysis of circadian pacemakers in nocturnal rodents. I. The stability and lability of spontaneous frequency. *J. comp. Physiol. A,* **106,** 223-252.

PITTENDRIGH, C.S. and DAAN, S. (1976b) A functional analysis of circadian pacemakers in nocturnal rodents. IV. Entrainment: pacemaker as clock. *J. comp. Physiol. A,* **106,** 291-331.

PITTENDRIGH, C.S. and DAAN, S. (1976c) A functional analysis of circadian pacemakers in nocturnal rodents. V. Pacemaker structure: a clock for all seasons. *J. comp. Physiol. A,* **106,** 333-355.

PITTENDRIGH, C.S., ELLIOT, J. and TAKAMURA, T. (1984) The circadian components in photoperiodic induction. In *Photoperiodic Regulation of Insect and Molluscan Hormones,* Ciba Foundation Symposium 104 (Ed. PORTER & COLLINS), pp. 26-47. Pitman, London.

PITTENDRIGH, C.S. and MINIS, D.H. (1964) The entrainment of circadian oscillations by light and their role as photoperiodic clocks. *Am. Nat.* **98,** 261-294.

PITTENDRIGH, C.S. and MINIS, D.H. (1971) The photoperiodic time measurement in *Pectinophora gossypiella* and its relation to the circadian system in that species. In *Biochronometry* (Ed. MENAKER), pp. 212-250. National Academy of Sciences, Washington, D.C.

PITTENDRIGH, C.S. and TAKAMURA, T. (1989) Latitudinal clines in the properties of a circadian pacemaker. *J. Biol. Rhythms,* **4,** 217-235.

POHL, H. (1972) Die aktivitatsperiodik von zwei tagaktiven nagern, *Funambulus palmarum* und *Eutamias sibiricus* unter dauerlichtbedingungen. *J. comp. Physiol.* **78,** 60-74.

REINHARD, P. (1985) Effect of lithium chloride on the lower range of entrainment in Syrian hamsters. *J. Interdiscip. Cycle Res.* **16,** 227-237.

RENCE, B.G., LISY, M.T., GARVES, B.R. and QUINLAN, B.J. (1988) The role of ocelli in circadian singing rhythms of crickets. *Physiol. Entomol.* **13,** 201-212.

REPPERT, S.M. and SCHWARTZ, W.J. (1983) Maternal coordination of the fetal biological clock *in utero. Science, Wash.* **220,** 969-971.

REPPERT, S.M. and SCHWARTZ, W.J. (1984) The suprachiasmatic nuclei of the fetal rat: characterization of a functional circadian clock using ^{14}C-labelled deoxyglucose. *J. Neurosci.* **4,** 1677-1682.

RICHARD, D.S. and SAUNDERS, D.S. (1987) Prothoracic gland function in diapause and non-diapause *Sarcophaga argyrostoma* and *Calliphora vicina. J. Insect Physiol.* **33,** 385-392.

RICHARD, D.S., SAUNDERS, D.S., EGAN, V.M. and THOMSON, R.C.K. (1986) The timing of larval wandering and puparium formation in the fleshfly *Sarcophaga argyrostoma. Physiol. Entomol.* **11,** 53-60.

RICHTER, C.P. (1970) Blood-brain barrier: its penetration by heavy water. *Proc. Nat. Acad. Sci., U.S.A.* **66,** 244 (abstract).

RING, R.A. (1967) Maternal induction of diapause in the larvae of *Lucilia caesar* L. (Diptera, Calliphoridae). *J. exp. Biol.* **46,** 123-136.

ROBERTS, B. and WARREN, M.H. (1975) Diapause in the Australian flesh-fly *Tricholioprocta impatiens* (Diptera: Sarcophagidae). *Aust. J. Zool.* **23,** 563-567.

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

ROBERTS, S.K. de F. (1962) Circadian activity in cockroaches. II. Entrainment and phase-shifting. *J. cell. comp. Physiol.* **59,** 175-186.

ROBERTS, S.K. de F. (1965) Photoreception and entrainment of cockroach activity rhythms. *Science, Wash.* **148,** 958-959.

ROBERTS, S.K. de F. (1974) Circadian rhythms in cockroaches. Effects of optic lobe lesions. *J. comp. Physiol.* **88,** 21-30.

ROCKEY, S.J. and DENLINGER, D.L. (1983) Deuterium oxide alters pupal diapause response in the flesh fly, *Sarcophaga crassipalpis. Physiol. Entomol.* **8,** 445-449.

SANDERSON, P.G. and CHARNLEY, A.K. (1983) The effect of diet on activity in male *Calliphora vicina* blowflies. *Physiol. Entomol.* **8,** 315-319.

SAUNDERS, D.S. (1965a) Larval diapause induced by a maternally-operating photoperiod. *Nature, Lond.* **206,** 739-740.

SAUNDERS, D.S. (1965b) Larval diapause of maternal origin: induction of diapause in *Nasonia vitripennis* (Walk.) (Hymenoptera: Pteromalidae). *J. exp. Biol.* **42,** 495-508.

SAUNDERS, D.S. (1966) Larval diapause of maternal origin. II. The effect of photoperiod and temperature on *Nasonia vitripennis. J. Insect Physiol.* **12,** 569-581.

SAUNDERS, D.S. (1967) Time measurement in insect photoperiodism: reversal of

a photoperiodic effect by chilling. *Science, Wash.* **156,** 1126–1127.

SAUNDERS, D.S. (1968) Photoperiodism and time measurement in the parasitic wasp, *Nasonia vitripennis*. *J Insect Physiol.* **14,** 433–450.

SAUNDERS, D.S. (1969) Diapause and photoperiodism in the parasitic wasp *Nasonia vitripennis*, with special reference to the nature of the photoperiodic clock. *Symp. Soc. exp. Biol.* **23,** 301–329.

SAUNDERS, D.S. (1970) Circadian clock in insect photoperiodism. *Science, Wash.* **168,** 601–603.

SAUNDERS, D.S. (1971) The temperature-compensated photoperiodic clock 'programming' development and pupal diapause in the flesh-fly *Sarcophaga argyrostoma*. *J. Insect Physiol.* **17,** 801–812.

SAUNDERS, D.S. (1973a) Thermoperiodic control of diapause in an insect: theory of internal coincidence. *Science, Wash.* **181,** 358–360.

SAUNDERS, D.S. (1973b) The photoperiodic clock in the flesh-fly, *Sarcophaga argyrostoma*. *J. Insect Physiol.* **19,** 1941–1954.

SAUNDERS, D.S. (1974) Evidence for 'dawn' and 'dusk' oscillators in the *Nasonia* photoperiodic clock. *J. Insect Physiol.* **20,** 77–88.

SAUNDERS, D.S. (1976) The circadian eclosion rhythm in *Sarcophaga argyrostoma* : some comparisons with the photoperiodic clock. *J. Insect 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. A,* **124,** 75–95.

SAUNDERS, D.S. (1979) External coincidence and the photoinducible phase in *Sarcophaga* photoperiodic clock. *J. comp. Physiol. A,* **132,** 179–189.

SAUNDERS, D.S. (1980) Some effects of constant temperature and photoperiod on the diapause response of the flesh-fly, *Sarcophaga argyrostoma*. *Physiol. Entomol.* **5,** 191–198.

SAUNDERS, D.S. (1981a) Insect photoperiodism: entrainment as a basis for time measurement. In *Biological Clocks in Seasonal Reproductive Cycles* (Ed. FOLLET & FOLLET), pp. 67–81. John Wright, Bristol.

SAUNDERS, D.S. (1981b) Insect photoperiodism - the clock and the counter: a review. *Physiol. Entomol.* **6**, 99-116.

SAUNDERS, D.S. (1982a) *Insect Clocks,* 2nd Edition. Pergamon Press, Oxford.

SAUNDERS, D.S. (1982b) Photoperiodic induction of pupal diapause in *Sarcophaga argyrostoma* : temperature effects on circadian resonance. *J. Insect Physiol.* **28**, 305-310.

SAUNDERS, D.S. (1986) Many circadian oscillators regulate developmental and behavioural events in the fleshfly *Sarcophaga argyrostoma. Chronobiol. Int.* **3**, 71-83.

SAUNDERS, D.S. (1987) Maternal influence on the incidence and duration of larval diapause in *Calliphora vicina. Physiol. Entomol.* **12**, 331-338.

SAUNDERS, D.S., HENRICH, V.C. and GILBERT, L.I. (1989) Induction of diapause in *Drosophila melanogaster* : photoperiodic regulation and the impact of arrhythmic clock mutations on time measurement. *Proc. Nat. Acad. Sci., U.S.A.* **86**, 3748-3752.

SAUNDERS, D.S. and LEWIS, R.D. (1987a) A damped circadian oscillator model of an insect photoperiodic clock. II. Simulations of the shapes of photoperiodic response curves. *J. theor. Biol.* **128**, 61-71.

SAUNDERS, D.S. and 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. and LEWIS, R.D. (1988) The photoperiodic clock and counter mechanism in two species of flies: evidence for damped circadian oscillators in time measurement. *J. comp. Physiol. A,* **163**, 365-371.

SAUNDERS, D.S. and THOMSON, E.J. (1977) Strong phase response curve for the circadian rhythm of locomotor activity in a cockroach (*Nauphoeta cinerea*). *Nature, Lond.* **270**, 242-243.

SAUNDERS, D.S., MACPHERSON, J.N. and CAIRNCROSS, K.D. (1986) Maternal and larval effects of photoperiod on the induction of larval diapause in two species of fly, *Calliphora vicina* and *Lucilia sericata. Exp. Biol. (Berl.),* **46**, 51-58.

SCHMID, H. and ENGELMANN, W. (1987) Effects of lithium, rubidium and tetraethylammonium chloride on the locomotor activity rhythm of *Musca domestica. J. Interdiscip. Cycle Res.* **18**, 83-102.

SCHULL, J., McEACHRON, D.L., ADLER, N.T., FIEDLER, L., HORVITZ, J., NOYES, A., OLSON,M. and SHACK, J. (1988) Effects of thyroidectomy, parathyroidectomy and lithium on circadian wheelrunning in rats. *Physiol. Behav.* **42,** 33-40.

SHAHEIN, A. (1986) Effect of intraspecific competition between larvae of *Calliphora erythrocephala* (Calliphoridae, Diptera). *Z. Angew. Zool.* **73,** 245-248.

SKOPIK, S.D., CAIN, W., PATEL, N.G. and NEWSWANGER, D.L. (1986) Photoperiodism in *Ostrinia nubilalis* : a new protocol for the analysis of the role of the circadian system. *J. Biol. Rhythms,* **1,** 145-150.

SMITH, P.H. (1983) Circadian control of spontaneous flight activity in the blowfly, *Lucilia cuprina. Physiol. Entomol.* **8,** 73-82.

SOLOMON, M.E. (1951) Control of humidity with KOH, sulphuric acid or other solutions. *Bull. Entomol. Res.* **42,** 543-554.

SOKOLOVE, P.G. and BUSHELL, W.N. (1978) The chi-squared periodogram: its utility for analysis of circadian rhythms. *J. theor. Biol.* **72,** 131-160.

STEEL, C.G.H. and LEES, A.D. (1977) The role of neurosecretion in the photoperiodic control of polymorphism in the aphid *Megoura viciae. J. exp. Biol.* **67,** 117-135.

STOFFOLANO, J.G., GREENBERG, S. and CALABRESE, E. (1974) A facultative imaginal diapause in the black blowfly, *Phormia regina. Ann. entomol. Soc. Am.* **67,** 518-519.

STOFFOLANO, J.G. and MATTHYSSE, J.G. (1967) Influence of photoperiod and temperature on diapause in the face fly, *Musca autumnalis* (Diptera; Muscidae). *Ann. ent. Soc. Am.* **60,** 1242-1246.

STRUHL, G. (1981) A gene product required for correct initiation of segmental determination in *Drosophila. Nature, Lond.* **293,** 36-41.

STRYER, L. (1981) *Biochemistry,* 2nd Edition. W.H.Freeman & Co., San Francisco.

SUTER, R.B. and RAWSON, K.S. (1968) Circadian activity rhythm of the deer mouse, *Peromyscus*: effect of D_2O. *Science, Wash.* **160,** 1011-1014.

SYZMANSKI, J.S. (1920) Aktivitat und Ruhe bei Tieren und Menschen. *Z. allg. Physiol.* **18,** 105-162.

TAKAHASHI, J.S. and TUREK, F.W. (1987) Anisomycin, an inhibitor of protein synthesis, perturbs the phase of a mammalian circadian pacemaker. *Brain Res.* **405**, 199–203.

TAKEDA, M. and MASAKI, S (1976) Photoperiodic control of larval development in *Plodia interpunctella.* In *Proc. U.S.-Japan Seminar Stored Product Entomol.* pp. 186–201. Manhattan, Kansas.

TAKEDA, M. and SKOPIK, S.D. (1985) Geographical variation in the circadian system controlling photoperiodism in *Ostrinia nubilalis. J. comp. Physiol. A,* **156**, 653–658.

TAUBER, M.J. and TAUBER, C.A. (1970) Photoperiodic induction and termination of diapause in an insect: response to changing daylengths. *Science, Wash.* **167**, 170.

TAUBER, M.J. and TAUBER, C.A. (1972) Geographic variation in critical photoperiodic and in diapause intensity of *Chrysopa carnea* (Neuroptera). *J. Insect Physiol.* **18**, 25–29.

TAUBER, M.J., TAUBER, C.A. and MASAKI, S. (1986) *Seasonal Adaptations of Insects.* Oxford University Press, Oxford.

TAYLOR, B. (1969) Geographical range and circadian rhythms. *Nature, Lond.* **222**, 296–297.

TAYLOR, B. and JONES, M.D.R. (1969) The circadian rhythm of flight activity in the mosquito *Aedes aegypti* (L.): the phase-setting effects of light-on and light-off. *J. exp. Biol.* **51**, 59–70.

THEILE, H-U. (1967) Formen der Diapausesteuerung bei Carabiden. *Verh. Dtsch. Zool. Ges. (Zool. Anz. Suppl.)* **31**, 358–364.

THEILE, H-U. (1971) Die Steuerung der Jahesrhythmik von Carabiden durch exogene und endogene Faktoren. *Zool. Jahrb. Abt. Syst. Oekol. Geogr. Tiere,* **98**, 341–371.

THEILE, H-U. (1977a) Measurement of day-length as a basis for photoperiodism and annual periodicity in the Carabid beetle *Pterostichus nigrita* F. *Oecologia (Berl.),* **30**, 331–348.

THEILE, H-U. (1977b) Differences in measurement of day-length and photoperiodism in two stocks from subarctic and temperate climates in the Carabid, *Pterostichus nigrita* F. *Oecologia (Berl.),* **30**, 349–365.

TOMIOKA, K. (1985) Residual circadian rhythmicity after bilateral lamina-medulla removal or optic stalk transection in the cricket *Gryllus bimaculatus. J. Insect Physiol.* **31**, 653-658.

TRUMAN, J.W. (1971). Circadian rhythms and physiology with special reference to neuroendocrine processes in insects. *Proc. Int. Symp. Circadian Rhythmicity* (Wageningen, 1971), pp. 111-135.

TRUMAN, J.W. (1973) Temperature sensitive programming of the silkmoth flight clock: a mechanism for adapting to the seasons. *Science, Wash.* **182**, 727-729.

TRUMAN, J.W. (1974) Physiology of insect rhythms. IV. Role of the brain in the regulation of the flight rhythm of the giant silkmoths. *J. comp. Physiol. A*, **95**, 281-296.

TYSHCHENKO, V.P. (1966) Two-oscillatory model of the physiological mechanism of insect photoperiodic reaction. *Zh. obshch. Biol.* **27**, 209-222.

UNDERWOOD, H. (1977) Circadian organization in lizards: the role of the pineal organ. *Science, Wash.* **195**, 587-589.

VAZ NUNES, M., KENNY, N.A.P. and SAUNDERS, D.S. (1989) The photoperiodic clock in the blowfly *Calliphora vicina. J. Insect Physiol.* (in press).

VAZ NUNES, M. and SAUNDERS, D.S. (1989) The effect of larval temperature and photoperiod on the incidence of larval diapause in the blowfly *Calliphora vicina. Physiol. Entomol.* (in press).

VAZ NUNES, M. and VEERMAN, A. (1979a) Photoperiodic time measurement in spider mites. I. Development of a two interval timers model. *J. comp. Physiol. A*, **134**, 203-217.

VAZ NUNES, M. and VEERMAN, A. (1979b) Photoperiodic time measurement in spider mites. II. Effects of skeleton photoperiods. *J. comp. Physiol. A*, **134**, 219-226.

VAZ NUNES, M. and VEERMAN, A. (1982a) External coincidence and photoperiodic time measurement in the spider mite, *Tetranychus urticae. J. Insect Physiol.* **28**, 143-154.

VAZ NUNES, M. and VEERMAN, A. (1982b) Photoperiodic time measurement in the spider mite, *Tetranychus urticae* : a novel concept. *J. Insect Physiol.* **28**, 1041-1053.

VEERMAN, A. and VAZ NUNES, M. (1980) Circadian rhythmicity participates in the photoperiodic determination of diapause in spider mites. *Nature, Lond.* **287**, 140–141.

VEERMAN, A. and VAZ NUNES, M. (1984) Photoperiodic reception in spider mite mites: photoreceptor, clock and counter. In *Photoperiodic Regulation of Insect and Molluscan Hormones,* Ciba Foundation Symposium 104 (Ed. PORTER & COLLINS), pp. 48–64. Pitman, London.

VINOGRADOVA, E.B. (1960) The experimental investigation of ecological factors involving imaginal diapause in bloodsucking mosquitoes (Diptera, Culicidae). *Entomol. Obozr.* **39**, 327–340.

VINOGRADOVA, E.B. (1974) The pattern of reactivation of diapausing larvae in the blowfly, *Calliphora vicina. J. Insect Physiol.* **20**, 2487–2496.

VINOGRADOVA, E.B. (1976) Embryonic photoperiodic sensitivity in two species of flesh flies, *Parasarcophaga similis* and *Boettcherisca septentrionalis. J. Insect Physiol.* **22**, 819–822.

VINOGRADOVA, E.B. (1984) *The Blowfly Calliphora vicina as a Model Insect for the Physiological and Ecological Investigations* (in Russian). Leningrad: "Nauka".

VINOGRADOVA, E.B. (1986) Geographical variation and ecological control of diapause in flies. In *The Evolution of Insect Life Cycles* (Ed. TAYLOR & KARBAN), pp. 35–47. Springer-Verlag, New York.

VINOGRADOVA, E.B. and ZINOVJEVA, K.B. (1972) Maternal induction of larval diapause in the blowfly, *Calliphora vicina. J. Insect Physiol.* **18**, 2401–2409.

WADDELL, B.C. (1984) Differential clock control of the circadian rhythms of eclosion and adult locomotor activity in *Calliphora stygia* (Diptera: Calliphoridae). M.Sc. thesis, University of Auckland, New Zealand.

WIEDENMANN, G. (1977a) Two activity peaks in the circadian rhythm of the cockroach, *Leucophaea maderae. J. Interdiscip. Cycle Res.* **8**, 378–383.

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

WILDE, J. de (1962) Photoperiodism in insects and mites. *Annu. Rev. Entomol.* **7**, 1–26.

WILDE, J. de, DUINTJER, C.S. and MOOK, L. (1959) Physiology of diapause in the adult Colorado beetle (*Leptinotarsa decemlineata*). I. The photoperiod as a controlling factor. *J. Insect Physiol.* **3,** 75–85.

WILLIAMS, B.G. and NAYLOR, E. (1967) Spontaneously induced rhythm of tidal periodicity in laboratory-reared *Carcinus*. *J. exp. Biol.* **47,** 229–234.

WILLIAMS, C.M. (1969) Photoperiodism and the endocrine aspects of insect diapause. *Symp. Soc. exp. Biol.* **23,** 285–300.

WILLIAMS, C.M. and ADKISSON, P.L. (1964) Physiology of insect diapause. XIV. An endocrine mechanism for the photoperiodic control of pupal diapause in the oak silkworm, *Antheraea pernyi*. *Biol. Bull., Woods Hole,* **127,** 511–525.

WILLIAMS, J.A. and NAYLOR, E. (1978) A procedure for the assessment of significance of rhythmicity in time-series data. *Int. J. Chronobiol.* **5,** 435–444.

WILSON, M. (1978) The functional organization of locust ocelli. *J. comp. Physiol. A,* **124,** 297–316.

WOLLNIK, F. and DOEHLER, K-D. (1986) Effects of adult or perinatal hormonal environment on ultradian rhythms in locomotor activity of laboratory LEW/Ztm rats. *Physiol. Behav.* **38,** 229–240.

WOLLNIK, F., GAERTNER, K. and BUETTNER, D. (1987) Genetic analysis of circadian and ultradian locomotor activity rhythms in laboratory rats. *Behav. Genet.* **17,** 167–178.

ZINOVJEVA, K.B. and POLYAKOVA, D.I. (1987) Effect of photorhythms and thermorhythms on the diel rhythm of eclosion in selected strains of *Calliphora vicina* R-D (Diptera, Calliphoridae). *Entomol. Obozr.* **66,** 236–246.

ZIMMERMAN, N.H. and IVES, D. (1971) Some photophysiological aspects of circadian rhythmicity in *Drosophila*. In *Biochronometry* (Ed. MENAKER), pp. 381–391. National Academy of Sciences, Washington, D.C.

ZIMMERMAN, N.H. and MENAKER, M. (1979) The pineal gland, a pacemaker within the circadian system of the house sparrow. *Proc. Nat. Acad. Sci., U.S.A.* **76,** 999–1003.

Addendum

GARNER, W.W and ALLARD, H.A (1920) Effect of the relative length of the day and night and other factors of the environment on growth and reproduction in plants *J. Agric. Res.* **18,** 553–606.

Appendices

These appendices contain the computer programs which were used to collect and analyse the data from the locomotor recorders. All of these were written with the assistance of Dr. R.D. Lewis (except the periodogram which was adapted from an existing listing by S.E.R. Bailey) for use on a BBC micro-computer. A wiring diagram is not given for the recorders as similar systems have been described in a number of previous papers (see Chapter 2).

The six programs necessary for the whole process were:

Appendix I. : *32CHS*, a program for testing the state of the detectors. In essence this ensured that the flies were able to break the beam, and that the event was being recorded by the computer. **Page 264**.

Appendix II. : *RECORD*, the data collecting program. This program records seven days data for 32 individual flies, storing all of the data as one long string. **Pages 265-266**.

Appendix III. : *RECOV32*, an initial data processing program. This creates separate files for each fly from the seven day string produced by the *RECORD* program. **Page 267**.

Appendix IV. : *JOIN32*, this joins together two consecutive seven day records for each of the 32 flies, all of which were produced by *RECOV32*. **Page 268**.

Appendix V. : *DUMP32*, this displays the fourteen day files (created by the previous program) on a computer screen, and screen-dumping allows the record to be printed out. **Pages 269-270**.

Appendix VI. : *PER32*, this is the periodogram analysis program. **Pages 271-274**.

```
   10MODE7
   12PRINTTAB(2,1)"This program is 32CHS"
   14PRINTTAB(2,2)"----------------------"
   20PRINTTAB(2,4)"This program allows you to check"TAB(2,5)"and alter the state
 of the sensors"TAB(2,6)"in the 32 channels."
   30PRINTTAB(2,8)"NB:It can only be used at the very"TAB(2,9)"begining of any r
un."
   40PRINTTAB(2,11)"You will be asked which channel you"TAB(2,12)"want.Decide an
d then press RETURN."TAB(2,13)"A row of 3 numbers will then be seen."TAB(2,14)"T
he first is the channel number,"TAB(2,15)"the second the state of the channel"
   50PRINTTAB(2,16)"and the third is the actual number"TAB(2,17)"of times the be
am has been broken."
   60PRINTTAB(2,20)"PRESS SPACE BAR TO CONTINUE"
   70REPEAT UNTIL GET=32
   80CLS
   90PRINTTAB(2,2)"The state of the channel can only be "TAB(2,3)"0 or 1.When th
e fly breaks the beam"TAB(2,4)"this number should change from 0 to 1."TAB(2,5)"T
his will probably be only momentary"
  100PRINTTAB(2,6)"--unless the fly is sitting in the"TAB(2,7)"path of the beam.
If the fly is not"TAB(2,8)"and the state is still 1 then the"TAB(2,9)"channel wi
ll have to be adjusted."
  110PRINTTAB(2,10)"This is done by changing the variable"TAB(2,11)"resistor for
 that channel until the"TAB(2,12)"value is once more 0."TAB(2,14)"The channel sh
ould be adjusted until"TAB(2,15)"the value is only just 0,and the fly"
  112PRINTTAB(2,16)"can be observed to change it to 1"TAB(2,17)"on breaking the
beam."TAB(2,19)"To select the next channel press S."
  120PRINTTAB(2,22)"PRESS SPACE BAR TO BEGIN"
  130REPEAT UNTIL GET=32
  140 MODE 2
  150 INPUT "CHANNEL NO.?"ch%
  160  TIME=0
  170 ?&FE62=&BF
  180 d%=0:f%=0:x%=0
  190 REPEAT
  200 ?&FE60=(ch%-1 AND &3F):x%=(?&FE60 DIV64) AND 1:IF x%=1 THEN f%=1 ELSE IF f
%=1 THEN f%=0: d%=d%+1:SOUND 1,-15,10,10
  210 PRINT TAB(10,10);ch%;"   ";f%;"   ";d%;"    "
  220 UNTIL INKEY$(5)="S"
  230GOTO140
```

```
  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$(ch%)=""
 120 NEXT ch%
 130 FOR iter%=1 TO 144
 140 TIME=0
 150 REPEAT
 160 FOR ch%=1 TO 32:?&FE60=(ch%-1 AND &3F):x%=(?&FE60 DIV64) AND 1:IF x%=1 THE
N f%(ch%)=1 ELSE IF f%(ch%)=1 THEN f%(ch%)=0: d%(ch%)=d%(ch%)+1
 170 NEXT ch%
 180 UNTIL TIME>59740
 190 PROCdisplay
 200 PROCstore
 210 NEXT iter%
 220 PROCsave
 230 NEXT day%
 240 ENDPROC
 250 END
 260 :
 270 DEF PROCinit
 280PRINTTAB(2,2)"This program is RECORD"
 290PRINTTAB(2,3)"_____________________"
 300PRINTTAB(2,5)"The function of this program is to record the data as it come
s straight"TAB(2,7)"from the infrared sensors. Before this can be done you shoul
d take this"
 310PRINTTAB(2,9)"disc out and turn the computer off and then on again. Now pus
h down the"TAB(2,11)"switch at the back of the disc drive (changing the machine
from 80 to"
 320PRINTTAB(2,13)"40 track) and place in drive 0 the disc marked 'ACORN 64K SI
DEWAYS RAM"TAB(2,15)"SUPPORT DISC'. Type in the command *BAS128, press return an
d fairly"
 330PRINTTAB(2,17)"quickly two lines of text will appear in the top left-hand c
orner of the"TAB(2,19)"screen. If this does not happen and an error message appe
ars turn the"TAB(2,21)"computer off and start again. Once the command has been"
 340PRINTTAB(2,21)'computer off and start again. Once the command has been obey
ed take this"
 350PRINTTAB(2,23)"disc out and (NB.!!) push up the switch at the back of the d
isc drive to"TAB(2,25)"return it to 80 track. Now you can load RECORD."
 360PRINTTAB(2,27)"The display will show all 32 channels at once: the top row b
eing, from"TAB(2,29)"left to right, ch1-8; the second row ch9-16 etc."
 370PRINTTAB(20,31)"PRESS SPACE BAR WHEN READY"
 380REPEAT UNTIL GET=32
 390 CLS
 400 ?&FE62=&FF
 410 DIM res$(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 xX,hX-464
 770 DRAW xX,hX-688
 780 MOVE xX,hX-696
 790 DRAW xX,hX-920
 800 DRAW xX,hX-914
 810 NEXT iX
 820 ENDPROC
 830 :
 840 DEF PROChisto
 850 hX=hX-(dayX*32)
 860 FOR iX=0 TO 7
 870 MOVE (iX*160)+2+iterX,hX
 880 DRAW (iX*160)+2+iterX,hX+SGR(dX(iX+chX))
 890 NEXT iX
 900 ENDPROC
 910 :
 920 DEF PROCdisplay
 930 hX=1000
 940 chX=1
 950 PROChisto
 960 hX=768
 970 chX=9
 980 PROChisto
 990 hX=536
1000 chX=17
1010 PROChisto
1020 hX=304
1030 chX=25
1040 PROChisto
1050 ENDPROC
1060 :
1070 DEF PROCstore
1080 FOR chX=1 TO 32
1090 IF dX(chX)>254 THEN dX(chX)=254
1100 res$(chX)=res$(chX)+CHR$(dX(chX))
1110 dX(chX)=0
1120 NEXT chX
1130 ENDPROC
1140 :
1150 DEF PROCerror
1160 CLOSE£0
1170 REPORT
1180 PRINT ERL
1190 END
1200 ENDPROC
1210 :
```

```
  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"
 140PRINTTAB(2,3)"_____ __"
 150PRINTTAB(2,6)"This program processes the raw data file that has come from t
he computer"TAB(2,8)"and separates out the data for each channel.The name for ea
ch 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 be on DRIVE 0,the channel data will b
e placed on"TAB(2,18)"DRIVE 2.Due to the number of files available on your disc
being limited"
 180PRINTTAB(2,20)"to 31 for each side the 32nd file will have to be put on DRI
VE 0."
 190PRINTTAB(15,30)"PRESS SPACE BAR WHEN READY"
 200REPEAT UNTIL GET=32
 210CLS:PRINT"Place disc in DRIVE 0"
 220 PROCpause
 230 *DR.0
 240*.
 250INPUT"Name of recorded data file?"n$
 260INPUT"First channel to be recovered"a%
 265INPUT"Last channel to be recovered"chs%
 270DIM res$(7)
 280A=OPENUP(n$)
 290ENDPROC
 300:
 310DEF PROCfrom_disc
 320*DR.0
 330FOR day%= 1 TO 7
 340PTR£A=((ch%-1)*146)+(4672*(day%-1))
 350INPUT£A,x$
 360res$(day%)=x$
 370NEXT day%
 380ENDPROC
 390:
 400DEF PROCto_disc
 410*DR.2
 412IF ch%>31 THEN GOTO415 ELSE GOTO420
 415*DR.0
 420chn$=n$+STR$(ch%)
 430CLS:PRINT"Channel ";ch%;" ";chn$;"   "
 440B=OPENOUT(chn$)
 450FOR day%= 1 TO 7
 460PRINT£B,res$(day%)
 470NEXT day%
 480CLOSE£B
 490ENDPROC
 500:
 510DEF PROCdisplay
 520 height%=900
 530FOR day%= 1 TO 7
 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)+S0R(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
```

```
   10REM PROGRAM JOIN32
   20MODE128
   30ON ERROR PROCerror
   40PROCinfo
   50PROCinit
   60PROCjoin
   70END
   80:
   90DEF PROCinit
  100DIM join%(32)
  110DIM res$(14)
  120CLS
  130*.0
  140PROCpause
  150CLS
  160*.1
  170PROCpause
  180CLS
  190*.2
  200 PROCpause
  210CLS
  220*.3
  230PROCpause
  240CLS
  250INPUT"Are all discs in correct drives? (Y/N) "a$
  260 IF a$="Y" THEN GOTO270 ELSE GOTO40
  270INPUT"Stem name of first files? "stem1$
  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
  380*DR.2
  390f$=stem1$+STR$(join%(file%))
  400B=OPENUP(f$)
  410FOR day%=1 TO 7
  420INPUT£B,res$(day%)
  430NEXT day%
  440CLOSE£B
  450*DR.3
  460s$=stem2$+STR$(join%(file%))
  470B=OPENUP(s$)
  480FOR day%=8 TO 14
  490INPUT£B,res$(day%)
  500NEXT day%
  510CLOSE£B
  520ENDPROC
  530:
  540DEF PROCwrite
  550*DR.0
  560n$=stemf$+STR$(join%(file%))
  570A=OPENOUT(n$)
  580FOR day%=1 TO 14
  590PRINT£A,res$(day%)
  600NEXT day%
  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
  745PRINTTAB(2,2)"This program is JOIN32"
  750PRINTTAB(2,3)"-----------------------"
  755PRINTTAB(2,8)"This program functions to join all 7 day files which have bee
n produced"TAB(2,10)"by the RECOV32 program.The first 7 days data must be on DRI
VE 2,and"
  760PRINTTAB(2,12)"the second 7 must be on DRIVE 3.The new files will be placed
 on DRIVE 0."TAB(2,14)"It is important to ensure that DRIVE 0 is empty of files,
in order"TAB(2,16)"that sufficient space will be available for the new files."
  780PROCpause
  790ENDPROC
  800:
  810DEF PROCjoin
  820FOR file%=1 TO files%
  830PROCread
  840PROCwrite
  850NEXT file%
  860ENDPROC
```

```
  10REM DISPLAY 32
  20PROCScreenDumpAssemble
  30REM USE CALL &A00 FOR SCDUMP
  40 ON ERROR PROCerror
  50 MODE 128
  60 PROCinit
  70 PROCread
  80 PROCdisplay
  90X=GET
 100IF GET =32 THEN VDU2:CALL&A00:VDU3
 110 END
 120:
 130 DEF PROCinit
 140 DIM res$(15)
 150PRINTTAB(2,2)"This program is DUMP32"
 160PRINTTAB(2,3)"_____________________"
 170PRINTTAB(2,10)"This program allows you to print out on the screen the 14 da
y files"TAB(2,12)"which you have created by combining two 7 day files from the 3
2"TAB(2,14)"channel system of locomotion recording."
 180PRINTTAB(2,16)"The display at the end of the program can be printed out on
paper"TAB(2,18)"if you press the space bar firmly."
 190PRINTTAB(20,20)"PRESS SPACE BAR WHEN READY"
 200REPEAT UNTIL GET=32
 210 CLS:INPUT"Name of file to be displayed? "n$
 220 INPUT"Dates of experiment? "date$
 230 INPUT"Protocol of experiment? "prot$
 240 INPUT"Time of begining of experiment? " time%
 250 PRINT"Place disc in drive and press SPACE":PROCpause
 260 INPUT"Total number of days? " days%
 270 ENDPROC
 280 :
 290 DEF PROCread
 300 A=OPENUP(n$)
 310 FOR day%= 1 TO days%
 320 INPUT£A,res$(day%)
 330 NEXT day%
 340 CLOSE£A
 350 ENDPROC
 360 :
 370 DEF PROCdisplay
 380 PROCtitle
 390 PROCbox
 400 PROChisto
 410 ENDPROC
 420 :
 430 DEF PROCtitle
 440 CLS:PRINT TAB(0,0);"Display for ";n$
 450 PRINTTAB(57,0);"Dates: ";date$;TAB(0,1);"Protocol: ";prot$;TAB(57,1);"Star
t time: ";time%
 460 ENDPROC
 470 :
 480DEF PROCbox
 490x%=80
 500FOR y%=896 TO (896-(64*days%)) STEP-64
 510MOVE x%,y%:DRAW x%+1152,y%
 520NEXT y%
 530y%=896
 540FOR x%=80 TO 1239 STEP 576
 550MOVE x%,y%:DRAW x%,y%-(64*days%)
 560NEXT x%
 570FOR x%= 80 TO 1232 STEP 48
 580MOVE x%,y%:DRAW x%,y%+16
 590NEXT x%
 600FOR y%= 1 TO days%
 610PRINT TAB(0,y%*2+2);y%
 620 NEXT y%
 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 ENDPROC
 790 :
 800 DEF PROCdraw
```

```
  810 d$=res$(day%+right%)
  820 FOR iter%=1 TO 144
  830 MOVE 4*iter%+x%,y%
  840 i$=MID$(d$,iter%,1)
  850 n%=ASC(i$)
  860 IFn%>60 THEN n%=60
  870 DRAW 4*iter%+x%,y%+(n%)
  880 NEXT iter%
  890 ENDPROC
  900 :
  910 DEF PROCerror
  920 CLOSE#0
  930 REPORT: PRINT ERL:PRINT ERR
  940 STOP
  950 ENDPROC
  960DEF PROCScreenDumpAssemble
  970 xpointlo=&70:xpointhi=&71:ypointlo=&72:ypointhi=&73:pixelvalue=&74:printer
byte=&75:bitcount=&76:mode0=&77:step=&78:OSWRCH=&FFEE:OSWORD=&FFF1:OSBYTE=&FFF4
  980FOR PASS=0TO3 STEP3
  990P%=&A00: [OPT PASS
 1000.ScreenDump LDA#&FF:STAypointlo:LDA#&3:STAypointhi
 1010LDA#0:STAmode0:LDA#4:STAstep:LDA#135:JSROSBYTE:TYA:BNELineGap:INCmode0:LSRs
tep
 1020.LineGap LDA#27:JSRPrinter:LDA#65:JSRPrinter:LDA#8:JSRPrinter
 1030.NewLine LDA#0:STAxpointlo:STAxpointhi
 1040LDA#27:JSRPrinter:LDAmode0:BEQModeAbove0:LDA#76:JSRPrinter:LDA#128:JSRPrint
er:LDA#2:JSRPrinter:BNENewColumn
 1050.ModeAbove0 LDA#75:JSRPrinter:LDA#64:JSRPrinter:LDA#1:JSRPrinter
 1060.NewColumn LDA#8:STAbitcount
 1070.ReadPixel LDX#xpointlo:LDY#0:LDA#9:JSROSWORD
 1080CLC:LDApixelvalue:BEQSetPrinterByte:SEC
 1090.SetPrinterByte ROLprinterbyte
 1100LDAypointlo:SEC:SBC#4:STAypointlo:BCSCheckColumnEnd:DECypointhi
 1110.CheckColumnEnd DECbitcount:LDAbitcount:BNEReadPixel
 1120.Print LDAprinterbyte:JSRPrinter
 1130.NextColumn CLC:LDAstep:ADCxpointlo:STAxpointlo:BCCCheckLineEnd:INCxpointhi
 1140 .CheckLineEnd LDAxpointhi:CMP#5:BEQEndLine
 1150.ColumnTop LDA#32:CLC:ADCypointlo:STAypointlo:BCCNewColumn:INCypointhi:BCSN
ewColumn
 1160.EndLine LDA#10:JSRPrinter
 1170.CheckEnd LDAypointhi:BMIEnd:JMPNewLine
 1180.End LDA#12:JSRPrinter:LDA#27:JSRPrinter:LDA#64:JSRPrinter:RTS
 1190.Printer PHA:LDA#1:JSR&FFEE:PLA:JSR&FFEE:RTS
 1200]NEXT PASS
 1210ENDPROC
```

```
  10REM PERIODOGRAM ANALYSIS
  20PROCScreenDumpAssemble
  30REM USE CALL &A00 FOR SCDUMP
  40MODE128
  50PRINTTAB(2,2)"This program is PER32"TAB(2,3)"_____"
  60PRINTTAB(2,6)"The function of this program is to analyse the locomotor data
 from the"TAB(2,8)"32 chan. recording system.The basis of this is the PERIODOGRA
M statistical"
  70PRINTTAB(2,10)"test.You will be asked the name of the file the data to be a
nalysed comes"TAB(2,12)"from and then the total length of that file-normally 14
 days.The "
  80PRINTTAB(2,14)"begining and end of the data to be analysed is defined by an
 interval"TAB(2,16)"number.These numbers refer to the 10min intervals the data w
as recorded"
  90PRINTTAB(2,18)"in.Each day has 144 such 10min intervals, therefore if you w
ant the "TAB(2,20)"first 2 days analysed then your first and last intervals are
1 and 288"TAB(2,22)"respectively."
 100PRINTTAB(2,24)"The periods that the progam checks the data for are also def
ined in these"TAB(2,26)"10min intervals-ie a period of 144=24hrs,138=23hrs etc."
 110PRINTTAB(20,30)"PRESS SPACE BAR WHEN READY"
 120REPEAT UNTIL GET=32
 130CLS:INPUT"Name of file to be analysed? "n$
 140INPUT"Total number of days?"days%
 150 INPUT"Start iter no? "start%
 160 INPUT"End iter no?" end%
 170DIM D%(2016)
 180DIM res$(15)
 190PROCrandomize
 200PROCsetscanspeed
 210CLS
 220pass=0
 230REPEAT
 240pass=pass+1
 250IF pass=1 GOTO400
 260CLS:PRINTTAB(3,0)"At this point the actual data is being loaded in order to
 be analysed."
 270 A=OPENIN(n$)
 280FOR day%=1 TO days%
 290 INPUT£A,res$(day%)
 300NEXT day%
 310CLOSE£A
 320FOR day%=1 TO days%
 330d$=res$(day%)
 340FOR iter%=1 TO 144
 350int%=(day%*144)-144+iter%
 360i$=MID$(d$,iter%,1)
 370D%(int%)=ASC(i$)
 380NEXT iter%
 390NEXT day%
 400 FOR P=B TO E
 410FOR I=1 TO P:F(I)=0:NEXT
 420PROCformestimate
 430IF speed=1 THEN 470
 440PROCdrawformest
 450IF GET=32 THEN VDU2:CALL&A00:VDU3
 460PROCprintformest
 470V(P)=V
 480IF pass=1 THEN V1(P)=V(P)
 490NEXT P
 500UNTIL pass=2
 510PROCfindvarmax
 520PROCconflimits
 530pass=0
 540MODE128
 550REPEAT
 560pass=pass+1
 570IF pass=1 GOTO 590
 580FOR I=B TO E:V1(I)=V(I):NEXT I
 590IF E-B=0 THEN 630
 600PROCdrawpergram
 610IF GET=32 THEN VDU2:CALL&A00:VDU3
 620PROCprintperio
 630UNTIL pass=2
 640P=permax:speed=2
 650PROCformestimate
 660PROCdrawformest
 670IF GET=32 THEN VDU2:CALL&A00:VDU3
 680PROCprintformest
 690CLOSE£0
 700END
 710:
 720DEF PROCScreenDumpAssemble
 730 xpointlo=&70:xpointhi=&71:ypointlo=&72:ypointhi=&73:pixelvalue=&74:printer
byte=&75:bitcount=&76:mode0=&77:step=&78:OSWRCH=&FFEE:OSWORD=&FFF1:OSBYTE=&FFF4
```

```
  740FOR PASS=0TO3 STEP3
  750P%=&A00: [OPT PASS
  760.ScreenDump LDA£&FF:STAypointlo:LDA£&3:STAypointhi
  770LDA£0:STAmode0:LDA£4:STAstep:LDA£135:JSROSBYTE:TYA:BNELineGap:INCmode0:LSRs
tep
  780.LineGap LDA£27:JSRPrinter:LDA£65:JSRPrinter:LDA£8:JSRPrinter
  790.NewLine LDA£0:STAxpointlo:STAxpointhi
  800LDA£27:JSRPrinter:LDAmode0:BE0ModeAbove0:LDA£76:JSRPrinter:LDA£128:JSRPrint
er:LDA£2:JSRPrinter:BNENewColumn
  810.ModeAbove0 LDA£75:JSRPrinter:LDA£64:JSRPrinter:LDA£1:JSRPrinter
  820.NewColumn LDA£8:STAbitcount
  830.ReadPixel LDX£xpointlo:LDY£0:LDA£9:JSROSWORD
  840CLC:LDApixelvalue:BE0SetPrinterByte:SEC
  850.SetPrinterByte ROLprinterbyte
  860LDAypointlo:SEC:SBC£4:STAypointlo:BCSCheckColumnEnd:DECypointhi
  870.CheckColumnEnd DECbitcount:LDAbitcount:BNEReadPixel
  880.Print LDAprinterbyte:JSRPrinter
  890.NextColumn CLC:LDAstep:ADCxpointlo:STAxpointlo:BCCCheckLineEnd:INCxpointhi
  900 .CheckLineEnd LDAxpointhi:CMP£5:BE0EndLine
  910.ColumnTop LDA£32:CLC:ADCypointlo:STAypointlo:BCCNewColumn:INCypointhi:BCSN
ewColumn
  920.EndLine LDA£10:JSRPrinter
  930.CheckEnd LDAypointhi:BMIEnd:JMPNewLine
  940.End LDA£12:JSRPrinter:LDA£27:JSRPrinter:LDA£64:JSRPrinter:RTS
  950.Printer PHA:LDA£1:JSR&FFEE:PLA:JSR&FFEE:RTS
  960]NEXT PASS
  970ENDPROC
  980:
  990DEF PROCsetscanspeed
 1000CLS
 1010INPUT"Legend for Diagrams? "l$
 1020PRINT"What is the shortest period to test for  "
 1030INPUT B
 1040PRINT"What is the longest period to test for  "
 1050INPUT E
 1060DIM F(E),V(E),V1(E)
 1070PRINT"Select 1 for Fast,2 for Slow route.":speed=GET:speed=speed-48:PRINT s
peed
 1080ENDPROC
 1090:
 1100DEF PROCformestimate
 1110CLS:PRINTTAB(0,0)"Calculating periodicity=";P
 1120FOR U=1 TO P
 1130FOR Y=U TO (end%-start%) STEP P
 1140X=D%(Y+ start%)
 1150F(U)=F(U)+X
 1160NEXT
 1170NEXT
 1180reps=(end%-start%) DIV P:rem=(end%-start%) MOD P
 1190FOR I=1 TO rem
 1200F(I)=F(I)/(reps+1)
 1210NEXT I
 1220FOR I=rem+1 TO P:F(I)=F(I)/reps:NEXT
 1230REM Select Highest F(I)
 1240IF speed=1 THEN 1300
 1250Z=F(1)
 1260FOR I=2 TO P
 1270X=F(I)
 1280IF X>Z THEN Z=X
 1290NEXT I
 1300REM CALC OF MEAN AND VARIANCE
 1310M=0
 1320FOR I=1 TO P:M=M+F(I):NEXT I
 1330M=M/P
 1340V=0
 1350FOR I=1 TO P:V=V+((F(I)-M)^2):NEXT I
 1360V=V/(P-1)
 1370ENDPROC
 1380:
 1390DEF PROCrandomize
 1400CLS
 1410PRINT "PUT IN DISC, PRESS SPACE"
 1420REPEAT UNTIL GET=32
 1430PRINT"At this point the data is being loaded to be randomised in order to p
roduce the confidence limits on the periodogram."
 1440A=OPENIN(n$)
 1450FOR day%=1 TO days%
 1460INPUT£A,res$(day%)
 1470NEXT day%
 1480CLOSE£A
 1490FOR day%=1 TO days%
 1500d$=res$(day%)
 1510FOR iter%=1 TO 144
 1520int%=(day%*144)-144+iter%
```

```
1530i$=MID$(d$,iterX,1)
1540D%(intX)=ASC(i$)
1550NEXT iterX
1560NEXT dayX
1570FOR iterX=1 TO (daysX*144)-2
1580YX=D%(iterX)
1590XX=iterX+RND((daysX*144)-iterX)
1600D%(iterX)=D%(XX)
1610D%(XX)=YX
1620NEXT iterX
1630ENDPROC
1640:
1650DEF PROCdrawformest
1660CLS
1670MOVE 100,100
1680DRAW 100,924:DRAW 1180,924:DRAW 1180,100:DRAW 100,100
1690MOVE 100,100+(F(P)*840/Z)
1700FOR I=1 TO P:GOSUB1980:DRAW X,Y:NEXT I
1710average=100+(M*824/Z):MOVE 100,average:PLOT 21,1180,average
1720VDU5
1730IF P>50 step=5 ELSE step=2
1740 FOR I=0 TO P STEP step
1750GOSUB1980
1760MOVE X,80:DRAW X,100
1770MOVE X-16,76
1780L$=STR$(INT(((I/10-INT(I/10))*10)+.5))
1790PRINT L$
1800NEXT
1810MOVE 400,40:PRINT"PERIOD PHASE"
1820MOVE 100,970:PRINT"FORM ESTIMATE,P= ";P;"  from ";1$
1830IF pass<>1 THEN 1850
1840MOVE 900,970:PRINT"RANDOMIZED"
1850FOR I=0 TO Z STEP2
1860Y=100+(I*824/Z)
1870MOVE 80,Y:DRAW100,Y
1880NEXT
1890A$="AMPLITUDE"
1900FOR I=1 TO 9
1910MOVE 10,I*50+300:PRINT MID$(A$,10-I,1)
1920NEXT
1930mean$=STR$((INT((M)*100))/100)
1940MOVE 1200,average+16:PRINT LEFT$(mean$,4)
1950VDU4
1960ENDPROC
1970:
1980Y=100+(F(I)*824/Z):X=100+(I*1080/P)
1990RETURN
2000:
2010DEF PROCprintformest
2020CLS
2030PRINT"PRINT OUT FORM ESTIMATE VALUES?  (Y/N)  "
2040INPUTA$:IF A$="N" THEN ENDPROC
2050PRINT;"PHASE","REPL.","AMPL."
2060FOR I=1 TO P:PRINT;I,STR$(reps+1),LEFT$(STR$((INT(F(I)*100))/100),4):NEXT
2070PRINT"PERIOD=";P;"MEAN=";M;"VARIANCE=";V
2080IF GET=32 THEN VDU2:CALL&A00:VDU3
2090ENDPROC
2100:
2110DEF PROCfindvarmax
2120varmax=0
2130FOR I=B TO E
2140IF V(I)>varmax THEN  varmax=V(I)
2150IF V(I)=varmax THEN permax=I
2160NEXT I
2170ENDPROC
2180:
2190DEF PROCdrawpergram
2200CLS
2210MOVE 100,100:DRAW 100,924:DRAW 1180,924:DRAW 1180,100:DRAW 100,100
2220I=B
2230GOSUB2540
2240MOVE X,Y
2250FOR I=B+1 TO E:GOSUB2540:DRAW X,Y:NEXT
2260VDU5
2270step=1:IF E-B>10 THEN step=2:IF E-B>20 THEN step=5:IF E-B>50 THEN step=10
2280FOR I=B TO E STEP step:GOSUB2540:MOVE X,80:DRAW X,100:MOVE X-26,76:PRINT ST
R$(I):NEXT
2290FOR I=0 TO varmax STEP 2
2300Y=100+(I*824/varmax)
2310MOVE 80,Y:DRAW 100,Y
2320NEXT
2330MOVE 20,Y+16:PRINT STR$(I-2)
2340A$="VARIANCE "
2350FOR I=1 TO 9
```

```
2360MOVE 10,I*50+300:PRINT MID$(A$,10-I,1)
2370NEXT
2380MOVE 100,970:PRINT"PERIODOGRAM,P=";B;" TO ";E,1$
2390MOVE 400,45:PRINT"PERIOD LENGTH IN TEN MINUTE INTERVALS"
2400IF pass<>1 THEN 2420
2410MOVE 900,970:PRINT"RANDOMIZED"
2420REM IF pass<>2 THEN 2660
2430y1=100+(Y1*824/varmax)
2440y2=100+(Y2*824/varmax)
2450y3=100+(Y3*824/varmax)
2460y4=100+(Y4*824/varmax)
2470MOVE 100,y1
2480PLOT 21,1180,y2:PRINT"95%"
2490MOVE 100,y3:PLOT 21,1180,y4
2500VDU4
2510SOUND 1,-15,90,20
2520ENDPROC
2530:
2540Y=100+(V1(I)*824/varmax)
2550IF pass=1 AND Y>924 THEN Y=924
2560X=100+((I-B)*1080/(E-B))
2570RETURN
2580:
2590DEF PROCprintperio
2600CLS
2610PRINT"PRINT OUT PERIODOGRAM VALUES? (Y/N) "
2620INPUTA$:IF A$="N" THEN ENDPROC
2630PRINT"PERIOD","VARIANCE"
2640FOR I=B TO E:PRINT;I,(INT(V1(I)*100))/100:NEXT
2650IF GET=32 THEN VDU2:CALL&A00:VDU3
2660ENDPROC
2670:
2680DEF PROCconflimits
2690sumx=0:sumy=0:ssx=0:ssy=0:sxy=0:n=(E-B)+1
2700FOR I=B TO E
2710sumx=sumx+I
2720sumy=sumy+V1(I)
2730ssx=ssx+I^2
2740ssy=ssy+V1(I)^2
2750sxy=sxy+V1(I)*I
2760NEXT I
2770mnx=sumx/n:mny=sumy/n
2780cxx=ssx-sumx^2/n
2790cyy=ssy-sumy^2/n
2800cxy=sxy-(sumx*sumy)/n
2810b=cxy/cxx
2820a=mny-b*mnx
2830resss=cyy-cxy^2/cxx
2840resms=resss/(n-2)
2850IF n=3 T=12.706
2860IF n=4 T=4.303
2870IF n=5 T=3.182
2880IF n=6 T=2.776
2890IF n=7 T=2.571
2900IF n=8 T=2.447
2910IF n=9 T=2.365
2920IF n=10 T=2.306
2930IF n=11 T=2.262
2940IF n=12 T=2.228
2950IF n>12 T=2.179
2960IF n>14 T=2.145
2970IF n>16 T=2.120
2980IF n>18 T=2.101
2990IF n>20 T=2.086
3000IF n>22 T=2.064
3010IF n>26 T=2.042
3020IF n>32 T=2.021
3030d=T*SQR(resms)
3040Y1=(a+d)+b*B
3050Y2=(a+d)+b*E
3060Y3=a+b*B
3070Y4=a+b*E
3080ENDPROC
3090:
```