Studies on the Circadian Locomotor Activity Rhythm in the Blow Fly, *Calliphora vicina*

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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

A study of circadian rhythmicity of the blow fly, *Calliphora vicina*, identified the role of locomotor activity as the 'hands' of the clock system, therefore providing a reliable gauge for the 'black box' approaches applied in the present investigation. Observations in constant conditions confirmed that the circadian rhythmicity of *C. vicina* is under the control of a self-sustained oscillatory system which is temperature-compensated and free-runs in constant darkness (DD) with a mean period length of 22.68 h. This, however, lengthened to a value greater than 24 h in continuous light (LL) below about 0.03 Wm^{-2}; above this intensity, locomotor activity became arrhythmic. Phase response curves (PRCs) for 1 h light pulses and temperature steps further described the periodic changes of sensitivity of such oscillatory system to these two main environmental Zeitgebers. The phase-shifts required for entrainment to a 24 h light/dark cycle were shown to be predictable by the light pulse PRC.

The splitting of the activity rhythm into two or more components, spontaneous or induced by light stimuli, provided strong evidence that the circadian rhythm of *C. vicina* is under the control of a coupled multioscillatory system. The attempt to interpret the observation of multi-components led to the development of a heuristic model which provided support for the multioscillatory hypothesis.

One of the main aims - a search for the anatomical locations of the photoreceptor(s) and the circadian pacemaker - was pursued via surgical treatments, including optic tract severance and complete removal of both optic lobes (lobectomy). Both treatments failed to destroy the activity rhythm or prevent entrainment to a

light/dark cycle. These results suggested that both the circadian pacemaker and the photoreceptor(s) are outside the optic lobes, possibly within the brain itself. Nevertheless, the elimination of exogenous responses to light stimuli observed in lobectomized flies suggested that the compound eyes and optic lobes may be involved in circadian photoreception.

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Glossary of Symbols and Terms

Aschoff's rule   The period of a free-running biological oscillation lengthens on transfer from DD to LL or with an increase in light intensity for dark-active (nocturnal) animals, but shortens for light-active (diurnal) animals.

Circadian time (Ct)   Time scale covering one full cycle of an oscillation.

Circadian rhythm   An endogenous oscillation with a natural period (τ) close to, but not necessarily equal to, that of a solar day (24 hour). The endogeneity of the oscillation can only be revealed if it is seen to persist in the absence of all environmental Zeitgebers.

Coupling   For a physical phenomenon, when two oscillators are connected and each affects the other **equally**, they are said to be coupled. In the context of circadian oscillation, the coupling between two oscillators indicates mutual interaction between the oscillators, therefore the driving force is not necessarily equal towards both directions.

Entrainment/Synchronisation   The coupling of a self-sustained oscillation to a Zeitgeber so that both adopt the same frequency ($\tau = T$) or that the frequencies are integral multiples. This is now adopted especially in referring to the synchronization of circadian rhythms.

Extraretinal photoreceptor   Indicates a photoreceptor other than the 'eye'. Other terms, such as 'extraocular' photoreceptor and 'extraoptic' photoreceptor are equivalent.

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Free-running period (τ) The natural period of an endogenous oscillator revealed in the absence of any Zeitgeber and at constant conditions.

Overt rhythm The measurable output of a circadian system, which is often exhibited as behavioural or physiological rhythms. The circadian locomotor activity rhythm in the adult blow fly *Calliphora vicina* is recorded in the present study.

Phase response curve (PRC) A plot of phase-shifts induced by a single perturbation (i.e. light pulse) at different circadian phases of an oscillator in free-run.

Range of entrainment Range of frequencies within which a biological oscillation can be entrained by a Zeitgeber. For circadian rhythms of most organisms, the range of entrainment about 21 - 27 hours.

Subjective day The first half of the circadian cycle (Ct0 - Ct12) where 'day' normally occurs.

Subjective night The other half of the circadian cycle (Ct12 - Ct24) where 'night' normally occurs.

Temperature compensation One of the most distinct properties found in most circadian systems, which suggests the apparent 'independence' of circadian period (τ) to temperature is gained by opposing processes with different temperature coefficients ($Q_{10}s$).

Temperature coefficients (Q_{10}s) Ratio of the period at one temperature to the period at a temperature 10° higher. When referred in the study of circadian rhythmicity, these are calculated as: period at $(t - 10)°$ / period at $t°$.

Transient(s) Cycles of non-steady-state following perturbation by a external stimulus before entrainment is achieved.

Zeitgeber/entraining agent The forcing oscillation which entrains a biological oscillator. In the natural environment the most important Zeitgeber is the daily light/dark cycle.

Wm^{-2} Measurement of irradiance. 1 Lux = 0.00146 Wm^{-2}

LD light/dark cycle

LL continuous light

DD constant darkness

τ natural period of a biological oscillator as revealed in free-running conditions

T period of Zeitgeber (i.e. LD 12:12, T=24 h)

ψ phase angle or phase relation

α activity time

ρ rest time

$+\Delta\phi$ advance phase-shift

$-\Delta\phi$ delay phase-shift

Q_{10} temperature coefficient

Ct circadian time

Zt Zeitgeber time

Chapter 1

Introduction

Part I.

General Introduction of Biological Rhythms

The earth's fluctuating environment plays an important role on the life style of almost all organisms living within its sphere. Among all the environmental changes, caused by planetary rotation, some appear to be rhythmic with a certain length of period for a full cycle, e.g. the daily light-dark cycle (24 hours), lunation (~29.4 days), the tidal cycle (~12.4 hours) and the seasonal cycle (a year). The physiological and biochemical events of most organisms are temporally organised with respect to these environmental cycles. Studies investigating phenomena involving such periodicity have been focused mainly on aspects including ontogeny, evolutionary history, adaptive significance, physiology, and biochemical regulation. Conceptual accounts concerning these aspects in the following paragraphs provide a general outline for the studies of biological rhythmicity.

1.1. Ontogeny and Evolutionary History of Biological Rhythmicity

The discovery of fossil corals provided evidence that the environmental periodicities have changed over the aeons. Physiological, morphological and

behavioural adaptations have been made by organisms through the evolutionary process to survive these changes. Naturally, the ability to identify environmental changes has become the focal point for organisms in their response to the environmental challenges. Tracing evolutionary history back to the beginning of life, failure to anticipate the environmental periodicities could have led to the extinction of many species. Such natural pressures, enforced on almost all organisms, emphasise the importance of the ability to anticipate periodic events, such as daylight, low tide, full moon and winter, in order to counteract the unfavourable periods and exploit the favourable ones. In response to natural selection, organisms have developed regulatory systems that function primarily to harmonise the environmental periodicities with appropriate, periodic alterations in physiology, biochemistry and behaviour. As Davis (1981) pointed out: "the ontogeny of circadian rhythms, as a set of biological questions, must, however, go beyond a simple cataloguing of many rhythms and their ontogenic changes which, although well documented as an emergence of rhythmicity during development, is indicated only by the scarcity of work on ageing and on changes that occur throughout the life of an organism." Progress in understanding the nature of temporal organisation and its ontogeny will depend on a greater knowledge of the circadian system, particular pacemaker mechanisms and the mechanisms of coupling among oscillators.

The rhythmicity observed in plants and animals commonly persists with no less vigour in laboratory constant conditions, therefore strongly implying its endogenous origin. Thus, these so called endogenous rhythmicities have become an important feature of almost all organisms. It is not surprising that the endogenous rhythmicities

have been built into the organisms' genome and are inherited like any other physiological characteristic (Brady, 1982a; Saunders, 1977). Working on circadian rhythms in the blow fly *Calliphora vicina*, Kenny (1989) suggested that developmentally, light or rather the transition between light and dark was not necessary for setting the oscillator into motion. This could be another indication of the endogeneity of such rhythmicity. The collective statement Dunlap (1992) drew to describe the fundamental nature of the biological time-measuring system - the cellular machinery that regulates the biochemical process over the course of a day is known as the biological clock, and its output as a circadian rhythm - says it all.

To generate such adaptive temporal programmes, the regulatory system must possess both the ability to register the passage of external time accurately and a mechanism to synchronise the internal programme to local time. Examining existing species, these survivors appear to have evolved successful strategies to predict the environmental periodicities as well as to utilise certain environmental changes (e.g. light intensity, temperature) as Zeitgebers. In general, synchronisation appears to be the most common and efficient strategy developed to cope with the fluctuating environment. Hence, a relationship has been established so that the environmental periodicities do not force an oscillation on the living system, instead, they merely serve to entrain an oscillation that is fully autonomous and innate to the organism concerned (Pittendrigh, 1965). The establishment of a specific, stable phase relationship between the endogenous oscillation and the environmental cycle sets the internal clocks to the local time, hence endogenous rhythms entrain to the

corresponding environmental Zeitgeber so that their periods become exactly equal to that of the Zeitgeber.

1.2. The Discovery and Recording of Endogenous Rhythmicity

The existence of rhythms was discovered when observing behavioural or physiological activities under natural environmental conditions. However these observations provided little information about the endogenous or exogenous nature of such rhythmicity. Early researchers must have presumed the existence of an endogenous oscillation which functions as a time-measuring system within an organism thereby governing biological processes and behaviours which appear to be rhythmic. Such cyclic processes in living organisms have been termed 'biological rhythms'. Since biological rhythms have been observed in organisms as diverse as unicellular organisms, plants and animals, it is not surprising to find the frequency spectrum is so broad that it ranges from some milliseconds to several years.

The natural period of the endogenous oscillation will only become manifest when the system is uncoupled from the environmental periodicities, hence, the rhythm is allowed to freerun. In practice, organisms are isolated and placed at constant temperature and in continuous darkness (DD) or constant light (LL) with all other possible Zeitgebers excluded. Under such conditions, the observed periodicity persists with a natural period (τ) close to, but usually deviating slightly from, the cycles to which it was previously synchronised. Numerous observations obtained under such conditions have led to the conclusion that the endogeneity of the

oscillation can only be revealed when it is seen to persist in the absence of environmental Zeitgebers.

Interest in biological rhythms can be traced back for more than 2500 years to the Greek poet, Archilochus who 'recognised' that rhythms governed human activity (in Aschoff, 1981). The term 'living clock' was first used by the French student, J. J. Virey to describe daily rhythms activity (in Aschoff, 1981). Records of these undoubtedly inbuilt 'rhythms of life' were first obtained in 1729 by the French astronomer De Mairan who was astonished by the persistence of the daily leaf movements of *Mimosa* plants in constant darkness (in Saunders, 1977). Experiments that demonstrated the fact that daily rhythms of activity are commonly controlled by endogenous circadian oscillators can be traced back to the work of Darwin (in 1880) who devoted a book to *'The Power of Movement in Plants'* (in Saunders, 1977). Two centuries after the first observation of endogenous rhythmicity, Anthonia Kleinhoonte, working on the plant *Canavalia*, discovered the periodicity, in constant conditions, deviates from 24 h (in Daan, 1982). However, the study of biological rhythms did not arouse much interest until the 1930s, when researchers became aware of the comprehensive importance of endogenous oscillations in the timing of biological processes. However, it is only in the last few decades that real progress has been made in the analysis of such rhythms, when, a large body of compelling experimental evidence has been obtained to support the idea that self-sustaining oscillations play an important role in the control of behavioural and physiological activities. Such internal clock systems control a vast array of periodicities and among

all the most frequently studied being .leaf movements of plants, the sleep-wake activity of mammals and the locomotor activity of insects.

1.3. Endogenous Rhythmicities - some general and well studied aspects

Like their exogenous counterparts, endogenous rhythms are constantly modulated by the fluctuating environment; this increases the difficulty in uncovering their characteristics in natural conditions. This situation has resulted in the demand for techniques and devices to record biological rhythms under laboratory conditions. Activity rhythms so recorded, with all environmental Zeitgebers excluded, can thereby be analysed for the study of endogenous rhythms. In the early years, the limitations of techniques and equipment restricted the recording of rhythms to those involving behavioural activities. In general, spontaneous locomotor activities such as running (walking), swimming and flight have been studied most commonly, because of the simplicity of recording such activity. It was only after the development of more advanced techniques that the recording of physiological rhythms was made possible. Thus, identification of some general characteristics has been made among the abundant activity rhythms collected from various organisms over the years. The endogenous rhythmicity has been found to originate from an innate basis and subsequently adapts itself to the environmental fluctuations. Driven by the self-sustained oscillatory system, the endogenous rhythms act as 'hands' of the internal clock and are valuable for their roles as gauges of the driving oscillation. Therefore, when a rhythm is seen to persist in the absence of any environmental cues, it indicates

the continuity of the clock function and illustrates the nature of the underlying system.

In almost all species investigated so far, the observed endogenous oscillations appear to exhibit the features one would expect to find in a system designed to measure the passage of time (Saunders, 1977). Such ability to keep time demands the possession of a time-measuring mechanism, namely, a clock. Man-made clocks mark the passage of time against equal segments of certain process occurring at a constant rate, for example, the fall of a weight on a chain (Brady, 1982b). As to living organisms, there are two possible means of keeping time - (1) rely on the exogenous periodicity of the environment, (2) or utilise the internal biochemical processes to measure time. The underlying mechanism of the latter is rather similar to that of a man-made clock, hence the time-measuring mechanism is driven by a clock that oscillates. The former, on the contrary, involves the utilisation of some non-oscillatory mechanism, such as an hour-glass type counter, which does exist in nature and has been proved to govern some of the periodic events in insects. These purely exogenous responses are merely direct reactions to the daily change of the environment and do not persist in the absence of environmental Zeitgebers. Such exogenous rhythms violate the basic characteristics of a circadian rhythm and will not be discussed in the present study.

Ever since man-made mechanical clocks were introduced in the fourteenth century, accuracy has been the most challenging task to keep good time. Inevitably, a biological clock must have undergone similar challenge before deriving its accuracy. The accuracy of the endogenous oscillator measured with respect to a particular

overt phase, such as the onset of activity, may be quite remarkable, particularly amongst higher animals (Saunders, 1977). It is not surprising, therefore, to find such accuracy a universal property of all biological clocks, since it is the prerequisite to restrict physiological and behavioural activities to a particular period of time. To fulfil this demand, a clock, man-made or biological, should be able to continue measuring time at the same rate under all conditions. Temperature compensation of the circadian rhythm is the best known example for such stability of the endogenous oscillatory system.

Although an endogenous oscillator is known as the 'internal clock' for its capacity to represent time on a physiological basis, there is no way to predict the phase of the external world from the phase of the internal oscillation. Hence, the internal oscillation would be of no use in coordinating the organism's activity with the periodically recurring events in the external world (Brady, 1982b). In order to have particular events performed at suitable times of the day, it is important that the internal clock synchronises its pace to the local time. Thus, the time-measuring system must contain a mechanism which serves as the link between the endogenous oscillation and the environment. Such a mechanism can detect certain types of environmental change and provide this information to the oscillator/pacemaker, thereby aiding the process of synchronisation. Concerning the functions of a biological clock, the following components are essential to assemble a clock that keeps good time all year round.

1) Pacemaker(s): the master regulating oscillator(s);

2) The input of local time information: a mechanism which includes both the detecting system (receptors) and the linkage to the pacemaker; and

3) The output of physiological or behavioural rhythms: the coupling that links the pacemaker to the 'hands' governing the performance which could be measured as overt rhythms.

Eskin (1979) proposed a conceptual model (Fig. 3.1.1.B) for the circadian system, in an attempt to illustrate the underlying mechanism of a circadian clock. Many of the components, both internal and external, are yet to be described, but this model outlines the basic linkages between the major components of the circadian system. The actual functioning mechanism is, of course, far more complicated than this simplified diagram suggests. However, it is useful as the foundation when designing 'blackbox' approaches.

1.4. Selective Advantages (Adaptive Significance)

While endogenous rhythmicities reflect their adaptation to the periodicities of the environment, many other activities, such as *Drosophila* larval pupariation, are not clock-controlled (Pittendrigh and Skopik, 1970). It has been noted that these rhythmic processes are of no ecological significance but only the reflections of a mechanism controlling some other adaptive rhythm. The existence of such phenomena leads to the assumption that the endogenous rhythmicity is performed only where it presents some selective advantages to the species concerned. In order to evaluate the existence of such endogeneity, it is important to take into

consideration the adaptive significance of the particular process that is to be fitted to the temporal order of the environment.

The adaptive significance of the regulatory system lies in two aspects - the restriction of these processes to a particular time of day, and providing a temporal framework for an internal organisation of various activities relative to each other (Pittendrigh, 1981). The idea of 'temporal organisation' outlines the configuration and provides feasible directions to the answer. When exposed to the environmental periodicity, the self-sustained oscillation adopts the period of the Zeitgeber and establishes a particular phase relationship to it. Therefore the oscillation achieves a steady-state relationship to the Zeitgeber and enables an organism to participate its activities into some kind of temporal order. Maintenance of such relationship enables a circadian clock to function as a device for the recognition of local time. 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 organisation of internal and external biological processes (Saunders, 1977).

The advantage of having an endogenous oscillator to control overt behavioural events rather than relying on direct (exogenous) responses to environmental signals lies in 'anticipation' and 'preparation' (Saunders, 1981). The former could be important in two folds: (1) interspecific relations, for example, the reduction of direct

competition between species feeding on the same food source; and (2) intraspecific relations - especially facilitation of contacts between the sexes during the reproductive period, the best example being the fruit fly *Dacus tryoni* (Tychsen and Fletcher, 1971). The latter allows organisms to initiate a slow physiological process prior to its occurrence so that the actual event can be performed at the 'right' time of the day or year. However, there are still some cases in which the selective advantage of a biological rhythm is not fully understood.

1.5. Circadian Rhythmicity

As the most important source of energy and the centre of the earth's rotation, the sun affects most life on earth, directly or indirectly. Daily changes in light and temperature have accompanied evolution throughout its course, and the forces of natural selection must always have operated differentially across day and night (Dann, 1982). Naturally, a solar day (24 h), both reliable and predictable, becomes the environmental periodicity to which most organisms synchronise their endogenous rhythms. Therefore, the 24-h rhythmicity has become an inseparable part of life on earth.

The term 'circadian rhythm', proposed by Halberg in 1959 (from Brady, 1982b), originally referred to any type of biological rhythm which had a period close to a solar day (24 hours). However, over the years, circadian rhythms have become defined as those which persist when all environmental periodicities are excluded, and in this free-running condition show a natural period which is close to but not necessarily equal to that of a solar day (24 hours) (Saunders, 1982). Hence, as all

other endogenous rhythms, the endogeneity of the circadian oscillation can only be revealed in the absence of all environmental cues.

Among all the endogenous rhythmicities, the best studied is the circadian rhythm which constitutes the main part of this thesis. The many characteristics a circadian rhythm exhibits, appear to be what is expected for an self-sustained oscillator. In other words, it possesses the properties one would expect for a biological clock (Saunders, 1977). Although most part of the nature of the circadian rhythm remains unknown, several general characteristics have been found shared by the majority of organisms. These characteristics are well documented and distinguish a circadian rhythm from a pure exogenous rhythm. The most distinct and notable property is their link with the cyclic changes of environment. Most other biological rhythms (e.g. heartbeat, spiracular opening and even some life-cycles) have no such temporal relationship with external cycles (Brady, 1974). The existence of such a link provides indication that could easily mislead to the assumption that circadian rhythms are merely direct responses to the environmental changes. However, this proposition failed to interpret the persistence of the circadian rhythm in constant conditions with a period close to 24 h. This characteristic has been applied as strong evidence to support the endogeneity of the circadian rhythmicity. One other feature that marks circadian rhythms off from other biological rhythms is their stability at different temperatures within the range for normal biological processes to take place. To sum up, among the many phenomena observed form various organisms, four prime characteristics have been applied to define the circadian rhythmicity.

(1) Circadian rhythms persist in the absence of external time cues with an endogenous period close to but never exactly the same as 24 h.

(2) Circadian rhythms are fairly accurate.

(3) Circadian rhythms are temperature compensated with a temperature coefficient (Q_{10}) approximately equal to 1.0.

(4) Circadian rhythms are fairly stable and will only entrain to environmental cycles within a narrow range, only a few hours from 24 h.

To demonstrate the characteristics stated above, a circadian system must consist of fundamental elements similar to any biological clock described previously. Hence, an endogenous oscillatory system, a mechanism linking the oscillator with the physiological processes it controls, and a photo-sensitive system through which the oscillator is entrained to the environmental light/dark cycle. In order to function practically, the underlying mechanism is surely not as simple as the possession of these three components. There is no doubt of the existence of a complicated mechanism which enables the mutual coupling between individual elements and results in a precise collective output thus ensuring the precision of a circadian rhythm. Despite the many theories and models proposed in attempts to interpret the underlying mechanism of a circadian system, the actual operation of the mechanism remains elusive. However the efforts devoted confirm one aspect of the underlying mechanism - the involvement in time measurement is nearly always mediated by circadian oscillations. The function of such particular pacemakers was predicted by Erwin Bünning as early as 1936, which is now firmly supported by experimental

evidence. Concerning the role they play in a circadian clock system for measuring environmental time, pacemakers function in two conceptually distinct ways:

(1) in providing for a proper phasing of the programme to the cycle of environmental change, they recognise local time; and

(2) in assuring an appropriately stable temporal sequence in the programme's successive events, they measure the lapse of time (Saunders, 1977).

Circadian rhythmicity has been observed as a universal phenomenon in living organisms at all levels (Rensing and Hardeland, 1990) including populations, organisms, organs or cells. The many common properties of circadian oscillators, observed at all levels, suggest that the clock is a single mechanism, preserved throughout evolution, which is capable of controlling all the different circadian functions (Roenneberg and Morse, 1993). Multicellular organisms seem to contain a whole population of circadian driving oscillations and an even more abundant array of driven rhythms which, in total, comprise the circadian system. In brief, the circadian systems in living organisms can be described as the outcome of an evolutionary adaptation to the 24 h time structure of the environment. Circadian timekeeping thus has profound consequences, not only ecologically, which is its adaptive function, but also behaviourally and physiologically (Dunlap, 1992). Broadly, it operates at three organisational levels: cell, tissue and whole animal - tissues depend on cells and whole animals depend on tissues, while behaviour can only be seen in whole animals.

1.6. The Fundamental Nature and Genetic Aspects of Circadian Rhythmicity

Early researchers (Pittendrigh, 1960; Roberts, 1960) had observed significant changes in the free-running period (τ) exhibited by individuals of the same species, and suggested that the range of τ is an expression of genetic influence. The idea is that functioning at a cellular level, the circadian clock gives out temporal information which acts as a signal to bring about changes in the behaviour of a cell, thus ensuring the occurrence of certain activities at appropriate times of the day. Dunlap (1993) made a more explicit assumption, in the context of circadian rhythms: clocks are built using proteins, the products of genes, and therefore the molecular gears and cogs of the biological clock can be isolated and studied through the identification of genetically altered strains with altered clocks. Directed by such a concept, the study of circadian rhythmicity involving genetic techniques has been focused primarily on the identification of the components responsible for the assembly/operation of the clock. Theoretically, to serve such purpose, genetics can be used as a fine dissecting tool and as a specific tag to determine what tissues are required for rhythmicity. Nevertheless, for years, analysis of biochemical mechanisms underlying circadian rhythmicity has been hampered by the apparent complexity of the system. This, as a consequence, resulted in the difficulty of designing definitive and unequivocal experiments aimed at identifying specific molecular events involved in the circadian clock mechanism. Among the many protocols involving the genetic approach, mutational analysis has been applied most frequently for it alters just one component of a complex system while keeping all of the other parts constant. In such a protocol, clock mutants with only one altered circadian parameter are isolated and analysed genetically, physiologically and biochemically in order to determine the biochemical

function encoded by the mutated gene. Strains bearing known mutations have been used, most extensively in *Drosophila* (Ewer *et al.*, 1988; Helfrich and Engelmann, 1983; Konopka and Benzer, 1971; Sehgal *et al.*, 1994; Wheeler *et al.*, 1993; Winfree and Gordon, 1977) and *Neurospora* (Aronson *et al.*, 1994; Dieckmann and Brody, 1980; Dunlap and Feldman, 1988; Gardner and Feldman, 1981; Loros *et al.*, 1989), to limit the number of different biochemical processes or pathways that might be required for the performance of rhythmicity. In the 1970s, attention was focused on the biochemical aspects of the circadian system, which consequentially brought about the proposition of a physiological model (Sweeney, 1974) suggesting that circadian rhythms were generated by a feedback mechanism.

Early research at the cellular and molecular levels of circadian systems was initiated in the attempt to answer three questions: What are the biochemical and molecular bases of the oscillator(s)? How is the oscillation, generated by the clock, dispersed within the cell to regulate metabolism and cell behaviour? And what is the mechanism underlying entrainment to an external Zeitgeber at a molecular level? The identification and isolation of clock genes in several organisms, together with results concerning feedback loops, have provided an answer to the first question. Further approaches to clock mutants that exhibit changes in τ , phase or sensitivity to environmental Zeitgebers have brought about better knowledge to the second question. As for the entraining mechanism, progress has been brought about by results of many recent reports, mainly in *Drosophila* (Edery *et al.*, 1994; Lee *et al.*, 1996; Myers *et al.*, 1996) and *Neurospora* (Crosthwaite *et al.*, 1995; Lakin-Thomas, 1992; Lakin-Thomas *et al.*, 1991). These experiments which involved the application

of various resetting factors (both photic and non-photic) resulted in the discovery of corresponding intracellular feedback cycles that are held responsible for the synchronization to the environmental light/dark cycle. Working on rats, Amir and Stewart (1996) suggested that, through an associative learning process, a non-photic cue which mimics the effect of light on the cellular elements within the SCN pacemaker of the rat can bring about entrainment of circadian rhythms. All this progress at the cellular level has enabled researchers to consider the three questions with a more integrated view.

In addition to his well known contributions to the understanding of the properties and physiology of circadian oscillators, Pittendrigh carried out some of the initial genetic selection experiments involving developmental rhythms. However, research work concerning this aspect did not attract much attention until Konopka and Benzer (1971) isolated three period mutants in *D. melanogaster*, which affect both the periodicity of eclosion and adult locomotor activity rhythms. This was followed by a series of reports, either on the same mutants or mutants of other species. Bruce (1972) recognised the single-gene that effects the circadian period of *Chamydomonas reinhardi*. Feldman and Hoyle (1976) used the frequency mutant in *Neurospora crassa* to distinguish between the 'clock' and the 'hands of the clock'. In plants, clock mutants were reported in *Arabidopsis thaliana* (Millar *et al.*, 1995). All these clock mutants have been used to identify genes known to affect the function of the clock and most results indicated that the circadian rhythm is coded for in the genome. Genes so identified include *Drosophila* period gene (*per*) (Konopka and Benzer, 1971), *Neurospora* frequency gene (*frq*) (Feldman and Hoyle, 1973) and the

golden hamster *Mesocricetus auratus* period gene (*tau*) (Ralph and Menaker, 1988). Results of extensive investigations on *Drosophila* single-gene mutations suggested that both period and phase are under genetic control and that gene products involved in maintenance of rhythmicity are also involved in determination of the phase of the rhythm (Konopka, 1981). More recent studies have produced evidence to support the hypothesis that *per* is a clock component (Edery *et al.*, 1994; Hardin *et al.*, 1990). Studies on *frq* (Aronson *et al.*, 1994; Crosthwaite *et al.*, 1995) also confirmed its role as encoding a central component in a circadian oscillator of the fungus. Therefore, the isolation of clock mutants and the availability of various genetic tools has given new insights into the study of circadian rhythms at both molecular and physiological levels.

Although genetic selection experiments have the advantage of producing mutants with altered properties to aid the study of basic principles used to organise the system, it is difficult to evaluate the contributions of the mutated genes to the observed changes in the system. Konopka (1979) proposed an alternative approach which involved chemical mutagenesis to induce mutations in single genes that affect basic properties of circadian oscillators, such as phase and periodicity. This approach provides a possible way of studying how a particular gene is involved in the operation of the oscillator. Furthermore, a mutated gene can act as a marker to indicate the location of the cells where the gene is expressed. For example, in higher organisms, clock mutants can be used to localise the pacemaker to specific organs or tissues and to identify specific cells or cell types that control various clock functions (Feldman, 1983). The single gene approach has already provided novel information concerning

the organisation of the circadian system controlling the timing of emergence and locomotor activity in *Drosophila* (Ewer *et al.*, 1988; Hardin *et al.*, 1990; Sehgal *et al.*, 1994; Vosshall *et al.*, 1994). Recently, great progress has been made in identifying genes controlled by the clock and in beginning to understand the mechanisms through which they are controlled. Considering the clock within the cell, observations are consistent with the suggestion that the fundamental feedback loop comprising the clock may be found at the level of intracellular regulation rather than intercellular communication (Dunlap, 1993). The increasing efforts devoted to this aspect will offer more possibilities for the understanding of clock mechanisms.

1.7. The Multioscillatory System

Circadian rhythms regulate a broad variety of physiological processes in organisms. Many functions of organisms, unicellular or multicellular, follow such a consistent daily periodicity, even in constant environmental conditions that it has been natural to postulate control by a single internal clock (Kohler and Fleissner, 1978). A decade ago, researchers still applied the result, obtained by McMurry and Hastings (1972) on the unicellular alga *Gonyaulax polyedra*, as an example to support the unitary circadian oscillatory system, which has been proved inadequate by Johnson and Hastings (1989). Although a single oscillator model is consistent with many aspects of circadian rhythms, particularly phase response curves, entrainment, and Aschoff's Rule (Gander and Lewis, 1979), other phenomena such as splitting of rhythms, spontaneous changes in free-running rhythms and after-effects remain unexplained. Moreover, circadian rhythms have been observed on multicellular

organisms, isolated tissues, and even single cells indicating the existence of numerous oscillators in a single organism.

Circadian activity rhythms sometimes split spontaneously into two or more components, each showing its free-running period, in constant laboratory conditions. This internal desynchronisation is considered as evidence that the circadian activity rhythm is the reflection of mutually coupled pacemakers; when the coupling is loose, the rhythm splits to express the temporal status of each self-sustained pacemaker. Pittendrigh (1960, 1967) reported a number of cases in nocturnal rodents in which, after prolonged constant illumination, the rhythm of locomotor activity split into two components. These components showed distinctly different frequencies and eventually desynchronised with each other at a new phase relation. Such findings indicate that several circadian oscillators control the overt rhythm of locomotor activity. The fact that such a phenomenon has been observed through a wide range of multicellular organisms led to the hypothesis of a multioscillatory system. This multioscillatory theory has been discussed from various points of view. One view suggested that a great variety of oscillators synthesise one overt rhythm (Pavlidis, 1969). A second hypothesis proposed a combination of two coupled oscillators with different properties controlling one overt rhythm (Pittendrigh *et al.*, 1958). The third hypothesis considered the multioscillatory system as a system with some equivalent oscillators each of which controls a separate overt rhythm (Hoffmann, 1971; Pittendrigh, 1960; Wever, 1973). Increasing evidence, mostly obtained in multicellular organisms, has suggested that the mechanism underlying the circadian rhythm is a multioscillatory system. Among the various approaches, applied on a

wide range of organisms to provoke internal desynchronisation, surgical treatment appears to be reported most frequently. In insects, examples of such studies are: cockroach, *Leucophaea maderae* (Page, 1978; 1981; Page *et al.*, 1977); mosquito, ground beetle, *Anthia sexguttata* (Fleissner, 1982); cricket, *Teleogryllus commodus* (Wiedenmann, 1983); and *Drosophila melanogaster* (Helfrich, 1987). Experimental results of studies on organisms from the unicellular alga, *Gonyaulax polyedra* (Johnson and Hastings, 1989; Morse *et al.*, 1994; Roenneberg and Morse, 1993), to plants (Hennessey and Field, 1992) and more complicated organisms, such as mammals, are available in favour of multioscillators governing the circadian system. In humans, internal dissociation and desynchronization of circadian rhythms were observed as clear indications of the multioscillatory system (Aschoff, 1969). In insects, this has been reported in *Leucophaea* (Page, 1983b), in *Sarcophaga* (Saunders, 1978), in *Blaps* (Köhler and Fleissner, 1978), in *Hemideina thoracica* (Christensen and Lewis, 1982) and in higher flies, *Calliphora stygia* (Waddell, 1984); *Drosophila melanogaster* (Dowse and Ringo, 1987; Helfrich, 1987), and the mosquito *Culex pipiens pallens* (Chiba *et al.*, 1993). Examples are also available in plants: stomatal opening and photosynthesis rhythms of bean plants (Hennessey and Field, 1992). This supportive evidence, therefore, leads to the dominant paradigm that most organisms, studied so far, exhibit multiple circadian rhythms under the regulation of a single oscillator or a group of tightly coupled oscillators that are possibly under the control of a 'master' oscillator. Theoretically, the next step should be carrying out sequential studies to determine the number of oscillators in a circadian system. This is, however, difficult both because the nature of these

oscillators is unknown and because different oscillators may be coupled with varying degrees of autonomy (Hennessey and Field, 1992). Nevertheless, some progress has been made on the marine alga *Gonyaulax polyedra* (Roenneberg and Hastings, 1991) in which the existence of two photoreceptors with distinct spectral responses, opposite τ-intensity relationships, observed in blue light and red light, were reported. This result led to the conclusion that in *G. polyedra*, light effects on period and on phase are mediated by different input pathways and are controlled by different mechanisms. Furthermore, studies at a cellular level have been providing ample evidence for the existence of multioscillator systems in the circadian makeup of organisms, which suggests the expression of circadian rhythms in an intact organism may depend to some extent on the coordination of oscillators in different cells and tissues. More recently, the multioscillatory model, Vilaplana *et al* (1995) proposed for the circadian system in rats, suggested that the oscillators may be grouped in sub-sets, each demonstrated slightly different frequencies distributed unimodally.

1.8. Clock Location

As described in many conceptual models (Eskin, 1979; Pittendrigh and Bruce, 1957) the circadian system consists of three major components, i.e. a central element which generates the near-24 h oscillation normally referred as the circadian pacemaker/oscillator, a pathway responsible for the distribuution of photic information to the pacemaker which is necessary for synchronisation to the environmental light/dark cycle, and the regulated system which is driven by the pacemaker to express the overt rhythms. In addition, these models also hypothesised

the mutual interactions between these elements. The search for the anatomical locations of the driving oscillators and the site of the photoreceptors, has been conducted as intensively as other aspects in the study of circadian rhythmicity. Surgical approaches have been applied most commonly when studying the anatomical locations of the circadian clock components. Theoretically, removal of a tissue which contains the pacemaker will result in arrhythmicity and the circadian rhythm should remain in the tissue itself when isolated from the rest of the body. Transplantation of such tissue will restore the rhythm to the recipient which was deprived of rhythmicity. The usual approach in the search for the relevant photoreceptors is to destroy or disconnect the candidate sites and observe whether the rhythm has been uncoupled from the Zeitgeber, i.e. the rhythm free runs in a light/dark cycle.

In the study of insect circadian system, enormous interest has been focused on the system controlling locomotor activity rhythms in cockroaches, because of their large size and ease of handling. In a series of papers which were amongst the first to claim the localisation of a driving oscillation in any animal system, Harker (1954; 1955; 1956; 1960) concluded that an autonomous endocrine clock in the suboesophageal ganglion controls locomotor activity in the cockroach *Periplaneta americana* and ocelli were the principal, if not the only, photoreceptor. These findings aroused great argument because subsequent researchers (Brady, 1967; Nishiitsutsuji-Uwo and Pittendrigh, 1968a, b; Roberts, 1966) failed to corroborate her results. Searches for the locations of circadian pacemakers and photoreceptors were then directed away from the suboesophageal ganglia to other parts of the central nervous system. In their series of experiments, Nishiitsutsuji-Uwo and Pittendrigh (1968a, b) obtained

contrasting evidence against Harker's claim by suggesting the bilaterally paired optic lobes as candidates for the circadian pacemaker in the cockroach. Since then, the optic lobes have been reported most frequently as circadian pacemakers (Fleissner, 1982; Loher, 1972; Page, 1982; 1983a, b; Tomioka and Chiba, 1986; Waddell *et al.*, 1990). In crickets, Tomioka and Chiba (1986) obtained conclusive evidence that the lamina-medulla complex, one of the main neuropiles comprising the optic lobes, contains a neural mechanism, i.e. a circadian pacemaker.

With respect to clock location, experimental results produced so far divide insects into two groups, depending on whether or not circadian locomotor rhythm persists after optic lobe ablation (bilobectomy). Species classified into the first group, i.e. with their circadian pacemakers located in the optic lobes, are mainly orthopteroid insects such as cockroaches, *Periplaneta americana* (Nishiitsutsuji-Uwo and Pittendrigh, 1968a, b), *Leucophaea maderae* (Page, 1978; Roberts, 1974), crickets, *Teleogryllus commodus* (Loher, 1972; Wiedenmann, 1983), *Gryllus bimaculatus* (Tomioka and Chiba, 1986; 1989) and the New Zealand weta, *Hemideina thoracica* (Waddell *et al.*, 1990). Similar results were obtained for a beetle, *Anthia sexguttata* (Fleissner, 1982). On the other hand, species that demonstrate no effect of bilobectomy on the persistence of the circadian rhythm are classified into the second group which includes moths and flies, for example, the mosquito *Culex pipiens* (Kasai and Chiba, 1987), the house fly, *Musca domestica* (Helfrich *et al.*, 1985) and the blow fly, *Calliphora vicina* (Cymborowski *et al.*, 1994). Experimental results indicated that the circadian pacemakers for locomotor rhythmicity of the blow fly, *C. vicina*, may lie within the brain tissue, and that the brain may also be the site for

extraoptic photoreception (Cymborowski *et al.*, 1994; Cymborowski and Korf, 1995). In other organisms, tissues so far identified as pacemakers are suprachiasmatic nuclei (SCN) of mammals, pineal gland of birds, and eyes of molluscs (see chapter 3 introduction). Efforts to localise the pacemaker resulted in the finding that in most animals circadian pacemakers are bilaterally represented in paired structures in the central nervous system.

Search for the relevant photoreceptors in insects has been directed to the ocelli (Harker, 1956), the compound eyes (Roberts, 1965; Nishiitsutsuji-Uwo and Pittendrigh, 1968a, b; Loher, 1972; Page *et al.*, 1977; Wiedenmann, 1983) and the extra-optic photoreceptor (particularly in the brain) (Dumortier, 1972). Moreover, bilobectomized blow flies (*C. vicina*) were reported to show no increase in activity near light-off, in comparison to the intact and sham-operated flies, which suggested that although compound eyes are not the principal or only photoreceptors for light entrainment in *C. vicina* they may still be involved in photoreception.

The complexity of the circadian system hampers the direct approach to the underlying mechanism as well as attempts to single out any portions of the system. Alternatively, the 'black box' approach has been applied commonly when studying circadian rhythmicity. In such an approach, knowledge of the concrete nature of the system is not essential, only the responses provoked by specific treatments are analysed. Decades of research on circadian systems have been based primarily on two different methods. One method is usually referred to as the formal analysis of rhythms, which describes the properties of both rhythms and the underlying mechanisms and finally aims at the development of adequate models of the system.

The other method is the physiological analysis of the underlying mechanisms, which aims to identify the anatomical locations of the components of the circadian system and to uncover the fundamental basis of the system at cellular level. The present study investigated both aspects of the circadian system in the blow fly *Calliphora vicina*.

. Part II.

Locomotor Circadian Rhythms - with special reference to insect circadian

locomotor activity rhythms

Although an overall concept of circadian rhythmicity was outlined in part I, a more

specific account, concerning especially the intensively studied aspects of insect

circadian rhythms, such as properties of a rhythm free-running in constant conditions

(constant darkness or constant dim light), temperature effects on locomotor activity

rhythms, the phase response curves (PRCs) and entrainment to 24 h light/dark cycles,

are given here in an attempt to build up both theoretical and experimental

background for the present study.

1.9. Circadian Rhythmicity in Insects

The study of circadian rhythmicity has been unified by the remarkable degree of

formal similarity among rhythms observed at all levels. However, the difficulty of

maintaining a consensus among researchers increased as the study of circadian

rhythmicity shifted from a descriptive to an analytical stage during the 1950s. The

identification and establishment of some general characteristics of circadian

rhythmicity have been interpreted as a reflection of the adaptive significance which, in

theory, can be found in any evolutionary process. Over the years, increasing evidence

has been produced to confirm that a broad variety of physiological and behavioural

phenomena are under the control of the circadian clock. In order to regulate events to

occur at the right time of the day or year, circadian clocks adapt themselves to take

account of the variation in day length with the changing seasons. Therefore, the overt rhythms so produced are usually restricted to particular times of a day. According to their daily active phase, which generally associates with raised locomotor activity or other physiological functions, insects are readily classifiable as diurnal, nocturnal or crepuscular. It is, therefore self-evident that circadian clocks play a significant role in the life of insects.

The behavioural responses of insects to various external stimuli were studied by earlier physiologists in relation to endogenous inputs from the physiological state of the animal (e.g. age, starvation), but only in the past few decades has this been extended to circadian time. In 1910, almost two centuries after the first experimental demonstration of an endogenous component, Forel reported the time sense in bees, which is probably one of the earliest reports on insect circadian rhythms. However, the work of Bünning (1935) on insect emergence rhythms is often regarded as a seminal study in insect clocks. Since then, studies have been focused on three general aspects:

1) The processes that circadian clocks control, and their adaptive significance.

2) The mechanism of entrainment.

3) The anatomical and physiological substrate for the generation of the oscillation.

Various strategies have been used to study the functioning of circadian rhythms. The 'black box' approach tries to draw conclusions concerning the clock mechanism by observing the reactions of the system after specific treatments. This approach has been used widely and the resulting models specify the ways in which light influences them (Johnsson and Karlsson, 1972; Pittendrigh and Dann, 1976a; Pittendrigh and

Dann, 1976b). Another, less successful, strategy is to follow up the physiological causes for observed circadian rhythms, with the expectation to arrive at the clock mechanism itself. Such results are frequently hampered by the difficulty to distinguish between the controlled process and the controlling clock mechanism. For example, the arrhythmicity observed after the removal or isolation of certain tissue could be interpreted as scarcity of the pacemaker or simply the result of a disconnection of the output pathway.

1.10. Insect Locomotor Activity Rhythms

In insects, circadian rhythms can be classified into three basic types, according to the nature of the rhythms observed: 1) behavioural rhythms: e.g. locomotor activity, feeding rhythms and sexual rhythms; 2) developmental rhythms: e.g. hatching rhythms, pupation rhythms and eclosion rhythms; 3) physiological rhythms: e.g. metabolic rhythms (Brady, 1974). Among such a broad variety, researchers have learnt to record locomotor activity for the measurement of insect circadian rhythms, mainly because of the simplicity of the recording and the minimal disturbance generated. These advantages have made locomotor activity the favourite parameter for the study of circadian rhythmicity.

In natural conditions, locomotor activity appears to be under the control of a self-sustained endogenous oscillation which is continually modulated by environmental fluctuations, mainly temperature and light/dark cycles. Studies on patterns of locomotor activity have not only provided evidence for the existence of such endogenous oscillation but also shown that the patterns of locomotor activity are a

reflection of the state of the underlying oscillation. This leads to the assumption that the daily rhythmicity of locomotor activity is the response of the internal clock to the external stimuli, hence the locomotor activity acts as the 'hand(s) of the clock'. Therefore, studying the hands of the clock at an individual level provides means of direct comparison with different outputs of the circadian system (Kenny and Saunders, 1991). However, like other organisms, the control of the temporal distribution of activity in insects is such a sophisticated process that one must be cautious when attempting to relate pattern or amplitude to the state of the clock (Pittendrigh, 1976). The only parameters of an overt rhythm that can be assumed as gauges of the state of the circadian oscillator are the period of the rhythm and its phase in steady-state (Page, 1985).

Although exceptions do exist, the field studies of Lewis and Taylor (1965) on 500 species of night flying insects suggested that enormous numbers of species are rhythmic in their locomotor activity. One of the first insect groups studied at an individual level was cockroaches (Harker, 1956). Their large size allows various recording devices to be effective and their role as pests made them obvious candidates for such an intensive study. Free-running rhythms have also been measured in the Coleoptera and Orthoptera, under a variety of constant conditions. In Diptera, extensive data are available mostly for *Drosophila*, studying the pupal eclosion rhythm at population level. Records of free-running locomotor activity rhythms at individual level have been obtained in *D. melanogaster* (Gillanders, 1993; Konopka and Benzer, 1971), *D. pseudoobscura* (Engelmann and Mack, 1978); the mosquitoes *Culex pipiens pallens* (Chiba, 1964), *C. pipens fatigans* (Wied) (Jones,

1976) and *Anopheles gambiae* (Jones *et al.*, 1967); the New Zealand weta *Hemideina thoracica* (Lewis, 1976); the blow flies *Phormia terraenovae* (Aschoff and von Saint Paul, 1982), *Calliphora stygia* (Waddell, 1984) and *C. vicina* (Kenny, 1989). Among the enormous number of locomotor activity rhythms recorded, patterns with single phases and sharp onsets are most commonly seen and perhaps the most convenient for the study of circadian rhythms, but it is by no means the only pattern of insect locomotor activity rhythm. The blow fly *C. vicina*, the species used in the present study, is one of the examples that show an unimodal diurnal pattern, with activity uniformly distributed throughout the light portion of the day. In the bimodal type, the second peak, in some cases, may be forced by the light-on signal (Jones *et al*, 1967; Brady, 1974), while in other cases, both peaks are truly endogenous and free run in constant conditions (Chiba, 1964). Other types of circadian activity pattern have been observed in Diptera, e.g. the tsetse fly, which restricts its locomotor activity to very brief flights, separated by long intervals (from Brady, 1982).

To facilitate the study of locomotor activity, various techniques have been developed to record activity rhythms at an individual level. Examples of such recording devices are: the archetypal rocking box, running wheel (Roberts, 1960), photocell/light-beam (Brown and Unwin, 1961), capacitance transducer, sound recording (Jones, 1964), flight mill, temperature differentials, and their modifications (for references see Brady, 1974). Devices that measure whole body movement, e.g. photocell/light-beam (Brown and Unwin, 1961), simplify the recording of locomotor activity to a matter of movement or no movement. Other devices, designed to record

only particular movements (e.g. walking or flying), have the advantage of reducing noise in the record, but disadvantage of missing parts of the activity (e.g. feeding). Moreover, it is worth noting the inseparability of spontaneous locomotor activity from feeding activity. The high correlation between these two activities infers that the degree of starvation may effect the intensity of activity thereby producing a 'masking effect' on the recorded frequency. However, only rarely have feeding and locomotion been examined independently and simultaneously in a single insect.

Owing to the significant maternal effect on larval diapause, most studies on *Calliphora vicina* have been focused on the photoperiodic responses to light/dark cycles at a population level (Richard and Saunders, 1987; Saunders, 1987; Saunders and Lewis, 1988; Vaz Nunes *et al.*, 1990; Vaz Nunes and Saunders, 1989; Vinogradova, 1974; Vinogradova, 1976; Vinogradova and Zinovjeva, 1972). Attempts to record and analyse individual adult locomotor activities of *C. vicina* were only initiated by Kenny (1989). Since evidence indicates that the circadian clock is involved in photoperiodism (Kenny and Saunders, 1991; Vaz Nunes *et al.*, 1990), the study of circadian rhythmicity will surely aid the understanding of photoperiodism.

1.11. Effects of Temperature

The temperature of the environment plays a crucial role in the life of insects. Although insects have developed some capacity for thermoregulation, their body temperature still depends strongly on the temperature of their environment (Roebroek *et al.*, 1990). In the natural environment, daily cycles of light intensity are

closely associated with cycles of temperature, the highest temperature often occurring in the early afternoon, and the lowest close to dawn. Therefore, it is not surprising that temperature cycles can act as important Zeitgebers although less 'strong' than cycles of light and darkness.

It has been noted as early as the 1920s that different constant levels of temperature have remarkably little effect on the free-running period of a circadian rhythm. Such stability in the face of different temperature levels is known as temperature compensation and it is evident that without it circadian oscillatory systems would be of little use as timekeepers. Although temperature compensation has been described as one of the most striking properties of circadian oscillations, it attracted comparatively little attention. The early researcher, Nicholson (1934), thought that the relationship between temperature and activity was so complicated that, from its very nature one could not hope to describe it completely by means of a simple formula. Working on the eclosion rhythm of *D. pseudoobscura*, Pittendrigh (1954) was the first to propose that temperature compensation of the free-running period (τ) is an essential functional prerequisite for biological time-measurement. Furthermore, Pittendrigh *et al* (1973) suggested that temperature compensation is just one example of a general homeostasis of the clock that is buffered against a variety of factors in the environment. Since then, temperature compensation of period has been established firmly as one of the characteristics of circadian rhythms (Gander, 1979; Roebroek *et al.*, 1990; Zimmerman *et al.*, 1968). Therefore, the effects of temperature on circadian rhythms must be studied more thoroughly to clarify the entraining mechanism to cycles of environmental temperature. During the 1970s,

interest in temperature compensation, as a distinguishing characteristic of the circadian rhythmicity, led to the development of several molecular models (Njus *et al.*, 1974; Sweeney, 1974) in attempts to interpret this phenomenon. The little information obtained, in spite of the efforts devoted, soon led to a decrease of interest. Thus, representative surveys no longer include temperature compensation or only refer to work done before about 1970 (Chiba *et al.*, 1993).

The steadiness through a wide range of constant temperatures must have struck researchers by the fact that it acts against the common rule for biochemical reactions - the speed is dependant on the ambient temperature - which has a Q_{10} (ratio of the period at one temperature to the period at a temperature 10° higher) approximately equalling 2. For the period of a circadian rhythm, however, the value of τ depends only slightly on the temperature at which it is measured. Q_{10} for τ varies between 0.8 and 1.3, generally only slightly more than 1.0, e.g. in *D. pseudoobscura* Q_{10}=1.02, in the cockroach *Leucophaea maderae* Q_{10}=1.04 (from Saunders, 1977). This essential invariance of τ does not therefore imply a true temperature independence or temperature insensitivity; it must derive from a temperature compensation mechanism in the oscillatory system (Pittendrigh, 1960). Hastings (1960), reported that circadian systems can be phase-shifted by temperature steps or pulses and suggested that the clock was able to distinguish between temperature differences and reset itself to the new steady-state temperatures. Pavlidis (1968) proposed a mathematical model for temperature compensation which also included phase-shifts by temperature steps and pulses. The fact that temperature compensation mechanisms work efficiently only within certain physiological or biochemical limits was also used to support

temperature compensation. Gardner and Feldman (1981) suggested that temperature compensation is the result of the interaction of two or more components, each of which is temperature-dependent. Chiba *et al* (1993), investigating the entrainability of mosquito flight and locomotor activity to temperature cycles, also reported that the circadian rhythm may consist of two rhythmic components, which are desynchronised in response to a shift of temperature cycle.

Sensitivity of the oscillations to temperature change has been demonstrated in a diversity of ways in several organisms. In natural conditions, temperature cycles rarely change drastically with amplitudes as large as those used in entrainment experiments. The fact that entrainment to temperature cycles of large amplitude does occur in warm-blooded animals (Eskin, 1971; Moore and Rankin, 1993), suggests that the clock mechanism is affected by the temperature stimulus indirectly and possibly via the peripheral senses. The involvement of multiple circadian oscillators in controlling insect circadian activity has been discussed since a classic, but still valuable, theory was put forward by Pittendrigh concerning the eclosion rhythm of *Drosophila*. Pittendrigh (1960) proposed a model in which a light-sensitive 'master' oscillator entrains a temperature-sensitive 'slave' oscillator, which controls the timing of eclosion. A similar case to this model may be seen also in the cockroach (Page, 1983b) which was reported to have two homogeneous circadian pacemakers distributed in the bilateral optic lobes, as well as a damped oscillator outside the optic lobes which is forced by or entrained to cycles of temperature. Working on the eclosion rhythm in *Drosophila*, Hamm *et al* (1975) first reported that the signal induced by the light can be influenced by temperature en route to the clock. By

comparing the light pulse PRCs obtained at different ambient temperatures, they showed that the effect of the light pulse was delayed when temperature was lowered. Njus *et al* (1977) reported that light and temperature had additive effects on the circadian rhythm of bioluminescence in *Gonyaulax polyedra*, either of which was otherwise ineffective when applied alone. Such result led to the presumption that the circadian system was effected by a synergistic action of light and temperature, which received support from the result of a later study (Broda *et al.*, 1989). More recently, Moore and Rankin (1993) found that although temperature cycles with a smaller amplitude failed to entrain the rhythm, the pattern of entrainment to light-dark cycle was modified and suggested that light and temperature interact before they reach the clock mechanism, i.e. a synergism of light and temperature effects the underlying circadian clock system.

The entraining effects of temperature changes have been studied most intensively in *D. pseudoobscura* in which they may be interpreted in terms of phase response curves (PRCs) and the generalised entrainment model. However PRCs for temperature steps or pulses have only been reported for a very limited number of species such as *D. pseudoobscura* (Zimmerman *et al.*, 1968) and *Hemideina thoracica* (Gander, 1979), therefore, the precise nature of the temperature compensating mechanism remains elusive. As circadian rhythms can be entrained to light perturbations, the sensitivity to sudden temperature changes at different phases of the cycle enables circadian rhythms to entrain to temperature cycles. Zimmerman *et al* (1968) showed that single non-recurrent temperature pulses and temperature steps, both up and down, caused phase-shifts similar in principle to those generated

by light. In their investigation, temperature steps-up caused only phase advances (+Δφ) while temperature steps-down caused only phase delays (-Δφ), magnitudes of both advance and delay depending on the perturbed phase. Since these changes in phase were stable, they suggested that temperature changes reset the circadian pacemaker itself (the A-oscillator) rather than some dependent system. Introducing low temperature pulses, Page (1990) obtained a PRC containing only phase delays and found that the low temperature pulses generated large phase shifts at phases where the pacemaker is relatively insensitive to light pulses. Therefore, he also concluded that low temperature pulses act directly on the pacemaker and not on the light entrainment pathway. Chiba *et al* (1993) suggested that the generalisation, the phase angle (ψ) difference between the environmental cycle and the entrained rhythm changes as a function of the free-running period, drawn by Pittendrigh (1981a) from studies on photic entrainment was applicable to thermal entrainment.

If the temperature compensation system functioned efficiently only when the rates of certain biochemical reactions are within certain limits, mutations which alter the rate of one of these reactions might alter the temperature range within which the system is compensated. Gardner and Feldman (1981) found that the period mutants of *Neurospora crassa* offer a unique opportunity to test a number of ideas about the temperature compensation mechanism of the clock.

1.12. Effects of Constant Light

Locomotor activity rhythms in many organisms have been found to persist in constant light (LL), providing that the irradiance is dim, as readily as in constant

darkness (DD), but with an altered period. Pittendrigh and Daan (1976a) suggested that the free-running period in continuous light (τ_{LL}) was determined by interindividual or interspecies variations, as well as on the light intensity. The free-running period in constant darkness (τ_{DD}) determines whether a light/dark cycle will entrain the rhythm by daily phase delays or advances. If τ_{DD} exceeds 24 h, the rhythm must be reset earlier, therefore a daily advance is required, i.e. entrainment will be effected mainly in the morning; whereas if τ_{DD} is less than 24 h, evening light will create the required phase delays. Aschoff (1979) noted a general difference between night-active and day-active animals in this respect which has become the so-called 'Aschoff's rule'. The idea was that $\tau_{LL} > \tau_{DD}$ upon transfer from DD to LL or with an increase in light intensity for dark-active animals, but $\tau_{LL} < \tau_{DD}$ for light-active animals. Although Aschoff's rule finds support from many vertebrate species, many insect species have been found to violate this rule, including the blow fly *C. vicina* (Hong and Saunders, 1994).

In addition to the effect of dim continuous light on the period of a free-running rhythm, continuous light of higher intensities (bright LL) has also been found to have effects on circadian rhythms. Constant bright light frequently inhibits the expression of circadian rhythmicity in organisms at all phyletic levels, and the intensity of light that suppresses rhythmicity is often surprisingly low (Enright, 1981). Bright LL has been reported to stop the circadian clock in *Drosophila* (Pittendrigh, 1966), the New Zealand weta, *Hemideina thoracica* (Lewis, 1976) and the house sparrow, *Passer domesticus* (Binkley, 1978). Lewis (1976) suggested that the clock is highly sensitive

to light and the loss of activity in constant light at a relative high intensity is the consequence of the stopping of the clock. However, as Aschoff (1981) pointed out, one has to be careful in interpreting the disappearance of a rhythm as a 'stopping' of the circadian clock as opposed to an 'uncoupling' of the overt rhythm or a desynchronisation among a multiple oscillators. Studying effects of different constant light intensities (0.25 lux - 500 lux) on the locomotor activity rhythm of rats, Vilaplana *et al* (1995) reported that the effect of light is manifested by a smoothing of the waveform of the activity rhythm, such an effect strengthens as the intensity increases. Furthermore, they suggested that if light has an inhibitory effect on the coupling between the oscillators that drive the overt rhythm, one can expect to find changes in the pattern of the motor activity circadian rhythm depending on the light intensities on the locomotor activity rhythm.

1.13. Phase Response Curves and Entrainment

1.13.1. Phase Response Curves (PRCs)

The biological clock system is thought to pass through an orderly series of physiological or physical states during the course of its circadian cycle (Peterson, 1980). This has proved to be a general property of all circadian oscillators. To proceed with the analysis of a circadian oscillator, it is important to know the phase relationship between the overt rhythm and the oscillator itself. The nature of the mechanism underlying circadian rhythms has been studied following this direction. The most straightforward approach is applying exogenous stimuli (e.g. light, temperature, drugs) to the endogenous oscillation and measuring the resulting phase-

shifts. The discovery that a single light pulse of short duration could shift the phase of circadian rhythms, free-running in constant conditions, brought about immense improvement in the analysis of entrainment, and has since been one of the most commonly applied protocols to generate light PRCs.

When exposed to an exogenous stimulus, the oscillatory system responds systematically, according to the circadian time at which the perturbation is applied; hence the sensitivity of the system to an external perturbation changes over the course of its circadian cycle. Such sensitivity is best characterised by a phase response curve (PRC) (Johnson, 1992). A PRC is a plot of phase-shifts caused by a single perturbation as a function of the phase of the oscillation. These phase-shifts can be of different magnitudes and directions, depending on both the intensity and the initial phase of the perturbation. The modern convention for phase response curves plots phase-delay ($-\Delta\phi$) shifts below the control (unperturbed) line and phase-advances ($+\Delta\phi$) above it. The fact that the magnitude of the phase shifts is dependent on the phase of the oscillation at the time of the perturbation suggests that the PRC can be used as a direct assay of the time course of the pacemaker. Hence, the PRC is a measure of the succession of phases through which the pacemaker passes in the course of its cycle (Pittendrigh, 1976). Since a phase-shift is considered to involve the central mechanism or pacemaker, studies of factors and conditions causing phase shifts should aid the understanding of the pacemaker. Therefore, information revealed by PRCs is considered more fundamental than other measurements of the overt rhythms.

A major function of a PRC is to provide predictions for steady state entrainment to 24-h light/dark cycles. In addition, PRCs can provide phase markers for the oscillator, probes for the clock mechanism, and gauges of oscillator amplitude (Johnson, 1992). Firstly, its role as a gauge of entrainment has provoked interest in generating PRCs for various stimuli in a wide variety of organisms. The fact that light is the most important Zeitgeber has led to the accumulation of many PRCs for light stimuli. A distinct generalisation emerging from these studies is that delaying phase-shifts occur in the early subjective night, advancing phase-shifts occur in the late subjective night and little or no phase-shifts occur during the subjective day. This generalisation holds true whether or not the overt rhythm peaks in the day, night, or at twilight (Saunders, 1977). The magnitude of phase-shifts is also a measure of the limits of entrainment. An oscillator that exhibits phase-shifts of larger amplitude is likely to entrain to T-cycles of a broader range (Johnson, 1992). PRCs have also been used as phase markers for the oscillator, and led to insights into essential features of circadian organisation. The phase of the circadian oscillator can be determined by measuring the PRC to light pulses, as the PRC describes the time course of light sensitivity of the oscillator. On the other hand, the phase of the rhythm itself is a function of both the phase of the oscillator and the phase angle difference between the oscillator and rhythm. Therefore, PRCs are better gauges of the phase (or phase angle) of the oscillator than is the rhythm (Johnson and Hastings, 1989). PRCs used as probes for the clock mechanism are usually those generated by chemical and drug stimuli. Analyses of the effects of chemicals upon both period and phase provide the possibility to uncover the biochemical mechanism of the pacemaker itself. Finally, as

gauges of oscillator amplitude, a PRC is particularly useful when studying the resetting mechanism and testing models.

In general, similar PRCs have been described for different rhythms among a number of insects, including the pupal eclosion rhythm of *D. pseudoobscura* (Dann and Pittendrigh, 1976a; Pittendrigh, 1960; Pittendrigh *et al.*, 1958; Pittendrigh and Minis, 1964) the egg hatch rhythm of *Pectinophora gossypiella* (Pittendrigh and Minis, 1971); locomotor activity of cockroaches *Leucophaea maderae* (Roberts, 1962); flying activity of *Anopheles gambiae* (Jones *et al.*, 1972); pupal eclosion of *Sarcophaga argyrostoma* (Saunders, 1976), and adult locomotor activity of *D. melanogaster* (Saunders *et al.*, 1994). Working on the dependence of the shape of PRC on τ, Daan and Pittendrigh (1976a) suggested that as τ shortens delay responses increase and advance responses decrease.

Winfree (1970) demonstrated two topologically different types of PRC which represent two types of phase-setting responses to light pulses. The amplitude of the phase-shifts depends on the strength of the signal: when light pulses were 'weak', phase shifts were small, when pulses were 'strong' phase shifts became abruptly larger. Weak resetting curves were called type 1 and strong curves type 0. Type 1-PRCs display relatively small phase-shifts and have a continuous transition between delays and advances, whereas Type 0 show large phase-shifts and a discontinuity (breakpoint) at the transition between delay and advance phase-shifts.

The exact configuration of the PRC varies not only between species but with the duration (Pittendrigh, 1960) and intensity (Chandrashekaran and Loher, 1969) of the light pulse. As the intensity and/or duration increases, PRCs go through two

transitions. Phase-shifting first changes from Type 1 to Type 0, but the circadian time of the transition between delay and advance shifts remains fixed. As the duration of the light pulse is increased further, a second transition occurs: the circadian time of the break point begins to shift to earlier times. This second transition has been interpreted as the clock stopping at Ct12 until the light pulse is terminated.

1.13.2. Entrainment

In natural conditions, the daily photoperiod is at any one time shortening or lengthening, so that the phase angle between dawn and dusk systematically changes. In consequence, the sensitivity of a circadian oscillation to the Zeitgeber changes with time so as to 'measure' the local time and synchronise to it. A series of single light pulses that covers the 24-h time scale, together with the resulting phase-shifts, reveals such sensitivity in a way that also describes the phase relationship between the oscillator and the Zeitgeber. The consequent plot shows that sensitivity and phase relationship are closely correlated and leads to the assumption that such cyclic change of sensitivity is a prerequisite of entrainment.

The fact that the free-running period of a circadian oscillator always deviates from a 24-hour cycle emphasises the importance of daily resetting. In each cycle, circadian oscillators make adjustments in response to the photic signals by adjusting period length to an exact 24 hour and having the overt event (e.g. the onset of activity) occurring at a particular phase of the light/dark cycle. This is consistent with the fact that in nature onsets of activity often coincide with dawn or dusk. Entrainment has been long defined as the phenomenon whereby a periodic repetition of light/dark or

temperature cycles cause an overt persistent rhythm to achieve the same period as the entraining cycle (Bruce, 1960). In brief, entrainment is the synchronization of a self-sustaining oscillator to the external light/dark cycle, so that the oscillator is reset daily by a small amount to keep the organism on an exact 24-hour periodicity.

By establishing a unique and steady relationship between the circadian oscillation and the environmental light/dark cycle, entrainment ensures adaptive occurrence of an overt phase with the appropriate part of the day. In practice, the underlying mechanism must consist of two parts: perception of light and phase adjustment of the circadian oscillation, the latter involving both period control and phase control. In steady state entrainment, a repeated light stimulus causes a phase shift in each cycle equal to the difference between the period of the oscillator (τ) and the period of the Zeitgeber (T). In steady state, this is achieved by the light pulse falling on that phase which produces a shift of exactly the required direction and magnitude. This basic principle for steady-state entrainment is summarised by the following equation:

$$\tau - T = \Delta\phi$$

In general, when T is shorter than τ, the oscillation phase lags the Zeitgeber and the light pulse must fall in each cycle during the late subjective night. A phase advance (+ $\Delta\phi$) is thereby induced to shorten the period of the oscillator so as to equal that of the driver. Conversely, when τ is shorter than T, the oscillation will phase-lead the Zeitgeber, the pulse will fall in the early subjective night and generate a phase delay (-$\Delta\phi$). According to the equation, it is self-evident that the 'amplitude' of the phase response curve (i.e. the maximum values of $\Delta\phi$) determines the limits of T to which a

circadian oscillator can entrain. The range of realisable T-values on either side of the natural period (τ) is called the 'primary range of entrainment' (Saunders, 1977). However, for different organisms, the 'limits of entrainability' vary quite widely. There is a rough generalisation to the effect that the more complex the organism the more difficult it becomes to entrain the rhythm to period lengths considerably different from 24 hours (Bruce, 1960). For most organisms, the range is 21 - 27 hour (Saunders, 1977; Brady, 1982).

As described in the previous section, PRCs not only provide predictions for steady-state entrainment to 24-h light/dark cycles, they also function as gauges of the entrainment mechanism. For entrainment to occur, it is essential that the PRC should have: (1) a region of negative slope which is less than -2, and (2) a point on the PRC where the phase shift equals τ - T. Hence, the PRC usually phase delays during the early subjective night and phase advances during the late subjective night. These two regions can be, but do not necessarily have to be, connected by a 'dead zone' during which light exposure does not result in a phase shift (Puchalski and Lynch, 1992). However, Johnson (1992) points out that it is not essential for a PRC to have both delay and advance regions. For example, if the free-running period is longer than 24 hours, a PRC exhibiting only advances will allow stable entrainment.

The process of entrainment to an environmental Zeitgeber is surely more complicated than what is observed in controlled laboratory conditions. Whatever the details of such a mechanism may be, it is clear that the effect of the daily light/dark cycle is to change the period of the circadian oscillator by an amount equal to τ - T,

which has been the basis of many models developed to simulate entraining mechanism.

Models for the entraining (resetting) mechanism

The essence of the entrainment mechanism lies in the ability of the clock system to 'reset' or to 'be reset'. Phase resetting has been studied extensively in a variety of organisms, especially insects and nocturnal rodents. Years of research on the resetting mechanism has resulted in the discovery of many resetting factors (light, temperature, drugs and chemicals) and the development of various models. Moreover, identifications of clock gene protein products, such as PER in *Drosophila* and FRQ in *Neurospora*, has brought about great progress in the understanding of the entraining mechanism. However, the mechanism itself remained elusive until the recent report on the activity of *frq* (Crosthwaite et al., 1995), which provided biochemical explanation of the resetting mechanism.

As one of the earliest researchers to work on insect circadian rhythms, Bünning (1936) proposed a model which divided each 24 hour period into two parts: the first 12 hours being the 'photophil' (light requiring phase) and the second 12 hours being the 'scotophil' (dark requiring phase). However, the failure of such a presumption that an endogenous oscillation has a period (τ) equalling the environmental period (T) can easily be predicted now that the equation of entrainment, $\tau - T = \Delta\phi$, has been established firmly. Pittendrigh and Bruce (1957) then proposed a conceptual model which defined four functional components in a circadian system: an oscillator, a photoreceptor for entrainment and two 'coupling' pathways, one mediating the flow

of information from the photoreceptor to the oscillator and the other between the oscillator and the function it controlled. For many years, this model has been the basis of many 'black box' approaches (for detailed account see chapter 3, introduction). A small advance was made when a feedback model was proposed to simulate the circadian rhythm of petal movement in *Kalanchoe* (Johnsson and Karlsson, 1972; Karlsson and Johnsson, 1972). Later, this was modified by Gander and Lewis (1979) to simulate the locomotor activity rhythm of the New Zealand weta *Hemideina thoracica*. Such single oscillator models, however, failed to interpret circadian phenomena such as spontaneous changes in the free-running period and the splitting of activity rhythms. Such inconsistency led to the development of multioscillatory hypotheses which suggested that the circadian rhythm was under the control of a population of coupled oscillators acting independently or interacting with each other. Furthermore, Pittendrigh and Daan (1976a) suggested that entrainment was due to a change in the phase relationship amongst circadian oscillators, therefore both the control mechanism of the overt oscillator and the relationship between the overt rhythm and the underlying oscillator/pacemaker should also be taken into consideration when developing a model. Although detailed knowledge of the precise physical nature of the clock components is not essential for the understanding of the overall control mechanism (Gander and Lewis, 1979), studies at a cellular level have provided insights into the nature of the biochemical activity underlying the circadian oscillatory mechanism. Consequently, clock genes have been identified in various systems (see 1.6 for detailed account), *per* in *Drosophila* and *frq* in *Neurospora* being the best documented. Moreover, many transcriptional pathways have been

reported to be oscillatory (Dunlap and Feldman, 1988; Ewer *et al.*, 1988; Hardin *et al.*, 1990). The fact that these identified genes regulate, directly or indirectly, their own expression suggested that the feedback regulation of transcription is likely to be a central component of the clock, whose instantaneous abundance, rate and direction of change establish circadian oscillation. Page (1994) (see Fig. 4.1.1) summarised results of these studies and described the oscillatory system as a negative feedback loop with several identified state variables whose levels can be adjusted to phase shift the clock, thereby, achieving entrainment.

Chapter 2

Studies on the Circadian Locomotor Activity Rhythms
in the Blow Fly *Calliphora vicina*

2.1. Aims

Experiments were mainly based on observations of the locomotor activity rhythm

of the blow fly *Calliphora vicina*, which has also developed a successful strategy to

overwinter as a diapausing larva - a form of dormancy. The induction of such a

photoperiodic response is triggered by the decrease in the ratio of daylength to

nightlength - the threshold being defined as the critical daylength/nightlength.

Inevitably, an intrinsic time-measuring mechanism must be involved to fulfil this

demand. Despite experimental difficulties, it has been shown that circadian

oscillations are involved in the control of photoperiodism in many insect species,

including *C. vicina* (Vaz Nunes *et al.*, 1990). The photoperiodic response and

maternally induced larval diapause have been studied intensively and has led to the

establishment of some fundamental information essential for the approach of the time

measuring system in this species, thereby, making it ideal material for the study of

circadian rhythmicity. It has been well documented that the photoperiodic regulation

of diapause in this species is largely maternal and the eggs and larvae show only a

slight sensitivity to daylength (Saunders *et al.*, 1986). Studies on circadian locomotor

activity rhythms in the adult fly were only initiated recently by Kenny (1989) who

found no differences between the behaviour of the sexes. Therefore, only female adults of *C. vicina* were used throughout all experiments in the present study. An additional advantage to investigate circadian locomotor activity rhythms in *C. vicina* is that adult flies display clear and persisting activity rhythms with very little 'noise' (Kenny and Saunders, 1991), therefore minimises the difficulties when analysing the recorded rhythm.

The complexity of the circadian system makes the direct access to the central mechanism almost impossible, therefore, confines the studies to the observation of peripheral rhythm driven by it. However, two measurable parameters, i.e. the free-running period (τ) and the phase response curve (PRC), can be considered as properties of the circadian pacemaker. In the present study, changes of τ were measured and analysed to indicate the response of the pacemaker to certain stimuli, such as light pulses and temperature changes. The following aspects of circadian rhythmicity in *C. vicina* were investigated: free-running activity in constant darkness (DD) and constant light (LL), temperature compensation of τ, effects of temperature steps and light pulses, entrainment to 24 hour light/dark cycles.

2.2. Materials and Methods

2.2.1. Stock Cultures

The strain of blow fly, *Calliphora vicina* Robineau-Desvoidy, used in all experiments was collected in Musselburgh, Scotland (55 °N), in 1984 and kept since then under constant laboratory conditions.

Adult flies were maintained in breeding cages in a constant temperature room at 25 ± 1°C, illuminated continuously by a fluorescent strip light (~700 lux). Each cage consisted of a sleeve made of white muslin gauze, a metal bottom tray with a sheet of white paper to facilitate cleaning and a metal rectangular frame (26×21×19 cm) to support the sleeve. Stock pupae were placed in a glass jar with a strip of tissue to provide the newly emerged flies a site to stretch their wings and harden their cuticles before they were capable of flying. About 200 - 300 pupae, collected on the same day, were placed in each breeding cage and the emerging flies were provided with granulated sugar and a jar of tap water on a constant basis, while the protein meal was only provided from day 4 after emergence. Defrosted beef muscle was used as the source of protein and was provided every other day before egg laying, and then daily after egg laying.

The eggs, collected daily, were left for 24 h to hatch in the same conditions as the adult fly, which consequently had not experienced any periodic changes in lighting regime prior to transfer to . First instar larvae were then transferred to a generous slice of beef placed on supplementary larval medium (see below) set in a plastic box

(26×10×9 cm). The number of larvae was restricted to about 400 in each culture to avoid over-crowding. To prevent the larvae from escaping and to provide a site for pupariation, the medium box with hatched larvae was set onto a bed of fine sawdust, contained in a larger tray (33×16×13 cm). The larval culture was then placed in a walk-in constant temperature room at 11°C in continuous darkness. The newly established larval culture was covered by a piece of glass to prevent the young larvae from wandering into the sawdust before they settled down to the medium. Larvae were then maintained in these conditions throughout their life span, feeding on the medium and beef muscle. When the larvae started to wander, the medium box was turned to lie on one side to help the larvae into the sawdust to dry out the excess body moisture. Newly formed puparia were sieved from the sawdust. Pupae collected on the same day were placed in breeding cages as described above.

For experimental cultures, the few flies emerging first were removed and after 24 h the unemerged pupae was discarded to ensure that all flies in the same cage had emerged within a 24 h period. This also ensured that experimental flies selected from these cultures had not experienced any periodic changes in lighting regime prier to transfer to experimental regime.

Preparation of Larval Medium

Ingredients of Larval Medium

Davis agar (Mackay and Lynn Ltd.)	20g
Dried milk powder (Millac; instant, spray-dried, separated milk, with added vegetable fat and vitamins A, C and D)	150g
Dried yeast (autolysed yeast powder)	12.5g
Water (Tap water)	2 litres

Method

The dry milk powder and yeast were mixed in a plastic tray. Water was added gradually and the mixture stirred gently into a smooth paste, avoiding large lumps. The powdered agar was added to about 200 ml cold water in a sauce pan and heated. Then the milk-yeast paste was stirred into the sauce pan. More cold water was added to make the total quality up to 2 litres, heating and stirring the mixture to prevent sticking. The liquid medium was then poured into plastic boxes (this recipe made approximately 5 portions, each filled the medium box to 3 cm) and left to set at room temperature. The medium was stored in the refrigerator at about 4°C.

2.2.2. Recording Locomotor Activity Rhythms

Several techniques have been developed to record insect locomotor activity, e.g. running-wheels, rocking actographs, light-beams, electrostatic fields and many other modifications (see chapter 2 introduction). Some of these devices record only certain aspects of the behaviour and thus result in the loss of some important components of

the activity, while others showed a gradually declining amplitude in activity peaks or a complete disappearance of the rhythm. It has been shown that different devices used for the same animal do not always give the same result, because locomotor activity may be influenced by various non-rhythmic elements in the environment. However, all of these devices share one common factor, namely the assumption that the daily pattern of locomotor activity was the result of an internal clock.

In the present investigation, infra-red recorders were selected to register locomotor activity because of the following advantages:

(a) this method measures whole body movements and thereby simplifies the recording to a question of motion or non-motion. (b) this method has been most commonly applied to species closely related to the blow fly; (c) it is capable of recording activity over a long period of time and producing clear results; and (d) unlike a running-wheel, which produces a rotary momentum, there are no further stimuli suspected to be responsible for the initiation of activity, thus a more realistic record is obtained.

The original Brown and Unwin (1961) recorder, with a thicker infra-red beam, tended to produce a 'noisy' record. To avoid this, an improved recorder with a miniaturised infra-red beam was applied.

Each recording device comprised: (Fig. 2.2.2.1)

- a Petri dish containing the experimental fly;

- a wooden platform with two sets of infra-red recorders on either side;

- a light tight box equipped with a water-jacketed fluorescent lamp as light source;
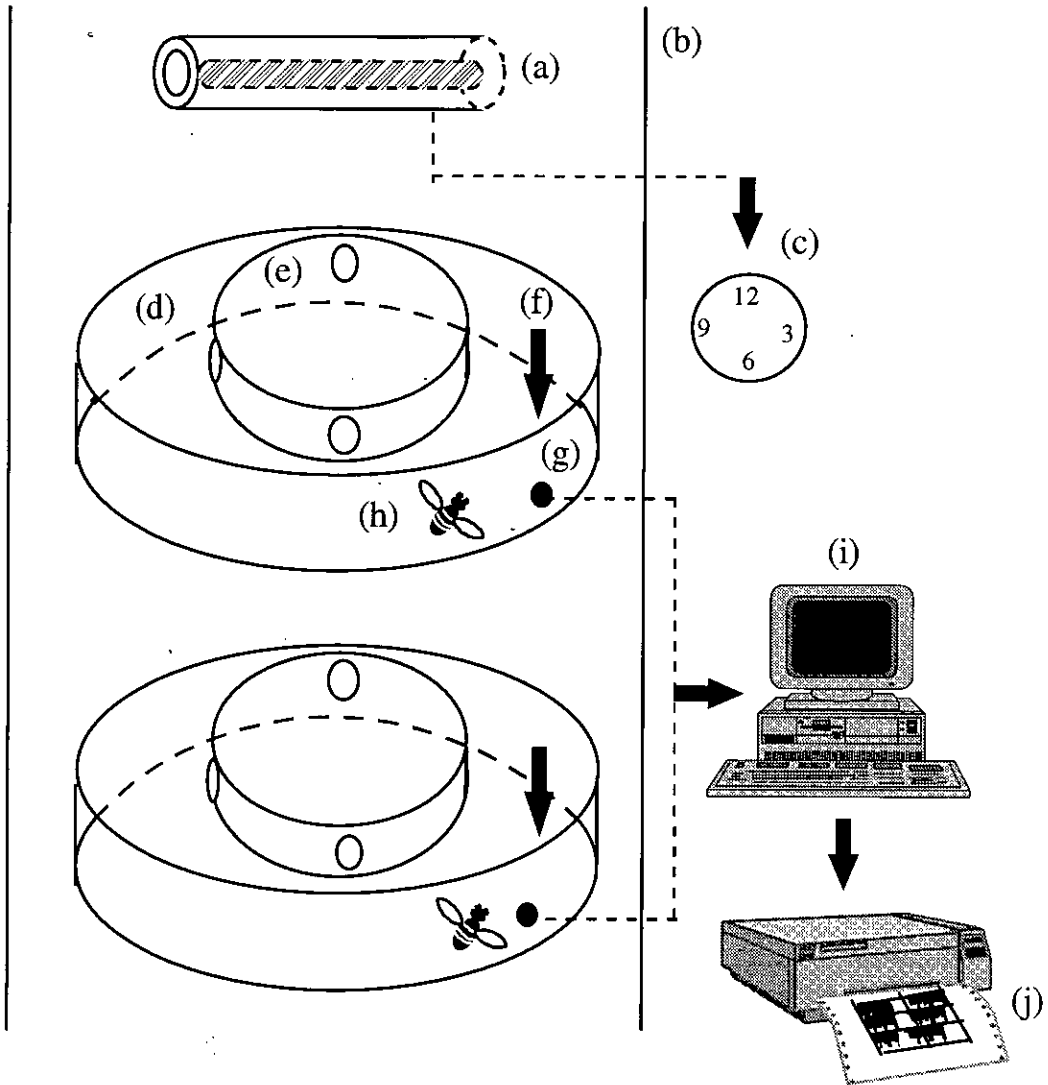
Fig. 2.2.2.1 The device for recording the locomotor activity rhythm of *C. vicina*. Each experimental fly (h) was placed in a device, consisting of a 9-cm Petri dish (d) and a 5-cm Petri dish (e), which provided a track for the fly's locomotor activity. Two such devices were mounted on each side of a wooden board (b) which was equipped with two sets of infra-red emitter (f) and detector (g). Such a wooden board was housed in a box illuminated by a fluorescent strip light (0.7 W/m^2) (a) which was connected to a 24 h timer (c) controlling the time of the light pulses or the duration of the light phase of a light/dark cycle. All light boxes were installed in a constant temperature room. The activity frequencies detected by the infra-red detectors were registered by a micro computer (i) at 10-minute intervals. Records of the activity rhythm were converted and printed as a double-plotted actogram (j) as described in section 2.2.2.

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- an independent timer controlling the length of light period which the experimental fly is exposed to; and

- a computer with recording programs for recording activity rhythms.

A 5 cm diam. Petri dish was glued to the centre of a large, 9 cm diam. Petri dish, to form a circular track. The inner dish contained cotton wool, soaked in 10% sugar solution, with some of the cotton drawn through lateral holes, evenly drilled along the inner dish, to provide a source of sugar and water for the fly. A single female of *C. vicina* was caught from the stock cage within 24 h of its emergence and placed within the track. Two such Petri dishes were mounted on a wooden platform which also provided support for the infra-red emitters (Radio Spares, type 306-077) and detectors (RS, type 306-077). On the upper part of the platform, underneath each of two parallel wooden bars, one emitter was arranged in a way that the infra-red light beam passed vertically across the side of the circular track and was received by the detector which was accommodated in a hole situated opposite the emitter on the bottom board. Such an assembly is called a 'channel'. Two platforms, namely four channels, were then enclosed in a light tight wooden box held in a constant temperature room at $20 \pm 0.5°C$. All experiments were carried out at this temperature unless otherwise stated. Each box was provided with a 4 W fluorescent lamp, water jacketed to suppress temperature rise when the light was on. The lamp in each box was connected to an independent timer which controlled the length of the light period to which the experimental fly was exposed. At the level of the experimental fly, irradiance from this lamp was about 0.7 Wm^{-2} (~480 lux) except in the light intensity experiment, when the irradiance of the light source was reduced. During the dark

phase of a light/dark cycle or in DD, all flies were handled in red light (5 W). Although there is no direct evidence that *C. vicina* can not see light of this wavelength, another dipteran, *Sarcophaga argyrostoma*, was found to be insensitive to red light (Saunders, 1982). Moreover, there was no indication from experimental results that *C. vicina* adults were in any way influenced by red light (Kenny, 1989).

2.2.3. Analysis of Locomotor Activity Rhythms (Fig. 2.2.3.1)

All locomotor activity experiments were recorded automatically by a BBC B-plus microcomputer. The experimental fly moved along the track and broke the infra-red light beam as it passed through it. This activity stopped the detector from receiving the beam at that particular moment. Such a signal was passed to the computer and was recorded as a datum of activity. Activity events were thus registered as the number of times the moving fly broke the infra-red light beam within successive 10-minutes intervals. The activities of experimental flies in individual channels were recorded separately by a program, **LOG32** (see below). The frequencies of activity in each 10-minute interval were plotted as a vertical line against a 24-hour time scale and could be seen on a VDU monitor. The recorded activity was saved onto a $5^1/_4$" floppy disc automatically every 24 h. Activities of the consecutive days were recorded in the same way and laid out in parallel time scales. Owing to the limited memory of the recording computer, the program needed to be restarted every 7 days. The raw data were then recovered and analysed by specially designed programs, **Recov32** and **Ctwork3**. The activity records were subsequently assembled into the
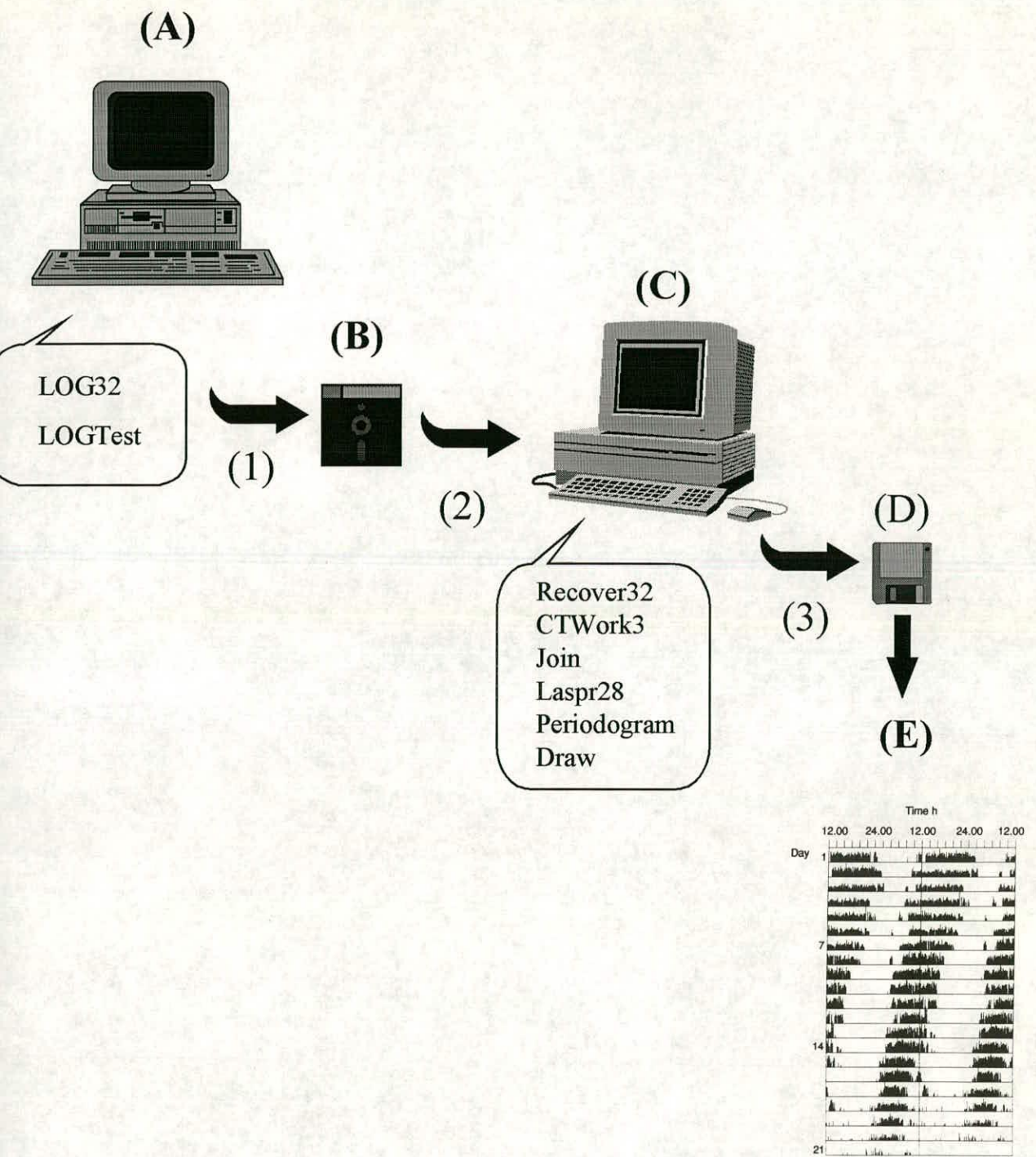
(A)

LOG32

LOGTest

(B)

(1)

(2)

(C)

Recover32
CTWork3
Join
Laspr28
Periodogram
Draw

(3)

(D)

(E)

Time h

12.00 24.00 12.00 24.00 12.00

Day 1

7

14

21

Figure 2.2.3.1. Flowchart of the data converting and analysing. Locomotor activity rhythms were recorded by a microcomputer (A) which then transfers data onto a 5 $\frac{1}{4}$" floppy disc (B) containing two programs, LOG32 & LOGTest. Such raw data were then converted from a DFS format to an ADFS format, which then can be analysed and edited by programs contained in a BBC Archimedes computer (C). All data files were save onto 3 $\frac{1}{2}$" floppy discs (D) and printed as double-plotted actograms (E), illustrated in this thesis.

conventional 'double-plotted' actogram format (Fig. 2.2.3.1). This showed a clearer activity pattern, because the records of consecutive days were displayed contiguously. Records longer than 7 days of the same experimental fly were analysed separately. A program, **Join**, was then applied to join actograms from successive 7 day periods.

Computer programs for recording activity rhythms

LOG32   This program registered activity frequencies at 10-min intervals continuously through a 7-day period and plotted the recorded data against a 24 h time scale which were shown on a VDU monitor. The registered data were stored in a 'Data' file temporarily then transferred onto a ADFS-formatted floppy disc on a daily basis. A number representing the 10-min interval was shown on the top of the screen, e.g. 1 represents the first 10-min interval and 144 the end of the first 24 hours.

LOGTEST   Used to test the channels and adjust the sensitivity of the infra-red detectors. When running the program, all channel numbers were shown on screen followed by the number 0 or 1. 0 represents active state while 1 shows an insensitive state to a stimulus.

Computer programs for data analysis

To analyse locomotor activity, a number of different techniques have been developed and tested in the past with the result that time-series analyses are most frequently applied. Care was taken when selecting the most appropriate analysis because the information required when using an ordinary statistical technique is simply not available for biological rhythms (Kenny, 1989). Before any analysis of data was undertaken it was ensured that the observations had been collected at regular intervals and in a wholly objective manner. Because of the large amplitude and relatively low 'noise' level, the rhythms displayed by *C. vicina* were found particularly amenable for analysis. Since the circadian pattern was often complicated, the free-running period detected by a statistical method such as the periodogram did not always agree well with the actual activity record. So an alternative method was adopted, in which τ was measured by fitting a straight line visually to activity onsets. Two main techniques were applied in this thesis: when the rhythm was clearly defined, '**Ctwork3**' was applied; alternatively, in the cases of arrhythmicity or unclear rhythms, '**Periodogram**' was applied. Programs used to analyse activity rhythms in this thesis are described as follows.

Recov32   This program was used to recover and divide raw data into individual channels then to save the data as an individual file.

Ctwork3   Converted and displayed the activity bands in the 'double-plotted' format. It calculated the mean amount of activity, the mean length of activity (α) and the free-running period (τ). It also calculated the value of the phase-shift, if a disturbing light pulse was given during the

experiment. For such purpose, a least-regression line can be drawn through the mid-points (Ct6) of the activity band by selecting the beginning and the end point of the daily activity band with the cursor. The slope of this line represented the period of the free-running rhythm. Two such regression lines, one prior to and the other after the light pulse, were drawn in order to calculate the value of the phase-shift induced by the light pulse (written by R.D. Lewis, modified by S.W. Gillanders, 1993).

At the end of each 7-day period (when the number on top of the screen reached 1008), the floppy disc ($5^1/_4$", DS, DD, ADFS-formatted) with automatically transferred data, was removed from the disc drive and replaced with another to record data for the next week. Data on the disc were then compiled by a program 'DFSReader' to facilitate transfer onto a DFS-formatted disc ($3^1/_2$", DS, DD). Then Recov32 and Ctwork3 were applied to analyse the records in individual channels. A double-plotted actogram was produced and printed at this stage. Finally, the Join program was used to join records from the consecutive weeks up to 28 days.

Periodogram

Although, as Enright (1981) pointed out, periodogram analyses can not separate out the components in such cases of non-stationarity, such defect can be overcome by selecting activity carefully (as described in Ctwork3). The version of the periodogram selected to analyse locomotor activity in the present study was originally devised by

S.E.R. Bailey at Manchester University in 1982, which subsequently modified by R.D. Lewis for the BBC Archimedes microcomputers. This program, has been a widely-used form of time series analysis partially because the calculation is relatively simple and without being any less accurate in the estimation of the period values (Kenny, 1989). It produces clear measures of the period of the rhythm. Furthermore, periodogram analysis can indicate whether the identified periods are significant. This is important when deciding if the result is reliable. In order to use the system for the analysis of the locomotor pattern of the blow fly, each record of each fly was considered as a single data 'string', containing the number of events in each 10 minute interval. The periodogram program was then able to analyse between any two units in the same string and within any range of periods. The periodogram analysis used here was based on Enright (1965), and (Williams and Naylor, 1978) and was used to calculate free-running periods (τ), duration of the 'active' phase per cycle (α) or arrhythmicity. The range of periods to be analysed was selected by typing in the numbers that represented the beginning and the end of the string of 10-min intervals to produce a periodogram in the waveform format with 95% confident intervals indicated. The periodogram so produced was saved as a draw file and edited (see below).

Computer programs for graphing

Laserpr28 This program was specially written to convert an actogram into a draw file to facilitate editing. (written by R. D. Lewis & C. Lewis)

Draw This program was used to edit actograms (re-scaling and text editing)

and periodograms and to produce the standard format of actograms seen in this thesis.

Statisticat A statistical program written to analyse data and convert into graphs as required. The curves (e.g. PRCs for light pulse or temperature steps) illustrated in this thesis were plotted by this program.

Powerpoint This program, designed mainly for producing slides, was applied to produce tables used in this thesis.

2.3. Experimental Protocols

2.3.1. Observations on Circadian Locomotor Activity Rhythms in Constant Darkness

When recorded in constant conditions a free-running activity rhythms is believed to represent the endogeneity of the oscillation. Moreover, the period length (τ) of such a free-running rhythm can be used as an indicator to determine whether a light/dark cycle will entrain the rhythm by daily phase delays or advances. The recording of locomotor activity rhythm reported so far has shown that free-running period is obtained most successfully under constant darkness (DD) or continuous light (LL) at constant temperature with other possible entraining agents excluded. However, according to previous studies (Kenny, 1989), the full intensity of illumination used in the present study will always result in apparent arrhythmicity in *C. vicina*. Owing to the difficulty in analysing arrhythmic activity, all recording of the present investigation was carried out in DD.

Large female flies were collected from the stock culture (rearing conditions see 2.2.1) and placed in recording device 24 hours after emergence. Sugar solution was checked weekly in dim red light to keep the disturbance to the least. Activity rhythms were recorded in constant darkness at 20 ± 0.5 °C for 4 weeks (method for recording see 2.2.2). Activity rhythms were than transformed into a double-plotted actogram to facilitate the analysis (see 2.2.3).

2.3.2. Assessment of the Effects of Temperature on Circadian Locomotor Activity Rhythms

Two experiments were carried out with the directions of temperature steps opposite to each other. From day 1 - 7, all flies in both experiments experienced the same constant conditions (constant darkness, 20 ± 0.5 °C). On day 8, the temperature was either raised (experiment I) or lowered (experiment II) by 5°C and maintained at that level for at least 7 days. Finally, the temperature was changed again by 10° (direction opposite to the first change) and maintained at that temperature until the end of the experiments. To exclude photic factors, all flies were kept in constant darkness throughout. Owing to limits of facility, it was not possible to control temperature in individual light boxes. Changes of temperature were operated through a centrally controlled device, i.e. all flies experienced the same temperature steps. When increased (steps-up) or decreased (steps-down) by 5°C, the ambient temperature settled at the new level within 30 minutes, whereas a further increase or decrease by 10°C was achieved within 1 hour. These changes of temperature were registered by a digital recorder (Rustrak). Lids of all light boxes were removed before the room temperature was changed so that the temperature inside the boxes could reach the new level rapidly and evenly. Inside one of the light boxes, a thermometer was placed at the level of the experimental flies to record the temperature in the box. Lids were replaced once the temperature inside the boxes had settled. To ensure that flies received no effective photic stimuli, dim red light (5 W) was used for illumination when changing the temperature.

2.3.3. Assessment of the Effects of Constant Light on Circadian Locomotor Activity Rhythms

Flies were allowed to free run in DD for 7 days, then exposed to constant light (LL) at reduced intensities for 7-11 days before a final free-run in DD. Experiments were carried out at a constant temperature (20 ± 0.5°C) throughout.

The reduced light intensities used in this experiment were achieved by wrapping paper of various thickness around the fluorescent tubes, with both ends of the tubes tightly wrapped in aluminium foil to prevent leakage of light. Light intensities were measured with a Tektronix J16 digital photometer in Wm^{-2} at the level of the experimental flies. The range of light intensities recorded by this method was from about 0.017 $Wm.^{-2}$ (~11.6 lux) to about 0.07 $Wm.^{-2}$ (~47.9 lux).

2.3.4. Determination of a Phase Response Curve (PRC) for One Hour Light Pulses

After an initial free-run (normally 7 days) in DD, flies were exposed to a single 1 h light pulse applied at various times, then remained in DD until the end of the experiment. Phase shifts induced by light pulses were calculated manually or by computer programs (see 2.2.3. Ctwork3) and plotted against the circadian time scale. To calculate the value (magnitude) of phase shifts, firstly, a regression line was produced through the midpoint of the activity bands recorded in DD. Then the predicted Ct6 was calculated by extrapolating this regression line to where the next activity band should occur. The difference between the predicted Ct6 and the mid

point of the actual activity rhythm, recorded after the light pulse, was defined as phase-shift, in this thesis.

2.3.5. Studies of Entrainment to 24 Hour Light/dark Cycles

Important parameters involved in entrainment include: light intensity, light pulse duration, and the period of the entraining cycle (T). In the present study, experiments were designed to test the first two parameters. Detailed procedures are described below.

Flies were exposed to light/dark (T=24) cycles of various L/D ratios after the initial DD free-run. The length of the light period varied from 1 to 22 hours. Some flies were also maintained in LL. Finally, all flies were put back in constant darkness. Light intensity was approximately 0.7 Wm^{-2} (~480 lux) in all light boxes. Time of light-on was set at 12.00 (local time) in all cases. Both light-on and -off were automatically controlled by timers connected to individual light boxes.

2.3.6. Application of LD1:23 as a Tool to Test the Multioscillatory Hypothesis

Earlier entrainment studies using very short photophases (LD 1:23) frequently revealed 'splitting' of the activity rhythm. In order to obtain more examples and to attempt a more systematic study, the same experimental procedure was followed (see 2.3.5) but using only LD1:23 as the entraining cycle in this experiment.

2.3.7. Studies of Entrainment to LD12:12 Cycles with Reduced Light Intensities

All flies were put in DD and allowed to free run for 7 days. Then a cycle of LD 12:12 was applied to all flies for a period of 11 days. The reduced light intensities used in this experiment were divided into 4 groups:

(1) close to the 'intensity threshold' (20 Wm^{-2}) found in the previous experiment (2.3.3.), below which most flies free ran;

(2) below the threshold;

(3) above the threshold which caused most flies to become arrhythmic (40 Wm^{-2});

(4) between the thresholds described in 1 and 3 (20 Wm^{-2} - 40 Wm^{-2}).

Actograms were examined at the end of the light/dark period to determine treatments for the next period. Flies appearing to entrain firmly were then given a new LD cycle with the same light intensity and ratio, but with the light coming on 7 hours later. The method used for reducing light intensity is described above.

2.4. Results and Discussion

2.4.1. The Free-running Rhythms

Studying the free-running period (τ) is a crucial part of any research on circadian rhythmicity for it is the most reliable indicator of endogeneity. Therefore, observing a circadian system releasing its endogeneity in constant conditions with all possible Zeitgebers excluded, is probably the best way to initiate the study of a circadian rhythm.

Patterns of free-running activity

Locomotor activity rhythms of newly emerged flies were recorded in DD at constant temperature (see 2.2.1). In most cases, the recorded activity rhythm showed a clearly rhythmic pattern with a period length always deviating from 24 h, therefore supporting the internal clock hypothesis.

When examined in detail, activity rhythms were classified into several types, according to the patterns. The majority (83%, N= 41) of flies displayed a uniform activity pattern and showed no changes in τ (type 1) throughout the recording period (Fig. 2.4.1.1), whereas some flies (7%) showed spontaneous changes in τ (type 2) (Fig. 2.4.1.2). Records classified into these two rhythmic groups were analysed and are discussed in the next section. A third type of activity rhythm which displayed a complex pattern with more than one rhythmic component (split) was observed in very few cases (5%) (Fig. 2.4.1.3). However, the significance of spontaneous splitting was self-evident since it has been used as evidence in favour of the multioscillatory
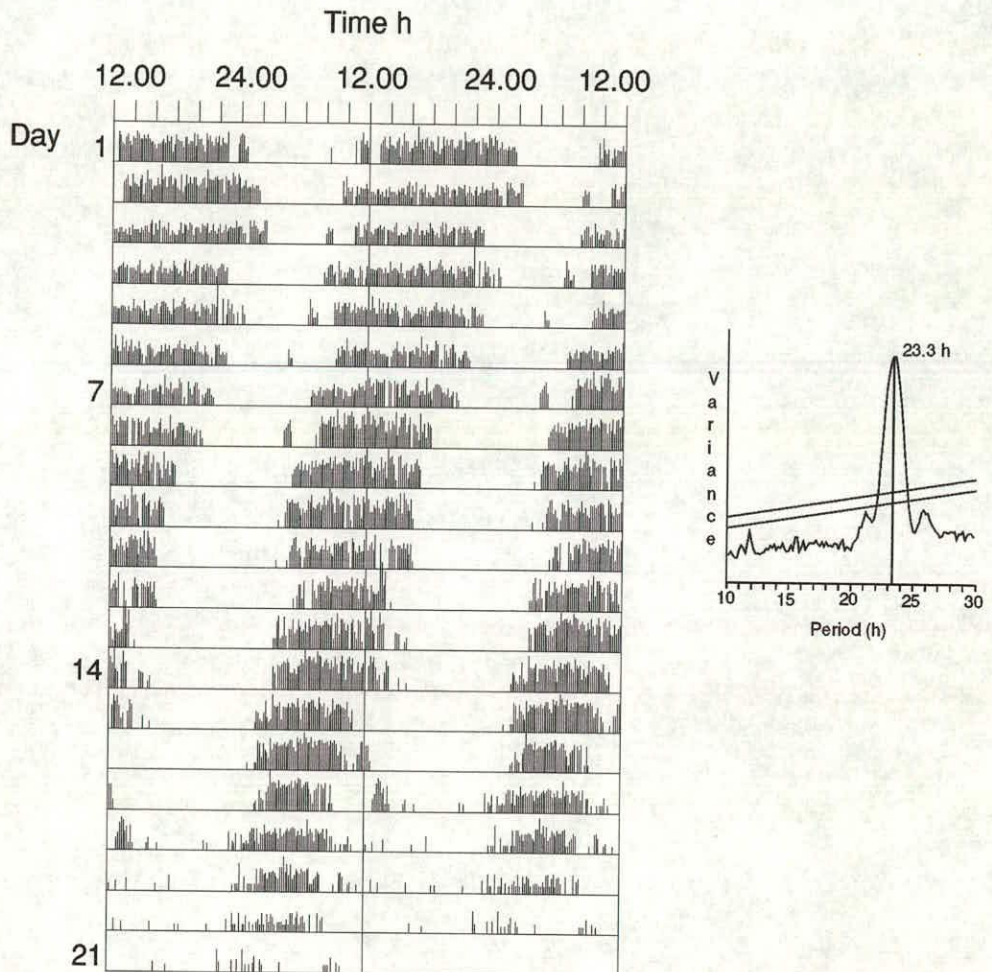
Figure 2.4.1.1. An example of type 1 activity pattern showing no spontaneous change in the free-running period. Result of periodogram analysis showed that the average period length (τ) over the recording period (21 days) was 23.3 h.
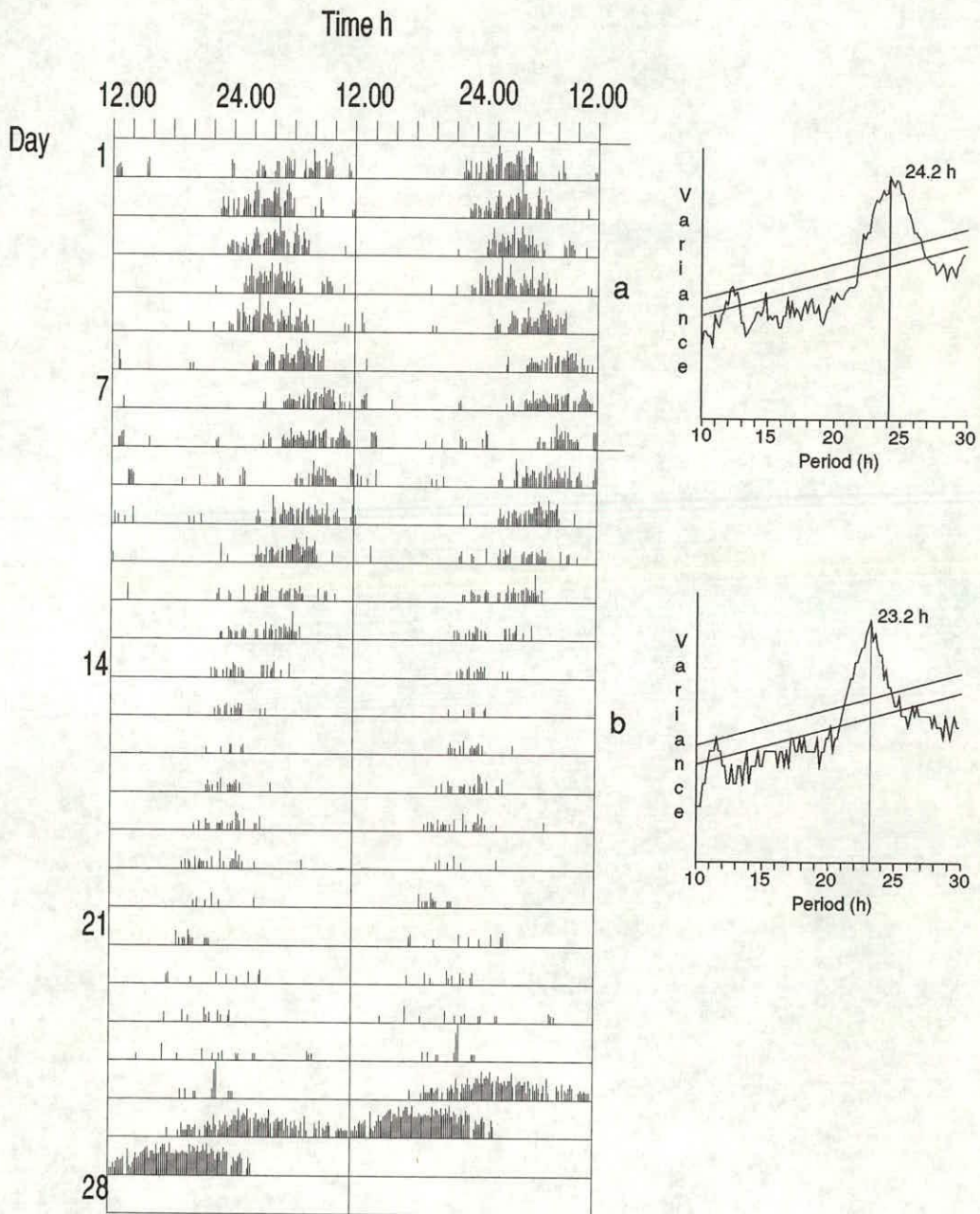
Figure 2.4.1.2. An example of type 2 free-running activity pattern - the activity rhythm changes free-running direction spontaneously during the recording period. In this case, the free-running period (τ) was initially greater than 24 h (a), which then shortened to less than 24 h spontaneously (b).
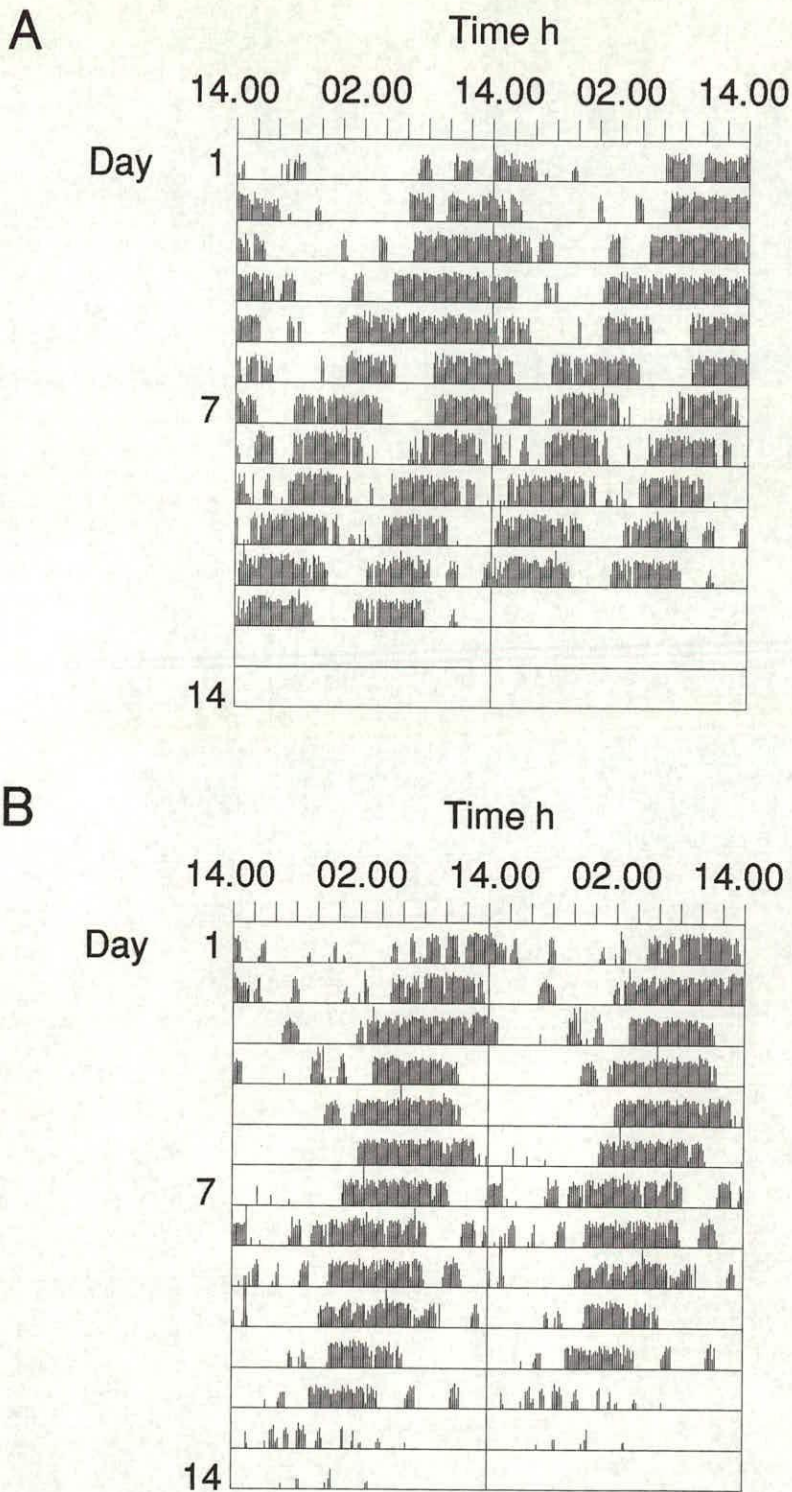
Figure 2.4.1.3. Examples of complex activity patterns. Such spontaneous split of activity rhythm into several components provides strong evidence in favour of the multioscillatory hypothesis. **A**: Rhythm splits to become bimodal on day 6. Both of the split components free-run with a similar period. **B**: The activity rhythm splits into several components, each free-run with different periods. These phenomena can be interpreted as the loosening of coupling between oscillators.

hypothesis for the circadian system. Such complex activity patterns will be discussed separately. Although the majority of flies displayed distinguishable rhythmicity, arrhythmicity was recorded in 2 flies (5%). One fly showed arrhythmicity for at least 17 days, and although activity from day 20-28 showed a tendency to form a rhythmic pattern, periodogram analysis failing to detect any real rhythmicity (Fig. 2.4.1.4). The other case began with a clear activity pattern but became arrhythmic after 6 cycles (Fig. 2.4.1.5).

Unimodal activity patterns

Despite the apparent difference between type 1 and 2 activity patterns, records obtained from both groups shared one generality - flies displayed only one main activity band which can normally be converted into a 'periodogram' with one distinguished peak. Such a pattern is therefore defined as unimodal activity.

Flies showing type 1 activity kept one free-running direction with a period (τ) normally shorter than 24 h (Fig. 2.4.1.1). However, although minor, some flies free-ran with a τ longer than 24 h throughout the experimental period (Fig. 2.4.1.6). The mean value of the free-running period (τ) over a recording period of at least 7 days was 22.68 $\pm$ 0.596 h (N=200) (Fig. 2.4.1.7). This compares with a mean τ of 22.53 $\pm$ 0.78 h (N=78) from an earlier study (Cymborowski *et al.*, 1993).

The analysis of τ revealed some overall information about the circadian rhythmicity. Further insight, i.e. analysing other parameters, such as active phase (α), is essential before reaching any conclusion. Active phases of the same individual, obtained from two periods, day 1-14 and day 15-28, were compared. Mean α was
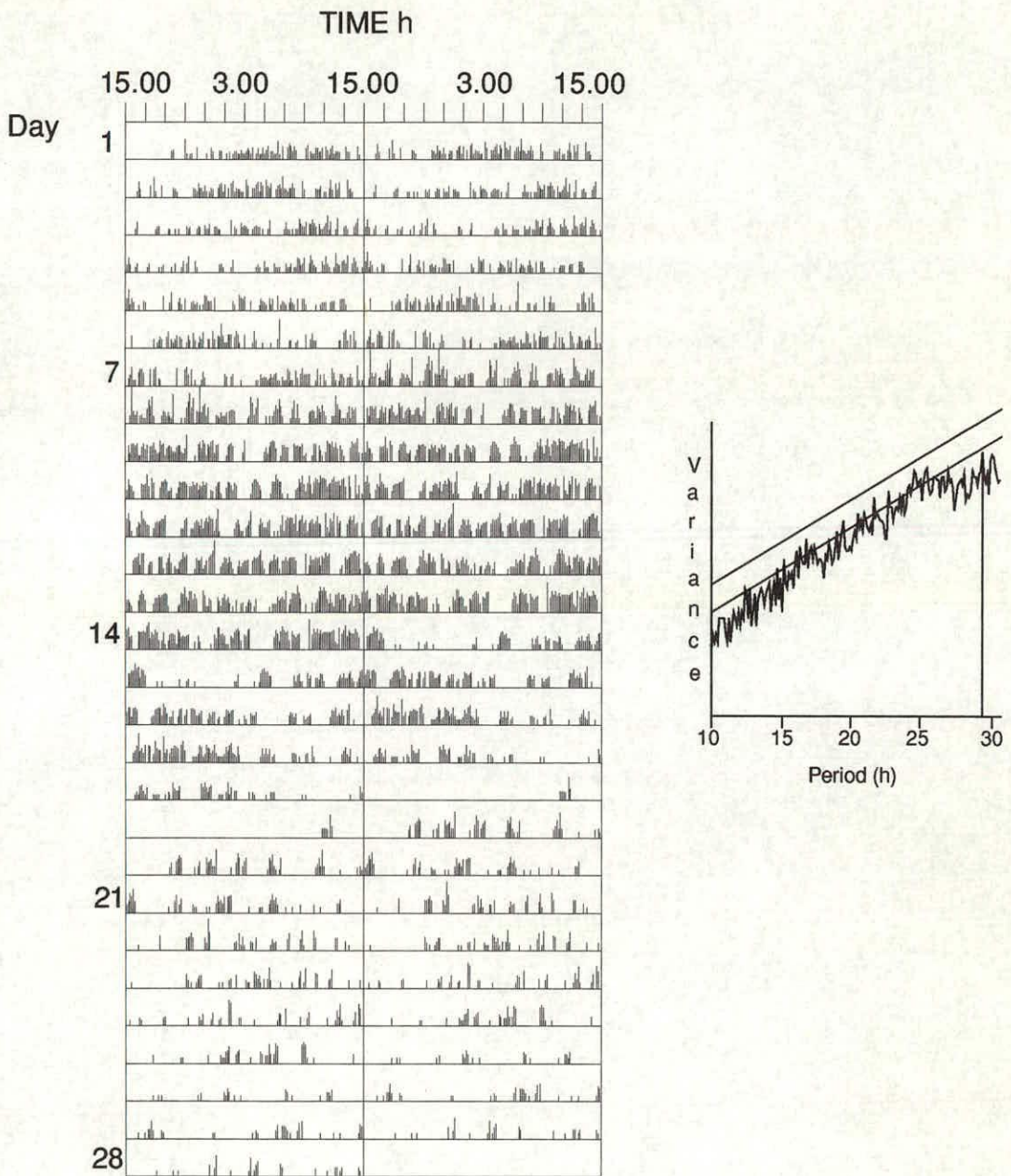
Figure 2.4.1.4. An example of arrhythmicity recorded in DD. Although activity between day 20 and 28 showed a tendency to form a rhythmic pattern, periodogram analysis failed to detect any real rhythmicity.
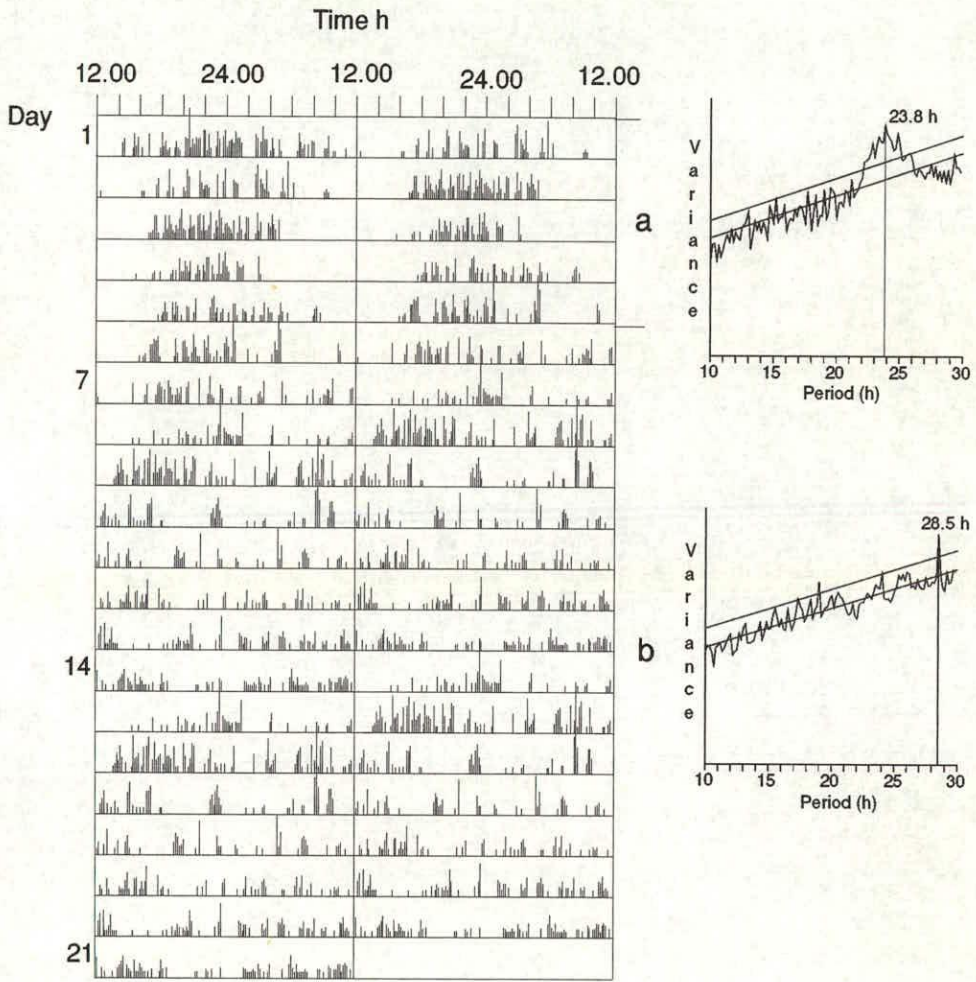
Figure 2.4.1.5. An example of arrhythmicity occurring spontaneously after several rhythmic cycles.
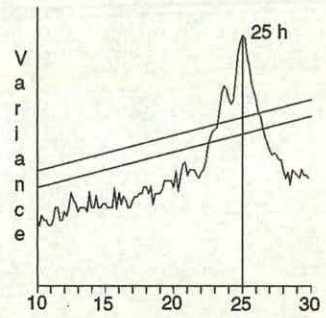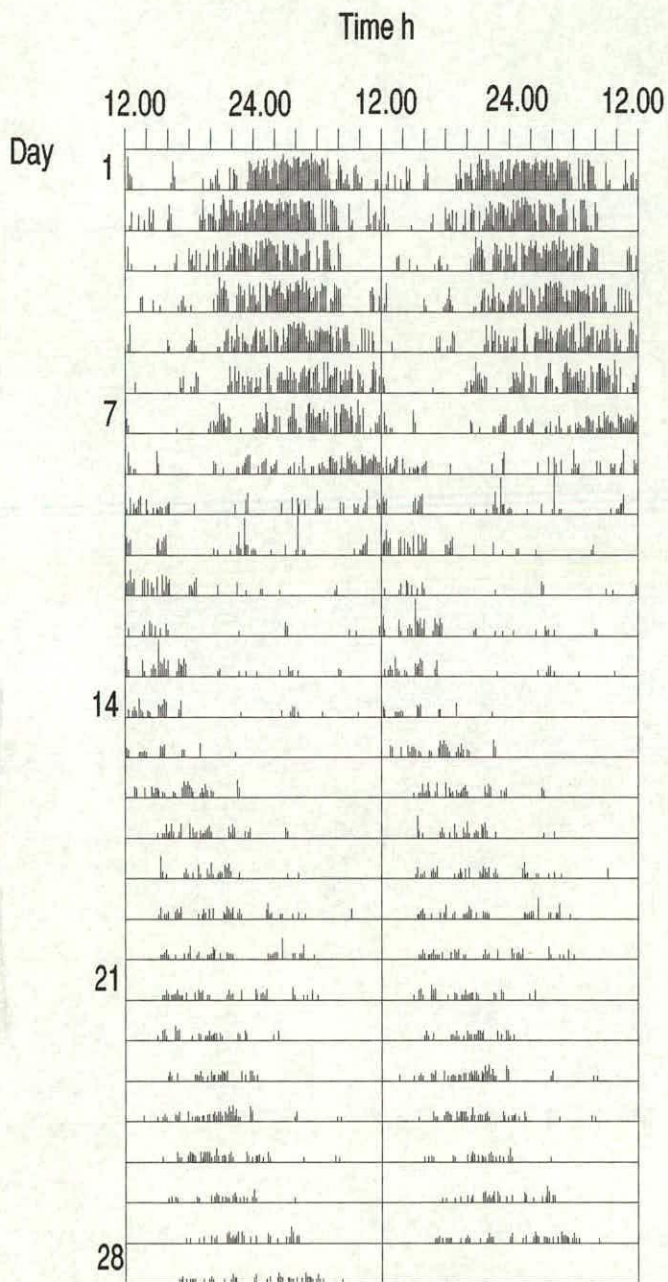
Figure 2.4.1.6. An example of type 1 activity pattern showing a free-running period greater than 24 h throughout the recording period.
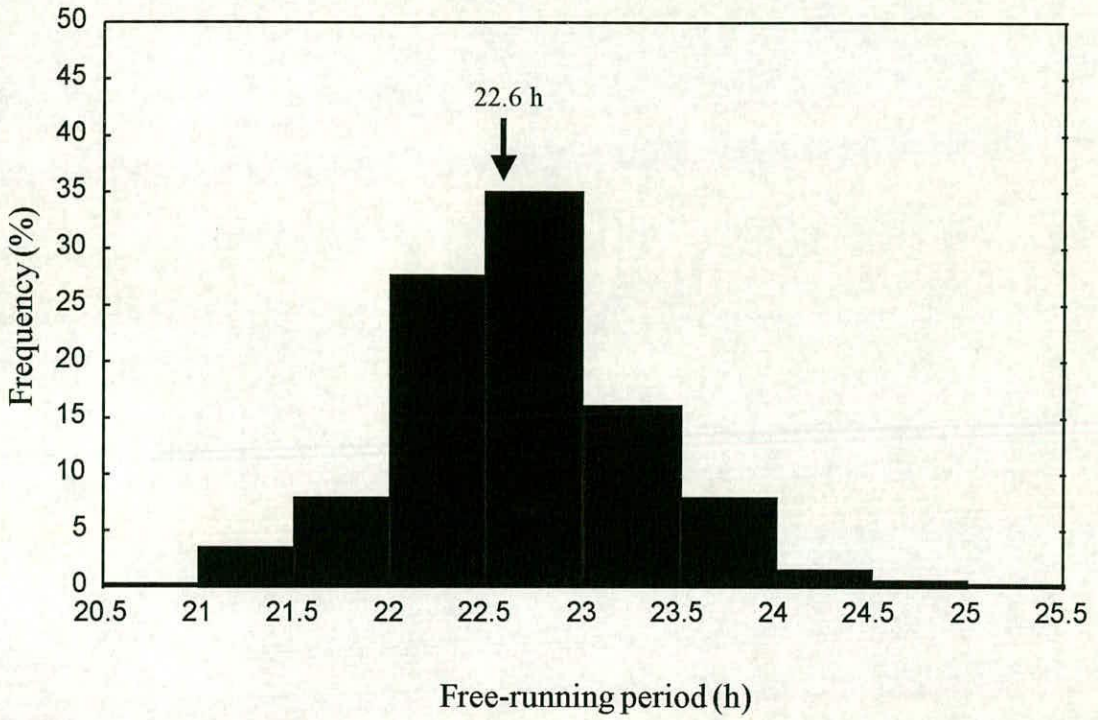
76

Figure 2.4.1.7. Free-running period of *Calliphora vicina* recorded over a period of at least 7 days in constant darkness (DD). The mean value of the free-running period (τ) = 22.6±0.596 h (N=200).

11.73 ± 2.267 h for days 1-14, and 11.67 ± 2.181 h for days 15-28. These values of

α were not significantly different (t=0.8825, df=16). However, it was previously

reported (Kenny, 1989) that in long recording (up to 50 days) activity rhythms

showed a slight lengthening of τ but shortening of α with an increase of age. This so

called 'ageing effect' of τ was discussed by Pittendrigh and Daan (1974) for rodents.

In their case, τ was observed to shorten as the animals aged. Furthermore, this result

suggested that the coupling between oscillators was tight in *C. vicina*. A gradually

lengthened α may indicate a loosely coupled oscillatory system.

Flies showing type 2 activity started with a free-running period either greater or

less than 24 h. Despite the initial free-running period, flies gradually changed their

free-running direction (τ < 24 h became τ > 24 h, or τ > 24 h became τ < 24 h),

which normally occurred after 4-6 circadian cycles (Fig. 2.4.1.2). When this

occurred, the activity rhythm did not appear to be reset to achieve its new steady

state. Instead, the period change occurred spontaneously with a daily phase change

which led the activity rhythm towards the direction of lengthening or shortening. This

type of phase change, without external forces, appeared to be different from the

phase-shift provoked by a perturbation which rarely changed the direction of the free-

running period. Spontaneous changes of τ during constant conditions have been

attributed to an interaction between different oscillators in a multioscillatory circadian

system (Eskin, 1971; Pittendrigh and Dann, 1976b). Therefore, the spontaneous

changes of τ observed in the present study provided strong evidence in support of the

multioscillatory hypothesis. According to this hypothesis, the various patterns depend

on the strength of the coupling among oscillators which can be affected by internal or external factors.

Complex activity patterns

Spontaneous splitting of activity rhythm has been reported most frequently in mammals which normally occurs fairly gradually, compared to the more abrupt split observed in insects (Kenny, 1989). In the present study, this is classified as complex activity patterns and was only observed in two flies (Fig. 2.4.1.3.). Their infrequent occurrence may be an indication that the oscillators in *C vicina* are normally tightly coupled. The actogram in Fig. 2.4.1.3.A. showed a free-running rhythm which started with a unimodal pattern ($\tau < 24$ h) which then split after 5 cycles into two components each free-running with a τ value similar to the one prior to the splitting. When this occurred, the activity band appeared to loosen (overall activity phase (α) gradually increased), which eventually led to the splitting. The actogram in Fig. 2.4.1.3.B. showed a more complicated type of splitting. The coupling was initially loose so as to display two components, each free-running with its own τ. For 4 cycles, the main activity band free-ran with a τ less than 24 h while the other ('slow') component free-ran with a τ greater than 24 h. The slow component joined the main activity band on day 5 whereupon a 'fast' component left the main activity band with $\tau < 24$ h. Such spontaneous splitting is different from splitting provoked by exogenous stimuli, which normally contains component(s) responding directly to the

stimuli. Comparisons between these two will be discussed in more detail in section 2.3.6.

2.4.2. Effects of Temperature

Although an immense part of present knowledge on circadian rhythmicity has been derived from experiments involving photic factors, studies on the effects of temperatures should be encouraged so as to approach circadian systems from a different angle, especially when an increasing number of reports (Hamm *et al.*, 1975; Njus *et al.*, 1977; Broda *et al.*, 1989; Moore and Rankin, 1993) have been suggesting a synergism of light and temperature effects on the underlying circadian clock.

As in most experiments involving photic factors, τ has been used to measure temperature effects on the circadian system. In the present study, experiments were carried out to record the locomotor activity rhythm of *C. vicina* at various constant ambient temperatures. The results indicated that τ showed rather little variation at the three temperatures (15, 20, 25°C) applied in the present study (Fig. 2.4.2.1 & Fig. 2.4.2.2). For temperature step-up experiments, the mean period length (τ) was 22.7 ± 0.338 h (N=34) at 20°C and 23.09 ± 0.464 h (N=34) at 25°C. For temperature step-down experiments, the mean period length (τ) was 22.62 ± 0.525 (N=20) at 20°C and 23.51 ± 0.671 (N=20) at 15°C. Although statistical analyses showed significant differences between the mean period lengths at different temperatures (Wilcoxon; T= 95.0, p < 0.001, N=34 for temperature steps-up; T=3.0, p < 0.001, N=19 for steps-
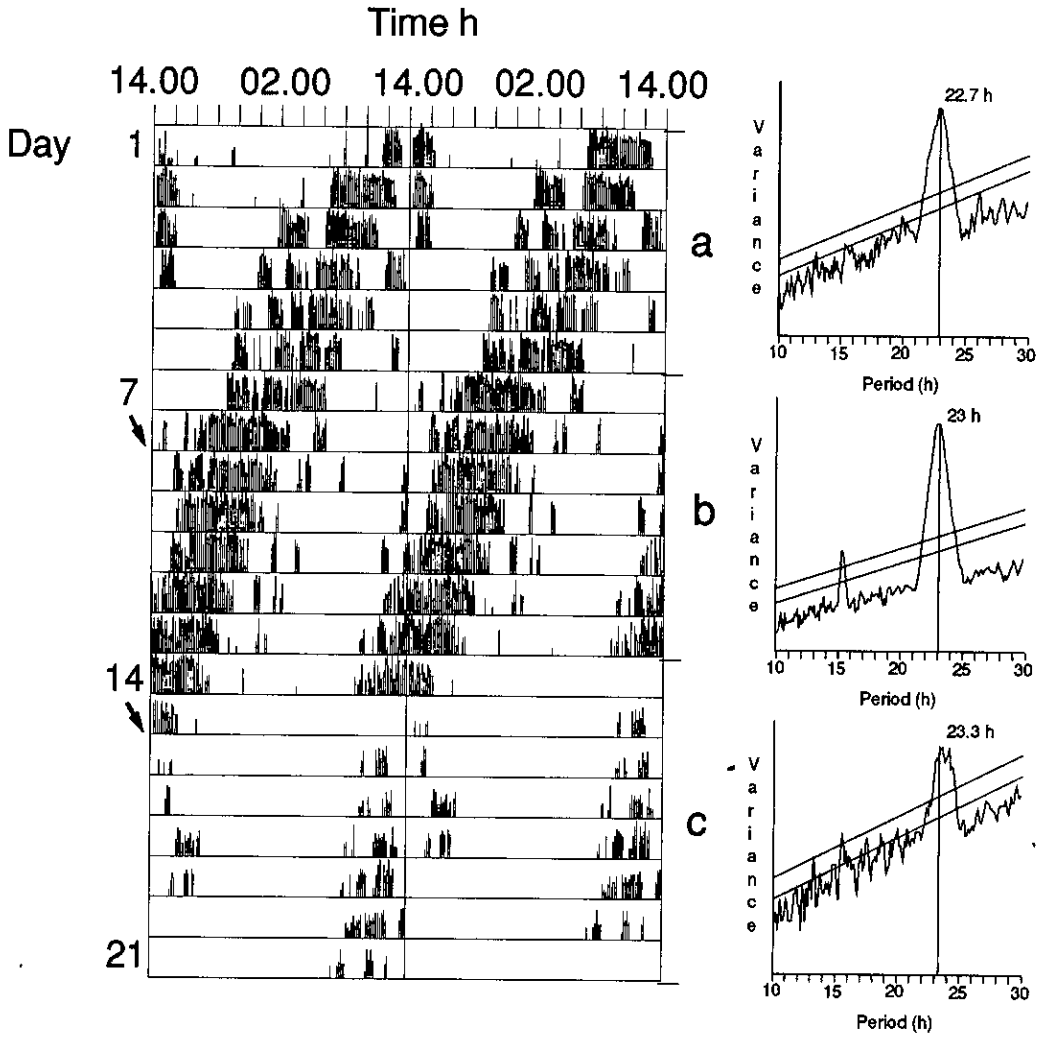
Figure 2.4.2.1. An example showing the effects of temperature changes on the activity rhythm in *C. vicina.* The activity rhythm was recorded in DD throughout the experiment. The fly experienced two temperature steps - firstly a step-up (20 - 25⁰C), then a step-down (25 - 15⁰C). The arrows indicate time of the temperature steps. Result of periodogram analyses showed little change in τ value.
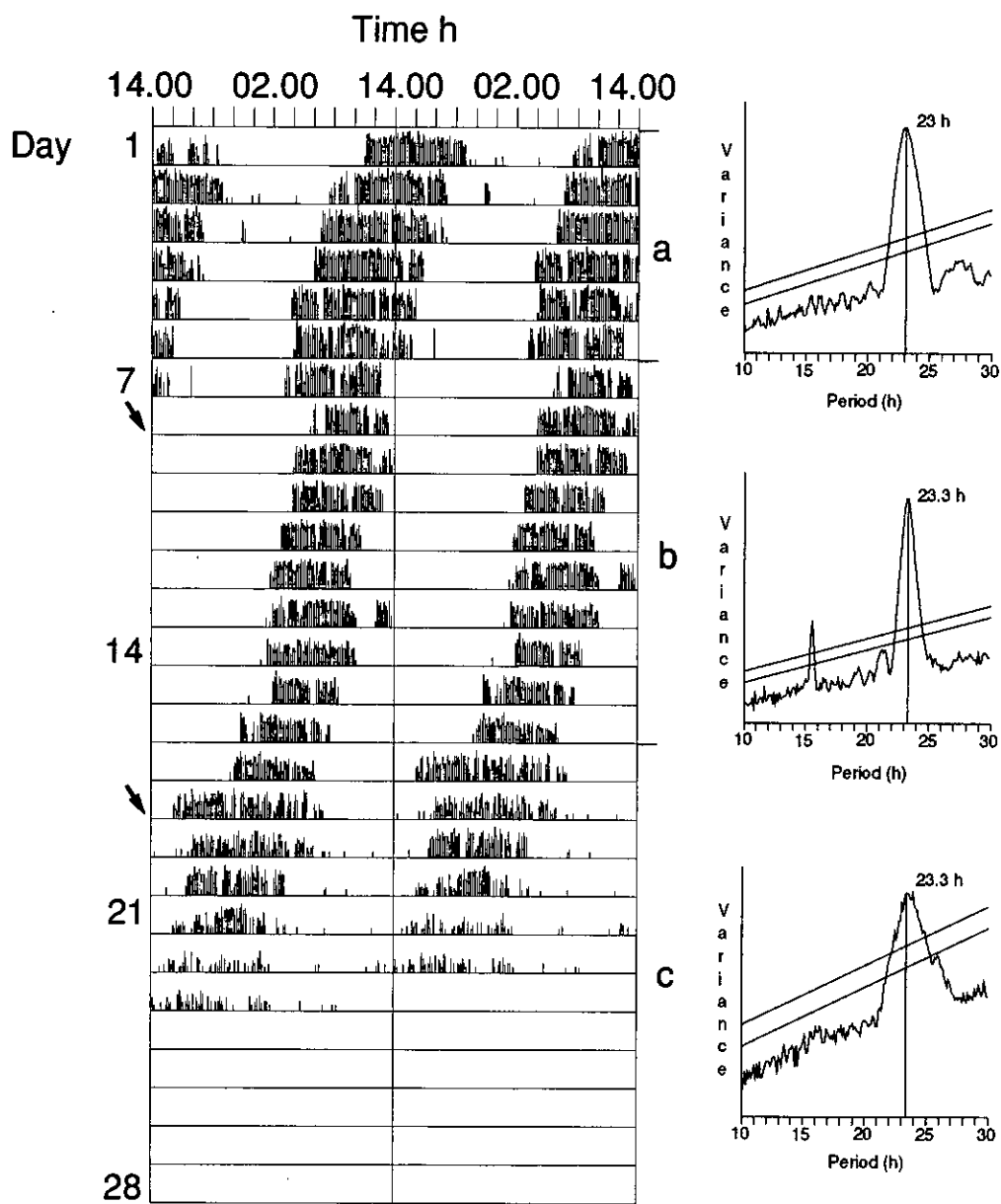
Figure 2.4.2.2. An example showing the effects of temperature changes on the activity rhythm in *C. vicina*. The activity rhythm was recorded in DD throughout the experiment. The fly experienced two temperature steps - firstly a step-down (20 - 15⁰C), then a step-up (15 - 25⁰C). The arrows indicate time of the temperature steps. Result of periodogram analyses showed little change in τ value. However, temperature steps-up generated advanced phase-shifts, whereas steps-down generated delayed phase-shifts. Moreover, the active phase shortened upon steps-down and lengthened upon temperature steps-up.

down), temperature coefficients (Q_{10}) derived from both temperature step-up and -down experiments were all very close to 1 (Table 2.4.2.1).

Furthermore, regardless of the direction of the temperature steps, most flies in both step-up (20 - 25°C) (71%, N=34) and step-down (20 - 15°C) experiments (84%, N=19) showed a lengthening of τ . Nevertheless, the direction of the temperature step seemed to affect the active phase (α) in a different way. In temperature steps-up experiments, most flies (65%, N=34) showed a lengthening of the active phase (α) (Fig. 2.4.2.1), whereas in temperature steps-down experiments, most flies (84%, N=19) shortened their α (Fig. 2.4.2.2). Further analyses were carried out in the attempt to explore possible correlations between changes of τ and changes of α, and temperature steps. Among flies that showed a lengthening of τ in temperature step-up experiments, the majority (71%, N=24) also showed a lengthening of α; whereas, in the same experiments, 50% (N=10) of flies showed a shortening of both τ and α. In temperature step-down experiments, among flies that showed a lengthening of τ the majority (88%, N=16) showed a shortening of α; whereas among the very few flies showing a shortening of τ in step-down experiments, 50% (N=2) of experimental flies also shortened their α.

Temperature steps-up and -down generated both phase-delays ($-\Delta\phi$) and phase-advances ($+\Delta\phi$) (Fig. 2.4.2.1 & Fig. 2.4.2.2). When plotting such phase shifts as a function of phase, both phase response curves for steps-up and -down showed the weak ('type 1') response (Fig. 2.4.2.3). The PRC for temperature steps-up consisted of a large portion of advanced phase-shifts and a small portion of delayed phase-shifts; whereas the PRC for steps-down consisted of a large portion of delayed phase-

Temperature treatment		N	α	τ	Q_{10}
I	20°C	34	10.37 ± 1.918	22.7 ± 0.388	0.9838 ± 0.0218
	25°C	34	11.48 ± 2.570	23.09 ± 0.464	
	15°C	15	7.34 ± 2.064	23.93 ± 0.511	1.0382 ± 0.0284
II	20°C	19	11.12 ± 1.802	22.62 ± 0.525	1.034 ± 0.0291
	15°C	19	8.65 ± 3.277	23.51 ± 0.671	
	25°C	3[*]			

* : sample size too small

Table 2.4.2.1. Results of temperature step-up and -down experiments. In experiment I, the temperature was firstly increased from 20°C to 25°C then followed by a 10° decrease down to 15°C. In experiment II, the temperature was firstly decreased from 20°C to 15°C then followed by a 10° increase up to 25°C.
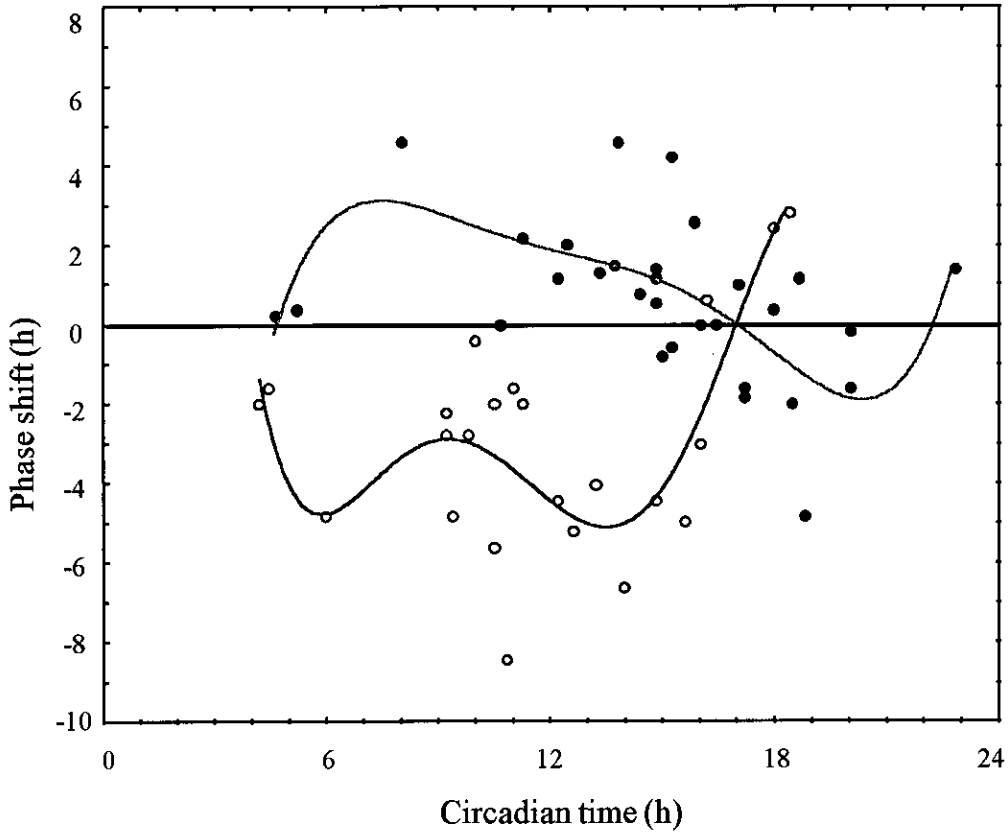
Figure 2.4.2.3. Phase response curves (PRCs) for temperature steps-up and -down. The solid dots were phase-shifts measured in temperature steps-up experiments (20 - 25°C). The open dots were phase-shifts measured in steps-down experiments (20 - 15°C). Both curves consist of advance (+Δφ) and delay (-Δφ) portions and appear to be mirror images. As with the light PRC (see Fig. 2.4.4.1), the existence of both advance and delay phase-shifts implies the entrainability of the activity rhythm to temperature cycles.

shifts and a small portion of advanced phase-shifts, namely, the two PRCs appeared to be 'mirror images' (Fig. 2.4.2.3). The PRC for temperature steps-down resembled the PRC for one hour light pulses (see Fig. 2.4.4.1) in that they both consisted of a large portion of delayed phase-shifts and a small portion of advanced phase-shifts; and that an intermediate area where a sharp change from delay to advance existed in both curves of the PRC - its general properties and how it has been applied as a basis to develop models for the circadian system, will be discussed in a later section (2.4.4).

2.4.3 Effects of Constant Light (LL)

Part I.

One of the fundamental properties of a circadian rhythm is that it persists (free-runs) in constant conditions, almost always in continuous darkness (DD) and often in continuous light (LL), provided that the intensity is below a threshold (Hong and Saunders, 1994; Saunders, 1982) with an altered period. Early researchers observed such a change of free-running period when transferring experimental animals from DD to LL (or vice versa), and noted a difference between diurnal and nocturnal animals. Such studies uncovered a generality called 'Aschoff's rule' which is widely applicable to vertebrate species. Aschoff's rule states that τ lengthens with an increase in light intensity for nocturnal animals ($\tau_{DD} < \tau_{LL}$), but shortens for diurnal animals ($\tau_{DD} > \tau_{LL}$). Nevertheless, in insects, many exceptions were found to violate this rule (Saunders, 1977).

Previous studies (Kenny, 1989) on *C. vicina* locomotor activity, showed that constant light at high intensity (0.7 Wm^{-2}) resulted in arrhythmicity which is difficult to analyse. Such an observation raised one question - is the arrhythmicity caused by LL truly endogenous or is it a masking effect due to the direct response to the exogenous stimulus? If the latter is responsible for such arrhythmicity, can the masking effect be eliminated by applying reduced light intensity? The present investigation was carried out, firstly, to find the intensity below which the activity rhythm persists in constant light, so as to test 'Aschoff's rule' and observe the features of the activity rhythm in constant light at reduced intensities.

After an initial free-run in DD (7 days), experimental flies were exposed to LL of various reduced intensities ranging from 0.01 Wm^{-2} to 0.07 Wm^{-2} for at least 7 days before transferring back to DD for a final free-run. Activity rhythms so recorded showed that most flies became arrhythmic when exposed to an irradiance higher than 0.03 Wm^{-2} (Fig. 2.4.3.1). Data obtained in this group were pooled with the results of a further experiment and will be discussed in the next section. Flies exposed to an irradiance between 0.03 Wm^{-2} and 0.02 Wm^{-2} showed an intermediate behaviour, going through a few days of arrhythmic behaviour before the rhythm resumed with a new τ value, usually greater than 24 h (Fig. 2.4.3.2). Lastly, flies exposed to an irradiance below 0.02 Wm^{-2} free-ran upon transfer to LL with a lengthened τ (Fig. 2.4.3.3). However, despite the intensity they were exposed to, all experimental flies
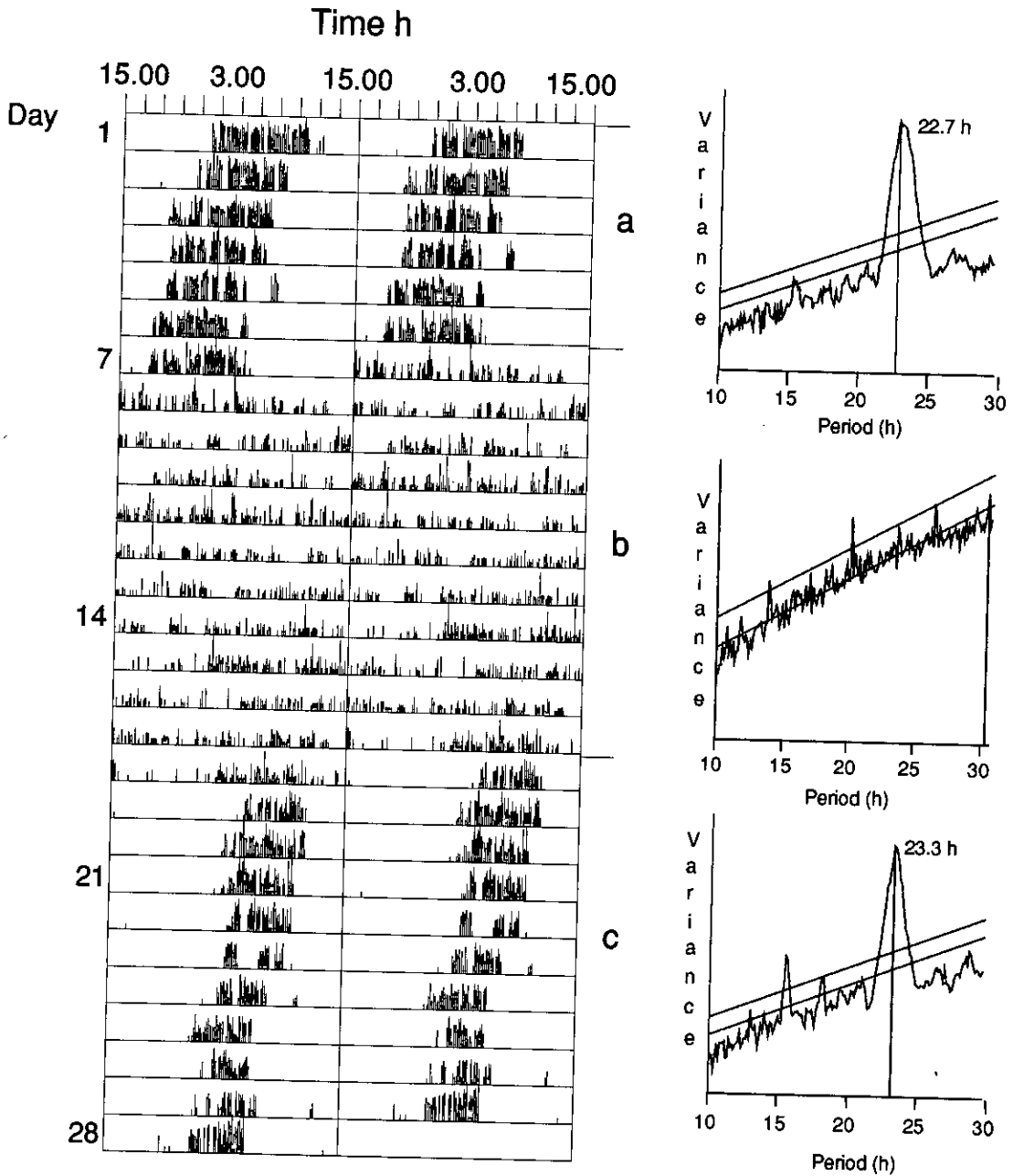
Figure 2.4.3.1. An example of arrhythmicity recorded in constant light (LL) of bright light intensity. The fly was held in DD for 7 days (a), then in LL at 0.033 Wm^{-2} for 11 days (b), and was finally returned to DD (c). Periodogram analysis failed to detect any real rhythm in LL. The fly showed little change in τ before and after the exposure to LL.
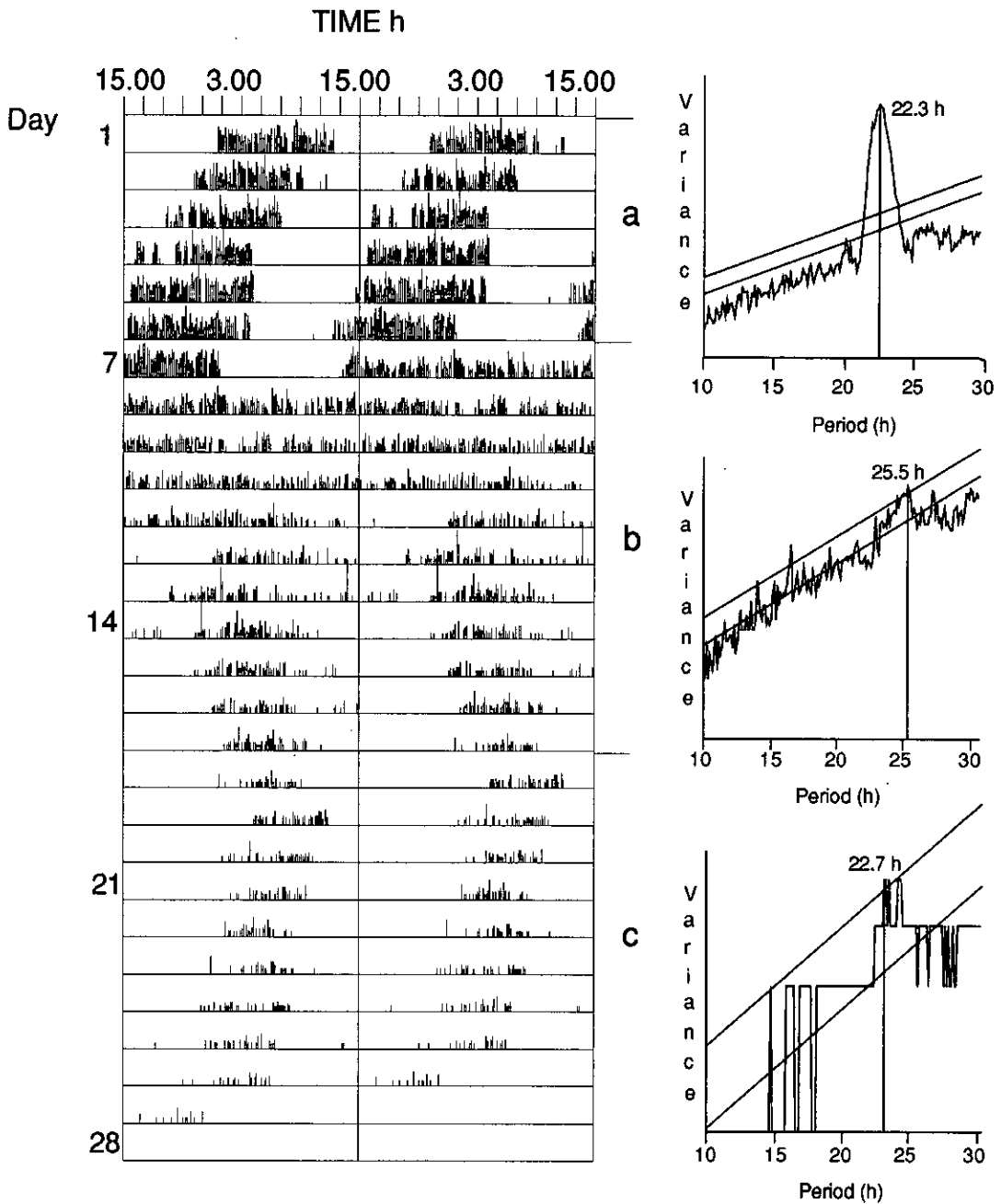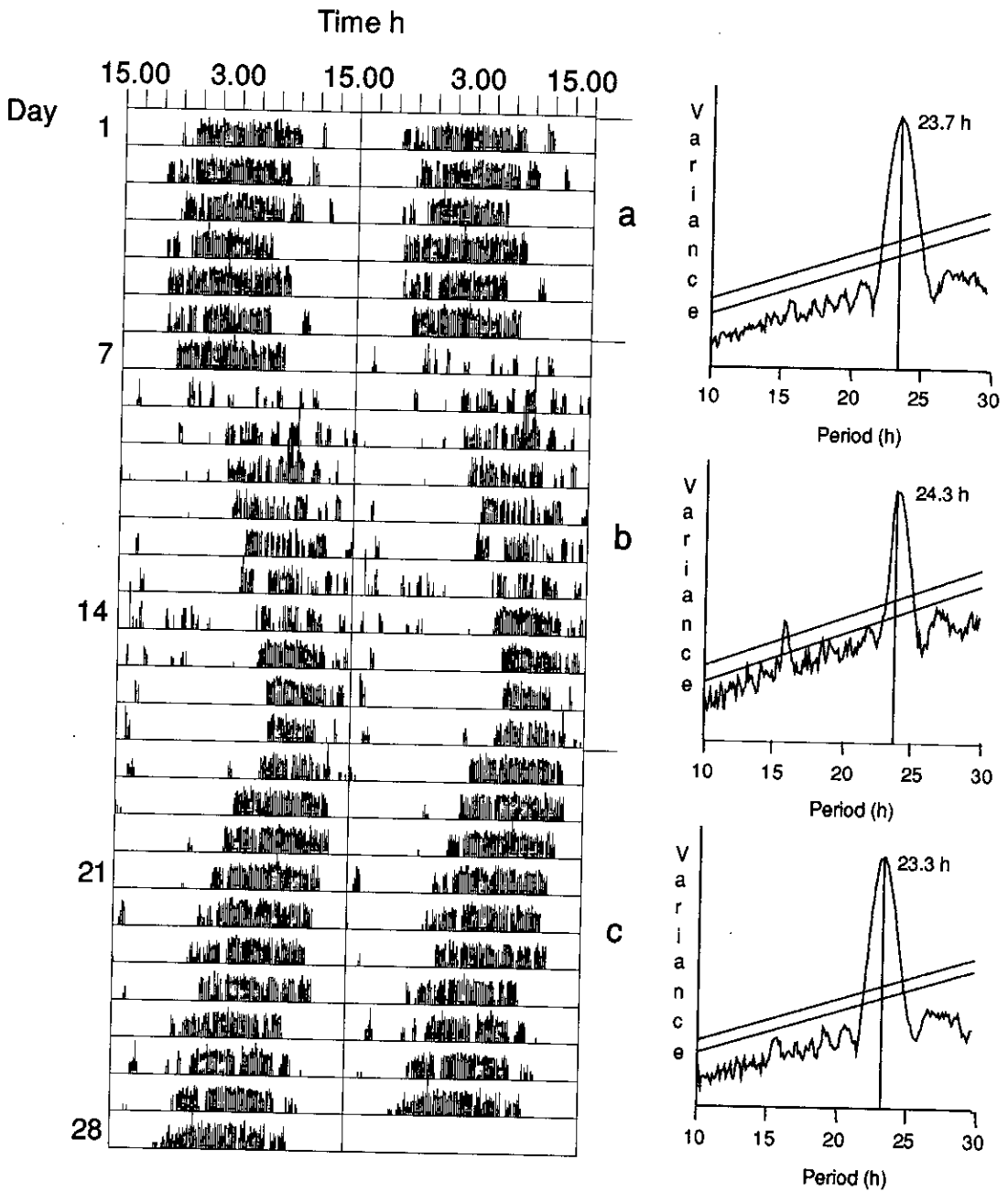
Figure 2.4.3.2. An example of intermediate behaviour recorded in constant light (LL). The fly was held in DD for 7 days (a), then in LL at 0.024 Wm^{-2} for 11 days, and was finally returned to DD (c). The fly became arrhythmic upon transfer to LL and remained in such state for 4 days before the rhythm was resumed with a lengthened τ (>24 h). τ varied little before and after the exposure to LL. The inability to produce a clear periodogram (c) was due to a paucity of data.

Figure 2.4.3.3. An example of the activity rhythm free-running in constant light (LL) of dim light intensity. The fly was held in DD for 7 days (a), then in LL at 0.018 Wm^{-2}, and was finally returned to DD (c). The fly changed its free-running direction upon transfer to LL, therefore τ was lengthened. τ was shortened again to a value close to that of the initial free-run when the fly was returned to DD.

shortened τ again when returned to DD. The intermediate pattern seen in Fig. 2.4.3.2 suggested that the circadian clock kept oscillating in LL and that the arrhythmicity seen in bright light is likely to be a masking effect of the exogenous stimulus.

All flies (N=13) exposed to dim LL (< 0.03 Wm^{-2}) appeared to be rhythmic. Mean free-running period for the initial free-run (τ_{DDi}) was 22.95 $\pm$ 0.56 h which lengthened to 24.61 $\pm$ 0.89 h (t = 5.0930, df = 12, p $\ll$ 0.001) (τ_{LL}) upon transfer to LL, then shortened to 22.97 $\pm$ 0.61 h (τ_{DDf}) when returned to DD for the final free-run. τ_{DDi} was not significantly different from τ_{DDf} (t = 0.1209). Further analysis showed that τ appeared to increase systematically with light intensity when transferred from DD to dim LL (Fig. 2.4.3.4.A) and decreased in a similar manner when returned from dim LL to DD (Fig. 2.4.3.4.B). The correlation between the increase of τ and the irradiance was shown in Fig. 2.4.3.5 which presents data for 29 flies exposed to LL at irradiances between 0.018 and 0.055 Wm^{-2}. In those flies that remained rhythmic in LL, τ increased with light intensity up to 14% increase. Most flies became overtly arrhythmic when exposed to an irradiance above 0.03 Wm^{-2}. However, a different result was obtained for the locomotor activity of the blowfly, *Phormia* (*Protophormia*) *terraenovae* in LL (Aschoff and von Saint Paul, 1982) which suggested that τ lengthened but did not depend in a systematic way on intensity of illumination within the range between 2 to 2000 lux. However, the conclusion is therefore that in *C vicina*, a diurnal insect, τ lengthens upon transferring from DD to LL ($\tau_{DD} < \tau_{LL}$) which violates 'Aschoff's rule'. Studies on the effects of continuous light on activity rhythms in Diptera are few (Hong and Saunders, 1994). However,
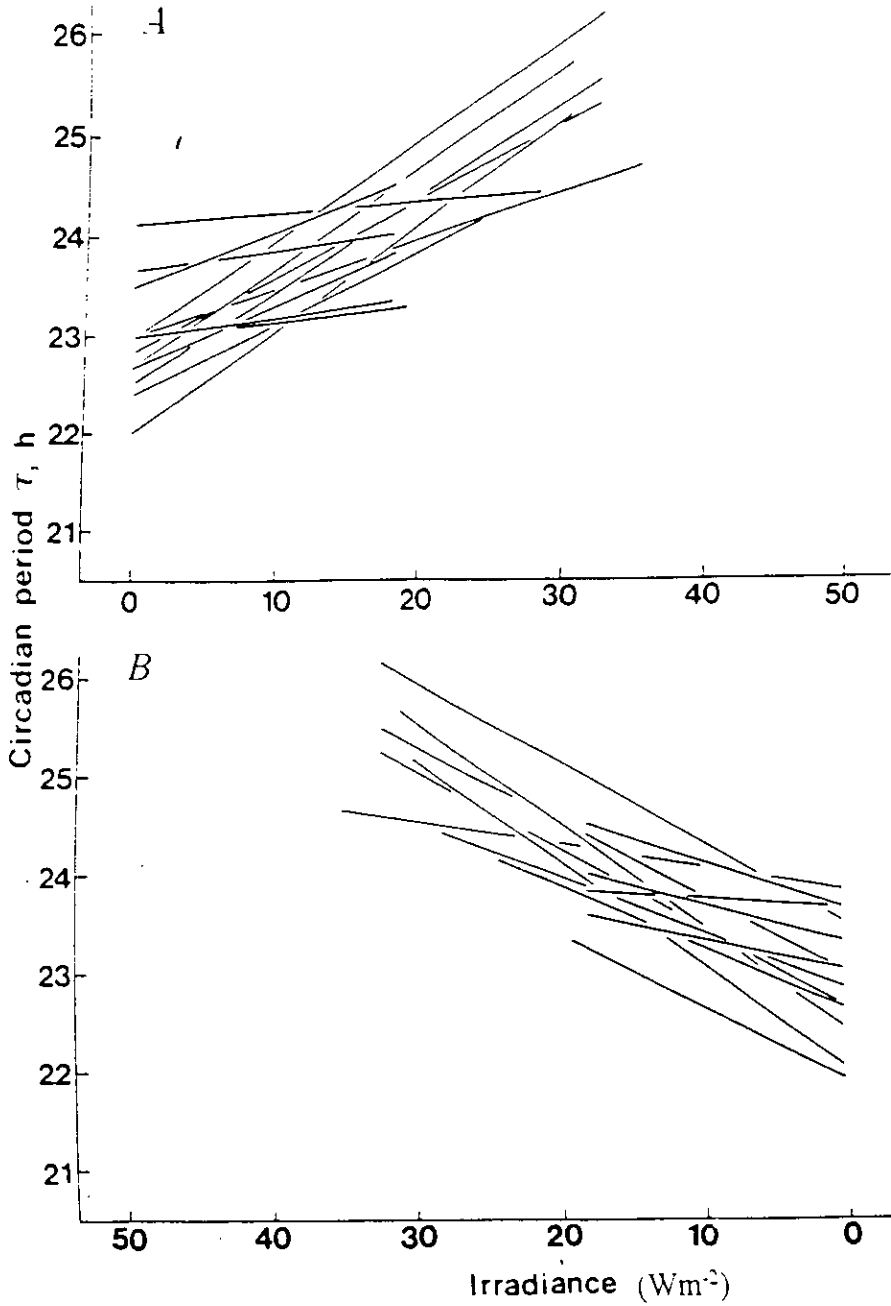
Figure 2.4.3.4. Changes in τ after transferring from DD to dim LL (<0.035 Wm⁻²) (A) and from dim LL back to DD (B). All flies (N=13) remained rhythmic in dim LL and showed a systematic increase in τ.
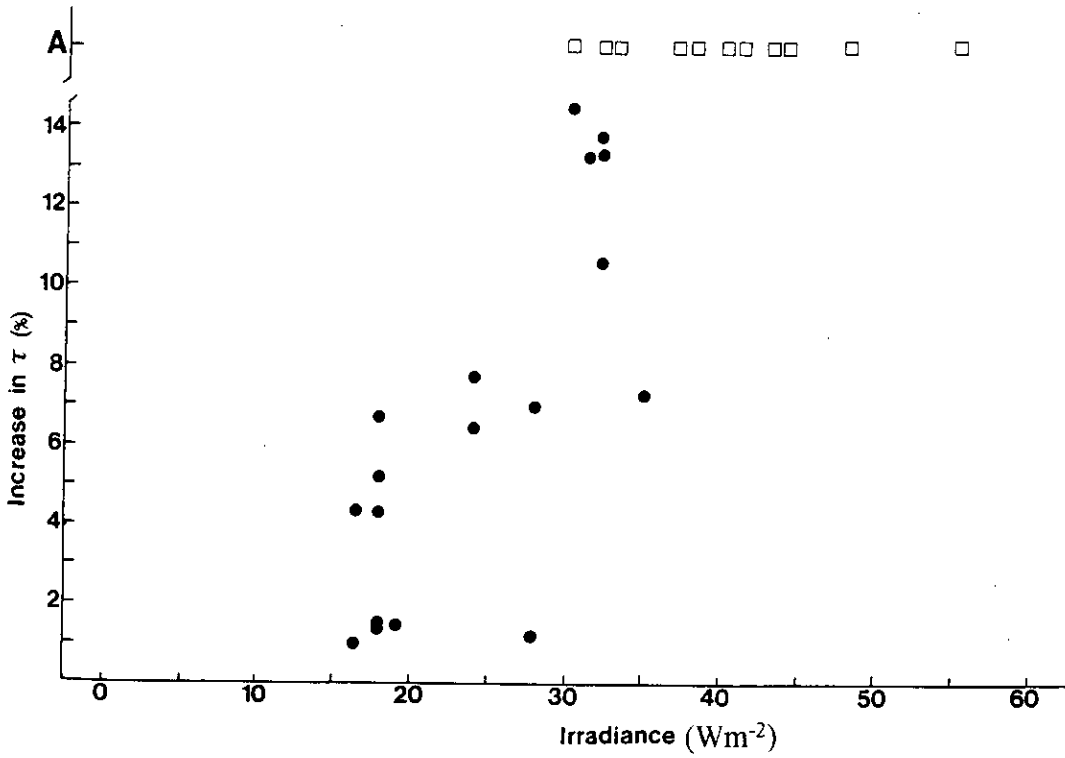
Figure 2.4.3.5. Increase in free-running period (τ) of *Calliphora vicina* upon transfer from DD to LL of various light intensities. A in x axis indicates arrhythmicity. Flies exposed to irradiance above 0.03 Wm^{-2} became arrhythmic ($\square$).

the lengthening of τ in LL has also been reported for the flight activity of two mosquitoes, *Aedes aegypti* (Taylor and Jones, 1969) and *Culex pipiens fatigans* Wied (Jones, 1976), and for the *Drosophila pseudoobscura* eclosion rhythm (Chandrashekaran and Loher, 1969; Winfree, 1974). Working on the unicellular alga *Gonyaulax polyedra*, Roenneberg and Hastings (1991) also reported that the lengthening of τ was a consequence of constant red light or a red light pulse. They also concluded that the magnitude of phase shifts decreased as intensity increased, the increase of intensity, however, resulted in stronger after-effects.

'Aschoff's rule' was further extended to cover two other parameters, the ratio of active phase to rest phase (α/ρ), and the total amount of activity per circadian cycle. To test the extended rule, Aschoff kept some vertebrate species in constant conditions and found that both parameters increased with increasing light intensity in diurnal animals, but decreased with light intensity in nocturnal animals. This is now well known as the 'circadian rule'. Results of the present investigation did not confirm this rule. Among the experimental flies which showed a lengthening of τ upon transfer to dim LL, the majority (68%, N=25) were found to shorten their active phase (α), while the others (32%) to lengthen it. It appeared that the lengthening of τ does not necessarily lead to the lengthening of α. In the cases of shortening, average active phase in DD (α_{DD}) was shortened from 9.08 ± 1.59 h to 7.17 ± 2.21 h (α_{LL}) on transferring to LL. In the case of lengthening, α_{DD} was lengthened from 9.08 ± 1.97 h to 11.41 ± 1.94 h on transfer to LL.

As to the total amount of activity, evidence has been obtained in many organisms that circadian rhythms decay in continuous light. In insects, population rhythms, such as pupal eclosion rhythm have been shown to be suppressed by often very low light intensities. Measuring the threshold for photosuppression in *D. pseudoobscura*, Winfree (1974) reported that pupal eclosion rhythm was suppressed by continuous light as dim as 0.01 erg cm^{-2}sec^{-1}. Furthermore, he suggested that the rhythm was suppressed faster and more completely by higher intensities. Results of the present investigation showed a similar suppression of the locomotor activity of *C. vicina* when experimental flies were exposed to bright LL (> 0.03 Wm^{-2}). Such suppression was eliminated and activity level was restored as soon as the fly was transferred back to DD (Fig. 2.4.3.1). On the other hand, the activity level of flies exposed to intensity below 0.02 Wm^{-2} did not appear to be suppressed in dim LL (Fig. 2.4.3.3).

Part II.

There are various ways in which a rhythm can be initiated after the fade-out of a previous rhythm or in the absence of a rhythm. Working on *Drosophila* pupal eclosion, (Pittendrigh, 1966) found that on transfer of cultures from LL to DD, the pupal eclosion rhythm resumed at a fixed circadian time. Pittendrigh, therefore, initially suggested that the prolonged light period, in excess of about 12 hours, constrained the circadian pacemaker thus 'freezing' the oscillator, provided that the intensity was sufficiently high. To test this hypothesis in *C. vicina*, experimental flies were allowed to free-run in DD for 7 days before exposure to LL at reduced

intensities ranging between 0.02 - 0.074 Wm^{-2}, then returned to DD at different times.

For the arrhythmic flies (N= 36) which were exposed to an irradiance between 0.033 and 0.074 Wm^{-2} (Fig. 2.4.3.6) the latency between the time the light went off and the time when the next bout of activity began was measured. These latencies varied between 3.6 and 22.4 hours and no correlation was found between the circadian time where light went off and the onset of activity. Moreover, the activity of some flies continued after light went off (Fig. 2.4.3.7) but appeared to be different from the light-rebound effect observed in another experiment (see Fig. 2.4.5.1). When extrapolating the activity rhythm in the final free-run back to the last cycle in LL, such prolonged activity coincided with the expected end of activity. This observation suggested the continuation of the oscillation in LL.

When exposed to dim LL ($\cong$ 0.02 Wm^{-2}), flies free-ran, as observed in the previous experiments (see Fig. 2.4.3.3 part I.), with a lengthened τ which was shortened again upon transfer to DD. Despite the time upon which flies were transferred from dim LL back to DD, the activity rhythm changed its free-running direction promptly at light-off (Fig. 2.4.3.8). However, the beginning of the next activity cycle did not appear to depend on the time of light-off, but on τ_{LL} and the amplitude of the phase advances which led to the shortening of τ . Together with the observations in the previous experiments (Fig. 2.4.3.7), these results led to an assumption that the underlying circadian pacemaker persisted through LL and that
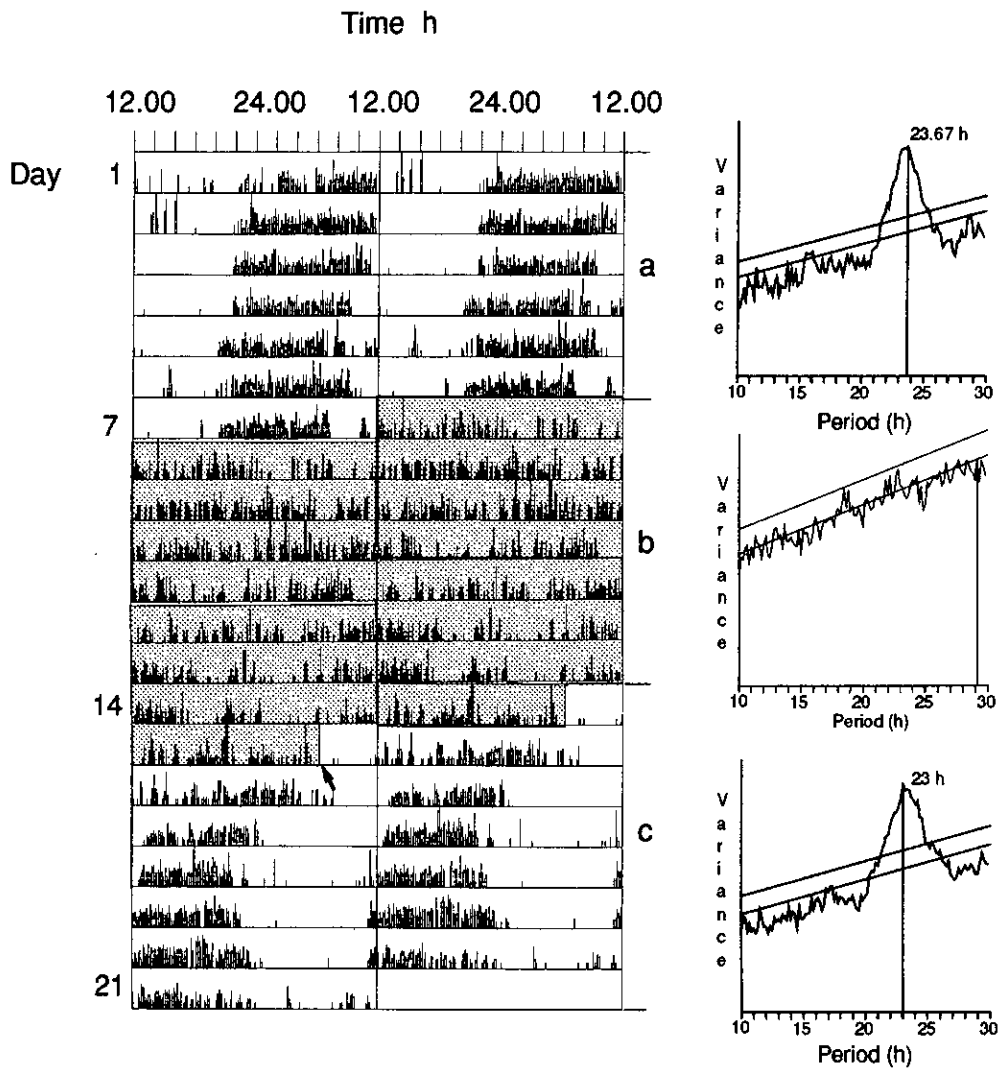
Figure 2.4.3.6. When exposed to LL of a reduced bright light intensity, the fly became arrhythmic (b), nevertheless, rhythmicity was resumed upon return to DD with a τ value (c) similar to that of the initial free-run (a). The fly was firstly held in DD (a), then exposed to LL at 0.055 Wm⁻² (b), and finally returned to DD at a selected time (arrow) on day 15 for the final free-run (c). No fixed correlation was found when measuring the latency between time of light-off and the onset of the next bout of activity.
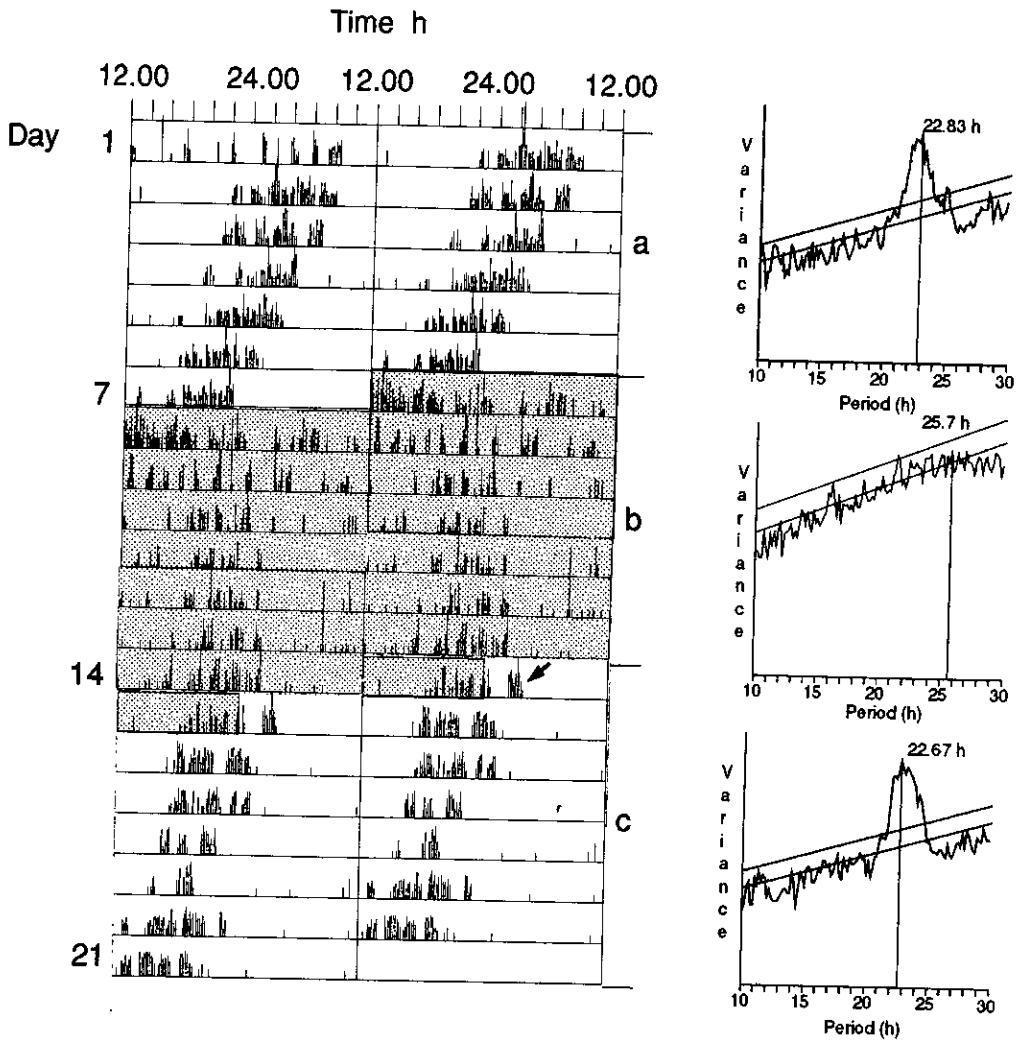
Figure 2.4.3.7. An example showing the activity rhythm continued after light-off. The fly was firstly held in DD (a), then exposed to LL at 0.033 Wm⁻² (b), and finally returned to DD on day 15 at selected time for the final free-run (c). The prolonged activity occurring after light-off (arrow) coincided with the expected end of activity when extrapolating the activity rhythm in the final free-run back to the last cycle in LL. Such observation together with the free-running observed in dim LL (Fig. 2.4.3.3 & 2.4.3.8) strongly suggested the continuation of oscillation in LL.
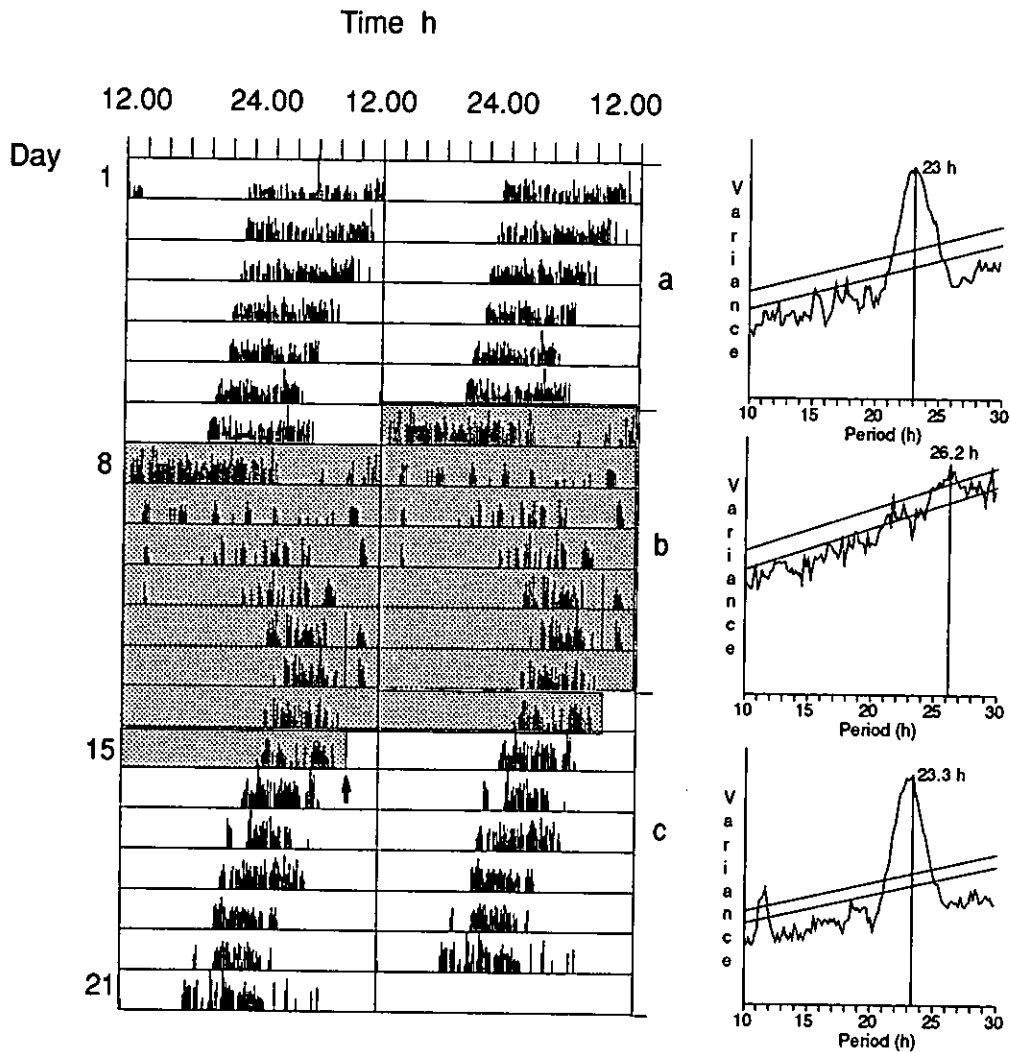
Figure 2.4.3.8. When exposed to dim LL, the fly free-ran with a lengthened τ (b) which was shortened, upon return to DD, to a τ value (c) close to that of the initial free-run (a). The fly was held in DD for the initial free-run, then was exposed to dim LL at 0.02 Wm⁻², finally, on day 15, was returned to DD at a selected time (arrow). The persistence of the activity rhythm in dim LL together with the result that no fixed correlation was found between the time of light-off and the beginning of the next activity cycle provide strong evidence that the circadian oscillation of *C. vicina* continues in dim LL (see also Fig. 2.4.3.3). However, time of light-off should not be excluded when deciding the beginning of the next activity cycle in DD. Because the beginning of the next activity cycle in DD appeared to depend on τ $_{LL}$ and the amplitude of the phase advance occurring upon return to DD. The latter is likely to be provoked by the light-off signal, thereby, may depend partly, if not solely, on the time of light-off.

the arrhythmicity seen in LL (at bright intensities) was the result of the 'masking effect' of an exogenous stimulus rather than the 'stopping' of the oscillator (clock). The possibility of time-keeping at all light intensities (bright or dim) was suggested by (Peterson and Jones, 1979) in an attempt to interpret the features of the activity rhythm of *Culex* in constant light. Evidence obtained in the present investigation in *C. vicina* appeared to support such a hypothesis.

2.4.4. The Phase Response Curve (PRC) for One Hour Light Pulses

The universality of the PRC is a strong indication that the time-measuring systems of eukaryotic organisms may share some fundamental characteristics (Beck, 1980). The first complete phase response curves for single stimuli, covering the whole period and including advanced and delayed phase-shifts, were those for *Drosophila* by (Pittendrigh and Bruce, 1957). Since then, researchers have stressed the importance of PRCs in that such analyses provide considerable information about the 'time course' of the circadian pacemaker, its 'structure' and the nature of entrainment to environmental light cycles. Although an increasing number of PRCs have been obtained for a wide variety of organisms, the concrete nature of the circadian system remains uncovered, and the above statements, first made several decades ago to draw attention to the importance of the PRC, still hold true, not only for the study of the *Drosophila* circadian system, but for the majority of such studies at all levels.

As the most important Zeitgeber, light has been applied most frequently to disrupt the performance of the circadian system to an extent that can be easily recorded and

analysed. Light pulses 'reset' the rhythm to a new phase, causing either advance or delay phase-shifts according to the circadian phase of oscillator subjected to the light perturbation. Attainment of a new phase by the overt rhythm, however, may not be instantaneous: the ultimate steady state may be achieved via a series of non-steady-state (transient) cycles. In general, the number of transients was greater in the case of phase-advances than with phase-delays. In other words, if the phase-shift required for entrainment exceeds the amplitude of the actual phase-shift, it will take more than one cycle to achieve the steady-state entrainment, i.e. the activity rhythm is seen to go through transients before entrainment. Moreover, when light pulses were applied, experimental flies experienced two transitions - from darkness to light (D to L) at light-on; and from light to darkness (L to D) at light-off. Early researchers (Aschoff, 1965b) had noted that both transitions provoke phase-shift but with opposite sign (in opposite direction). In his attempt to differentiate between effects of transitions with opposite signs, Aschoff (1965a) pointed out the importance of the α/ρ ratio (the ratio of activity time to rest time) which can be caused by transitions between light and darkness. His conclusion was therefore that neither onset or nor end of activity represents a useful phase for measuring phase shifts of the oscillator, rather, the midpoint of activity was suggested as a reference point for the real phase of the oscillator. Therefore, the midpoint of activity was used as a reference point when analysing activity rhythms recorded in the present study.

In the present investigation, one-hour light pulses were applied at selected times to experimental flies after their initial free-run in DD. Before applying a light pulse, τ in DD for an individual fly was calculated so as to predict the circadian phase

illuminated by the pulse. This enabled application of light pulses at all circadian phases. The phase-shifts so obtained were plotted against circadian time (Fig. 2.4.4.1). The PRC for one-hour light pulse contains a large portion of phase-delays (-Δφ) occurring between Ct4 - Ct20, a small portion of phase-advances (+Δφ) occurring between Ct20 - Ct2 and an insensitive area (dead zone) between Ct2 - Ct4 where light pulses caused very slight or no phase-shifts. Phase-shifts changed direction from delay to advance between Ct18 - Ct20. This coincided with one interesting phenomenon observed in LD 1:23 entrainment experiments. In such an experiment, when the initial one hour light pulse fell between Ct18 - Ct20, the light/dark cycle failed to entrain the fly; instead, it caused splitting of the activity rhythm (Fig. 2.4.6.5). This phenomenon will be discussed in more detail in later sections (2.4.6 & 2.5).

Daan and Pittendrigh (1976) proposed a model which successfully predicts τ in mammalian circadian systems. Their model used a light PRC to predict the velocity ($1/\tau$) of the oscillator on the basis that constant light speeds up the oscillator in the time corresponding to the advance portion and slows it down in the delay region of a PRC. Thus, if the advance portion of a PRC greatly exceeds the delay portion, periods will become shorter with increasing light intensities, whereas the periods of circadian clocks that have a PRC with a dominant delay portion will be lengthened. The two facts obtained in the present study - light PRC for *C. vicina* had a dominant delay portion and $\tau_{DD} < \tau_{LL}$ - were consistent with Daan & Pittendrigh's hypothesis. This can also be approached from another angle by applying the basic principle of steady-state entrainment: $\tau - T = \Delta\phi$. The average τ value of *C. vicina* obtained in
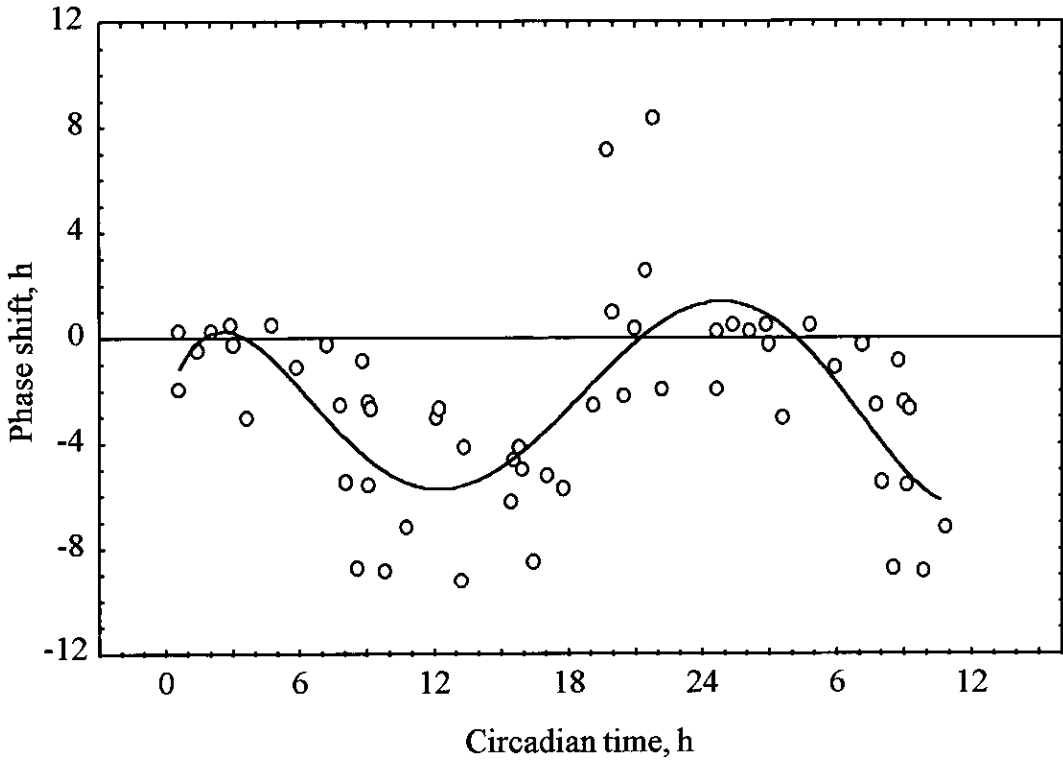
Figure 2.4.4.1. Phase response curve (PRC) for 1 hour light pulse. Phase-shifts provoked by a one hour light pulse were plotted against circadian time, which illustrates the periodic change on the sensitivity of the circadian oscillation to light stimuli in *C. vicina*. Little or no phase-shifting occurred when the light pulse fell in the area between Ct0 and Ct6 (early subjective day), therefore, it is commonly referred as the 'insensitive area'. Light pulses falling between Ct6 and Ct18 (late subjective day and early subjective night) cause delayed phase-shifts (-Δϕ), whereas, light pulses falling in the late subjective night (Ct20 - Ct24) often cause advanced phase-shifts (+Δϕ). Light pulses perturbing the intermediate area (Ct18 - Ct20) where delayed phase-shifts give way to the advanced phase-shifts often result in splitting of the activity rhythm (see Fig. 2.4.6.5 & 2.4.6.6)

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previous experiments (see 2.4.1) was less than 24 h, thereby, according to the equation, delayed phase-shifts ($-\Delta\phi$) are required for entrainment to a 24 h light/dark cycle. As shown in Fig. 2.4.4.1, the dominant delaying phase-shifts appeared to serve such a purpose. Furthermore, as a diurnal insect, *C. vicina* is active during the day thus will be entrained mainly by the light signal at dawn (sunrise).

2.4.5. Entrainment to 24 h Light/dark Cycles

The fact that the endogenous period of almost all circadian systems studied so far deviates from the solar day (24 h) makes synchronisation an important task for an organism to exploit the environmental light/dark cycle. Thus, organisms developed an innate 'temporal programme' which measures local time by environmental fluctuation (e.g. daily light/dark cycle) to ensure a successful synchronisation. Although such a complicated mechanism is not entirely understood, phase-resetting has been proposed to play the key role and has attracted enormous attention. The light PRC obtained in *C. vicina* (see 2.4.4) provided concrete information on how the circadian system responds to pulses perturbing at different circadian phases. Further observations on how the circadian system entrains to a light/dark cycle, i.e. how steady-state entrainment can be achieved by repeated phase-resetting, is the main aim of this section.

For the investigation of activity rhythms, it is important to begin with observing the patterns of locomotor activity in the photoperiod which most closely mimics the natural light/dark cycle (Kenny, 1989). Therefore, in the present study, experimental flies were exposed to 24 h light/dark cycles of various L/D ratios, ranging from LD

1:23 to LD 23:1, after an initial free-run in DD. All flies were then returned to DD for a final free-run. Results showed that all flies exposed to light/dark cycles with a light phase ranging from 4 to 20 hours became firmly entrained (Fig. 2.4.5.1). However, when exposed to a very short (LD 1:23 or 2:22) (Fig. 2.4.5.2.A) or a very long (LD 23:1 or LD 22:2) (Fig. 2.4.5.6) photophase within the light/dark cycle, some experimental flies failed to entrain. Although occurring infrequently, splitting of the activity rhythm was observed in flies exposed to LD 1:23. Such interesting observations aroused particular interest and led to extensive experiments reported in the next section (2.4.6).

Some general features were observed in activity rhythms entrained to light/dark cycles with short light phase: α shortened upon exposure to light/dark cycle; activity began several hours (2 - 8 h) before light-on, exceeded the light phase and extended to the dark phase. Figure 2.4.5.3 showed an example of firm entrainment to LD 4:20. The light phase of the first light/dark cycle perturbed Ct19 - Ct23, caused advanced phase-shift and led to three cycles of transients before entrainment was achieved. Active phase was shortened from 11 h to 7.5 h on transfer to the light/dark cycle. The entrainment collapsed as soon as the fly was transferred back to DD.

In contrast to the above, flies exposed to light/dark cycles with long light phase (12 - 20 h), restricted their activity to the light phase but with brief and intensive bouts of activity occurring immediately after the light went off (light rebound) (Fig. 2.4.5.1). Such an after-effect was likely to be a direct response to the light-off signal. This was also observed in previous studies (Kenny, 1989) and was interpreted as the sensitivity to the light-dark transition. In the present study, *C. vicina* appeared to be
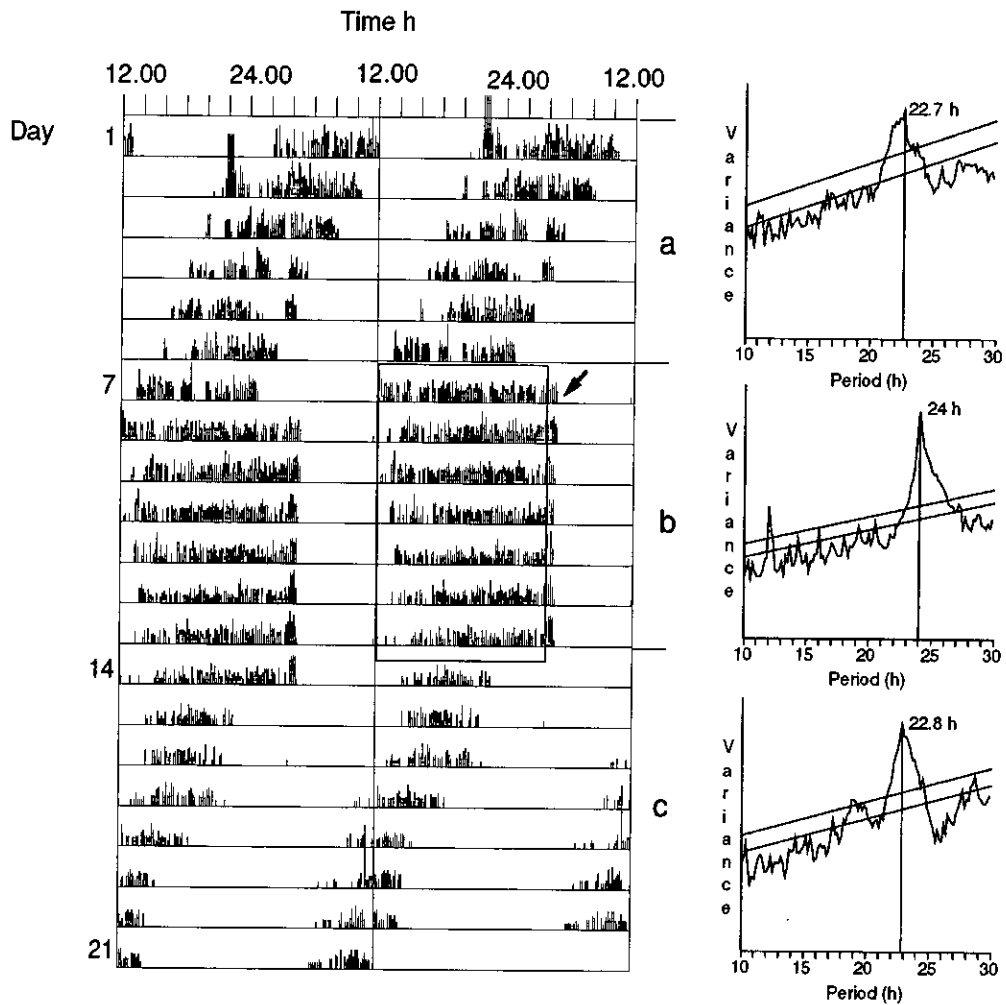
Figure 2.4.5.1. An example of actogram showing firm entrainment to a 24 hour light/dark cycle. The fly was held in DD for the initial free-run (a), exposed to a 24 hour light/dark cycle (LD 16:8 in this case) (b), then returned to DD for the final free-ran (c). The illustrated periodograms show τ values for the appropriate periods. The 'light-rebound' (arrow) effect was only observed in flies exposed to a light/dark cycle with long photo phase (>12 h light).
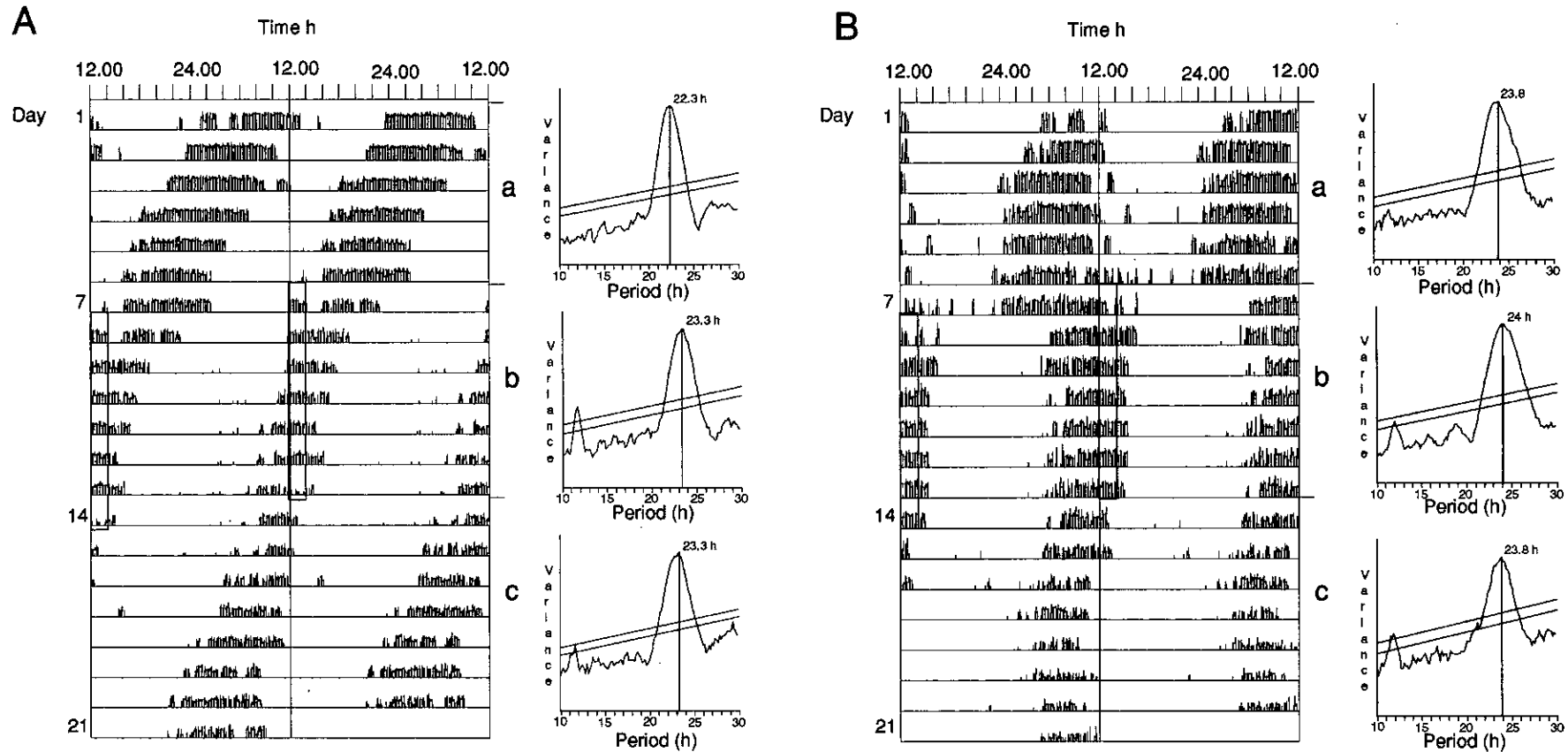
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Figure 2.4.5.2. Actograms of two flies responding differently to LD 2:22. In A, no phase shift occurred when the first light pulse perturbed the insensitive area (Ct0), the activity rhythm free-ran in LD 2:22; whereas, in B, the first light pulse perturbed the beginning of the subjective night (Ct12), delayed phase-shifts occurred and led to entrainment to LD 2:22.
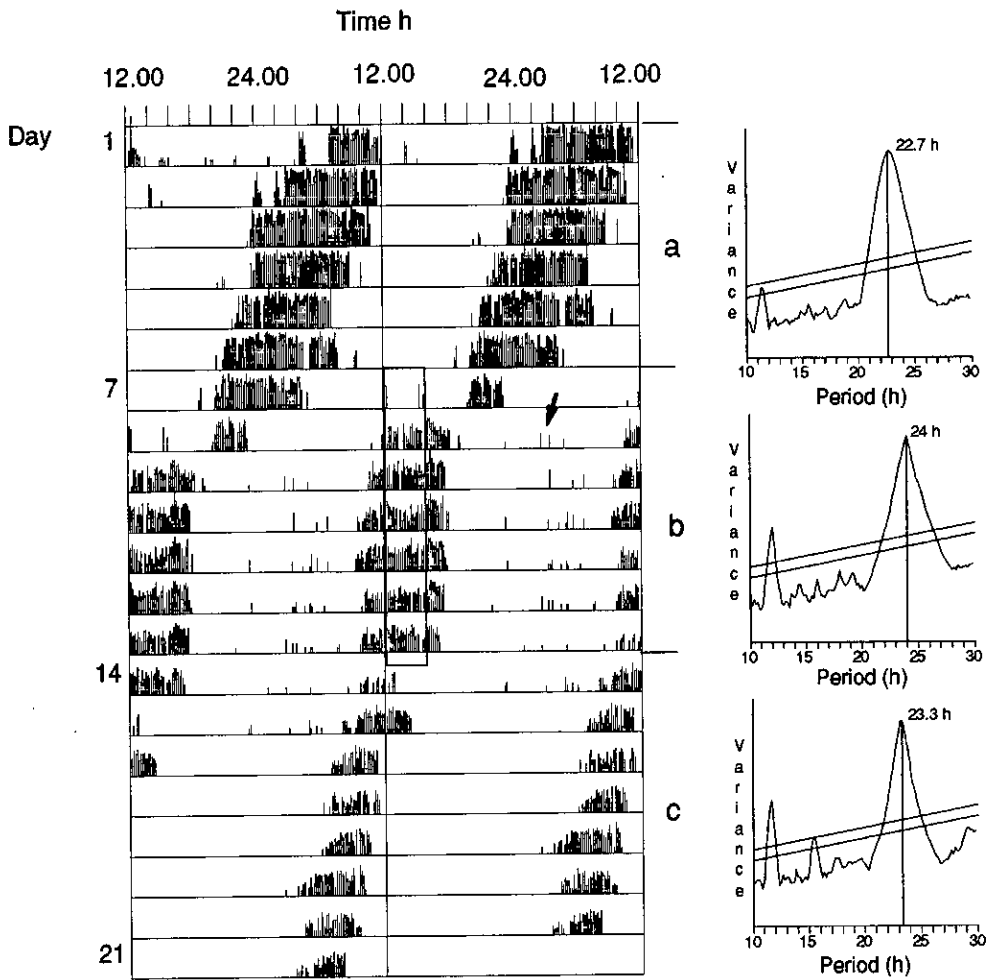
Figure 2.4.5.3. An example showing entrainment to a light/dark cycle with a short photo phase. After the initial free-run in DD (a), the fly was exposed to LD 4:20 (b), then was returned to DD for the final free-run (c). The first light pulse perturbed Ct19 - Ct23 and provoked advanced phase-shifts, thereby, leading to the entrainment. The shortening of active phase (α) during LD was noticeable. Small bouts of activity (arrow) occurring several hours after the main activity also appeared to entrain to the light/dark cycle.

more sensitive to dusk, since this was only observed at light-off. In a later investigation (see chapter 3), such response to the exogenous stimulus was eliminated by surgical removal of optic lobes. Moreover, when entertainment to light/dark cycles with long light phase occurred, lengthening of α was observed in all cases which then shortened again on transfer back to DD. As shown in Fig. 2.4.5.1, the active phase was lengthened from 12.2 h to 16.1 h when the fly was exposed to LD 16:8. Such lengthening of α to a duration close to that of the light phase appeared to be a direct response provoked by the long light phase. Although changes to the length of α were observed in flies entrained to light/dark cycles with either long or short light phase, the role that such an exogenous effect plays in entrainment is still unclear. Nevertheless, it should be taken into consideration when interpreting the mechanism of entrainment.

Results of the previous PRC experiment supported the well known fact that a light pulse can provoke both delay and advance phase-shifts, depending on the circadian phase where the pulse perturbed. Therefore, it is expected that a light/dark cycle can entrain the activity rhythm by phase-leading or phase-lagging it. Fig. 2.4.5.4. shows examples of entrainment to one photoperiod (LD 6:18 in this case) via either phase-delays (A) or advances (B). Actogram A showed that the light phase perturbed Ct10 - Ct16 and caused phase delays; whereas, in B, light phase perturbed Ct16 - Ct22 and advanced the activity rhythm. Moreover, it appeared that the delayed phase-shift led to immediate entrainment while transients took place before entrainment was achieved if the activity rhythm was phase led by the light pulse. This is consistent
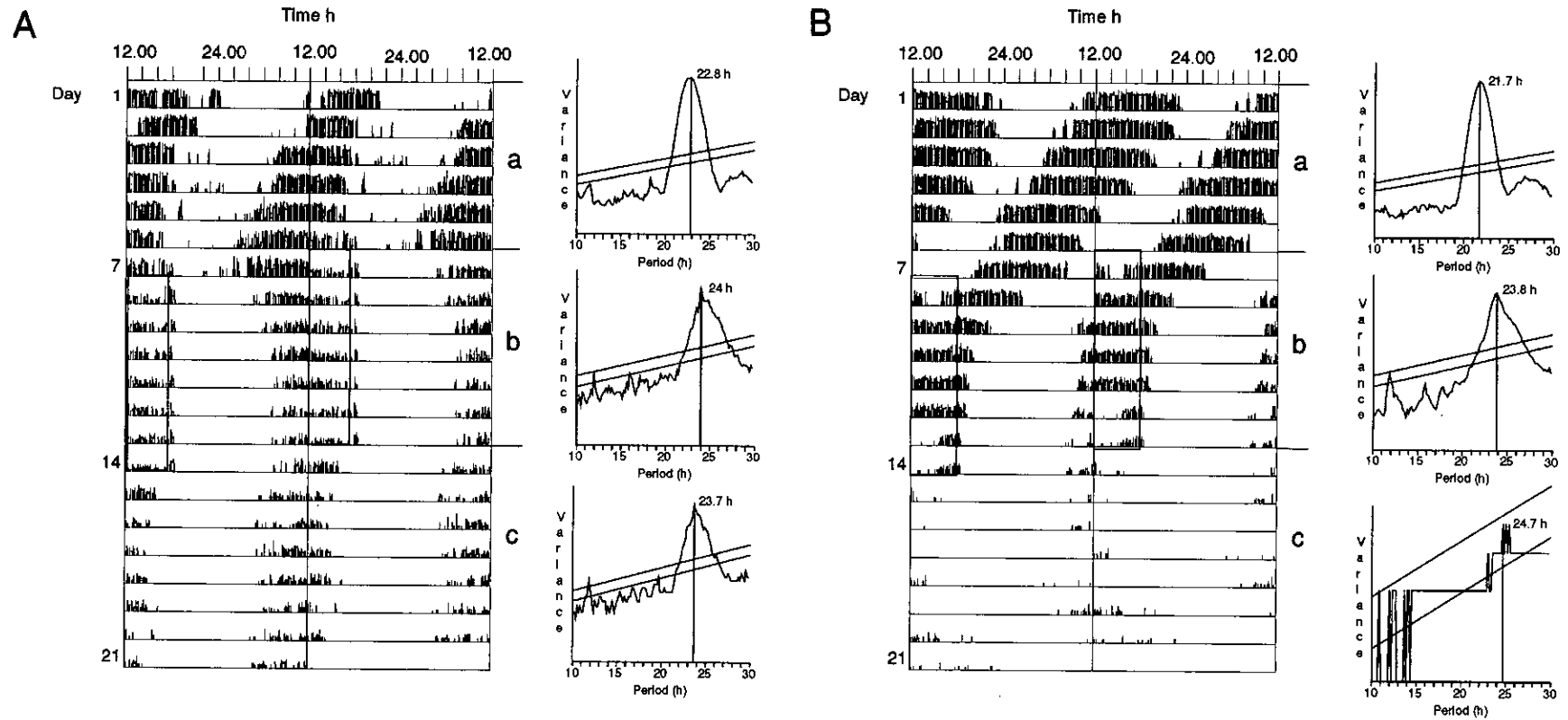
Figure 2.4.5.4. Entrainment to a light/dark cycle of the same LD ratio may be achieved via delaying phase-shifts (A) or advancing phase-shifts (B), depending on the circadian phase where the first light pulse perturbed. Both flies were entrained to LD 6:18. The first light pulse perturbed Ct10 - Ct16 and provoked phase delays in fly A, whereas, in fly B, Ct16 - Ct22 was perturbed and led to phase advances.

with the fact that the PRC for *C. vicina* has a dominant delay portion, namely, the limit for the amplitude of a delayed phase-shift is greater than that for an advanced phase-shift. Therefore, entrainment is more likely to be achieved in one cycle if delayed phase-shifts are required, while in the case of advances, several cycles of transients may be required to achieve the total amount of phase-shift needed for entrainment.

Fig. 2.4.5.2 shows actograms of two flies exposed to LD 2:22. According to periodogram analysis, fly A failed to entrain to the light/dark cycle and free-ran throughout the experimental period, whereas fly B appeared to entrain immediately to the light/dark cycle. Nevertheless, both flies free-ran with a period similar to that of their initial free-run on transfer back to DD. These two records were obtained from flies in the same light box, i.e. the photic conditions both flies were exposed to was exactly the same. The only difference was the circadian phases of the oscillations when the flies encountered the first light pulse on day 8. Calculations for fly A showed that the light pulse perturbed Ct0 - Ct2 (the insensitive zone), thus causing almost no phase-shift (but arousing a direct response to the light); whereas, for fly B, the light pulse perturbed Ct12 - Ct14 (early subject night) causing a delayed phase-shift which resulted in immediate entrainment.

When exposed to LD 22:2, flies either failed to entrain (Fig. 2.4.5.5.B) or entrained weakly (Fig. 2.4.5.5.A). The activity rhythm appeared to be arrhythmic when the fly was exposed to LD 23:1 (Fig. 2.4.5.6). Such arrhythmicity disappeared when the fly was transferred back to DD and was likely to be an exogenous effect of the long photoperiod similar to that observed for LL (Fig. 2.4.3.1). Although the
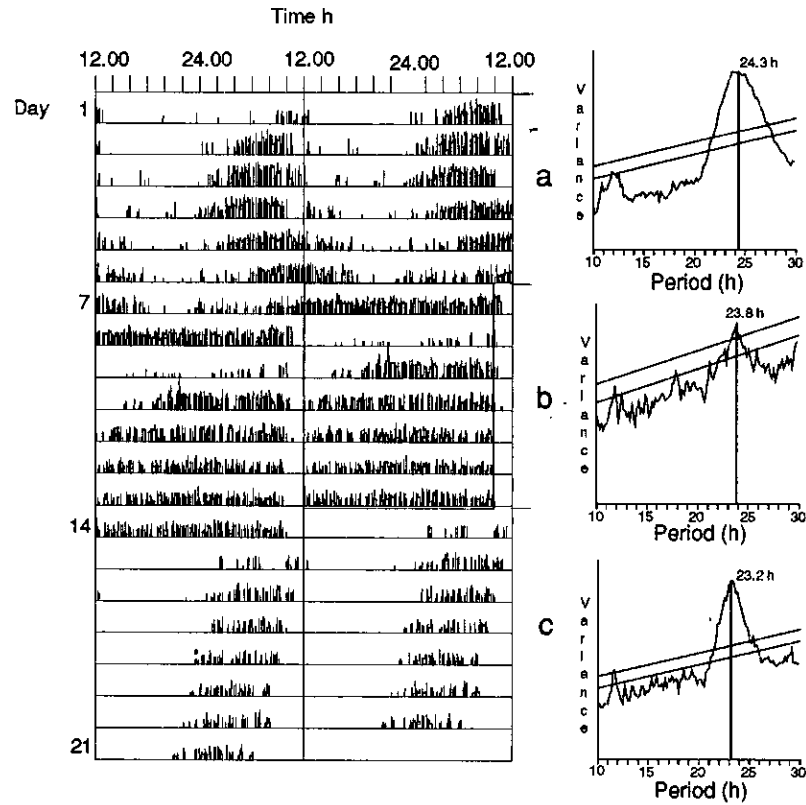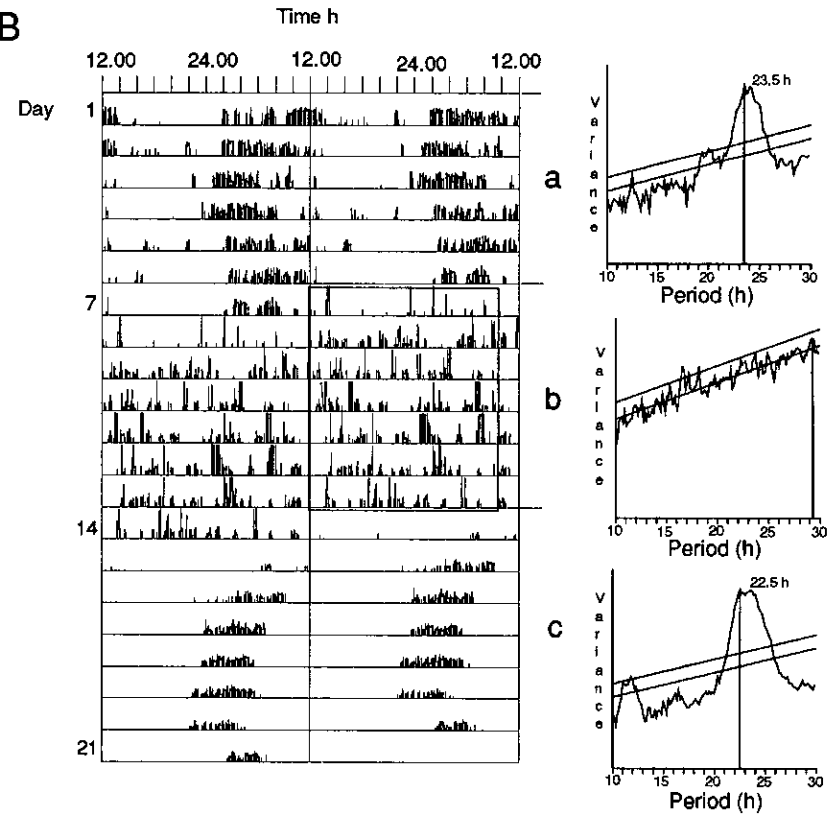
Figure 2.4.5.5. Actograms of two flies responding differently to LD 22:2. Periodogram analyses of period b showed different results - fly A was entrained weakly while fly B became arrhythmic. Nevertheless, rhythmicity was resumed as soon as the flies were transferred back to DD for the final free-run (c).
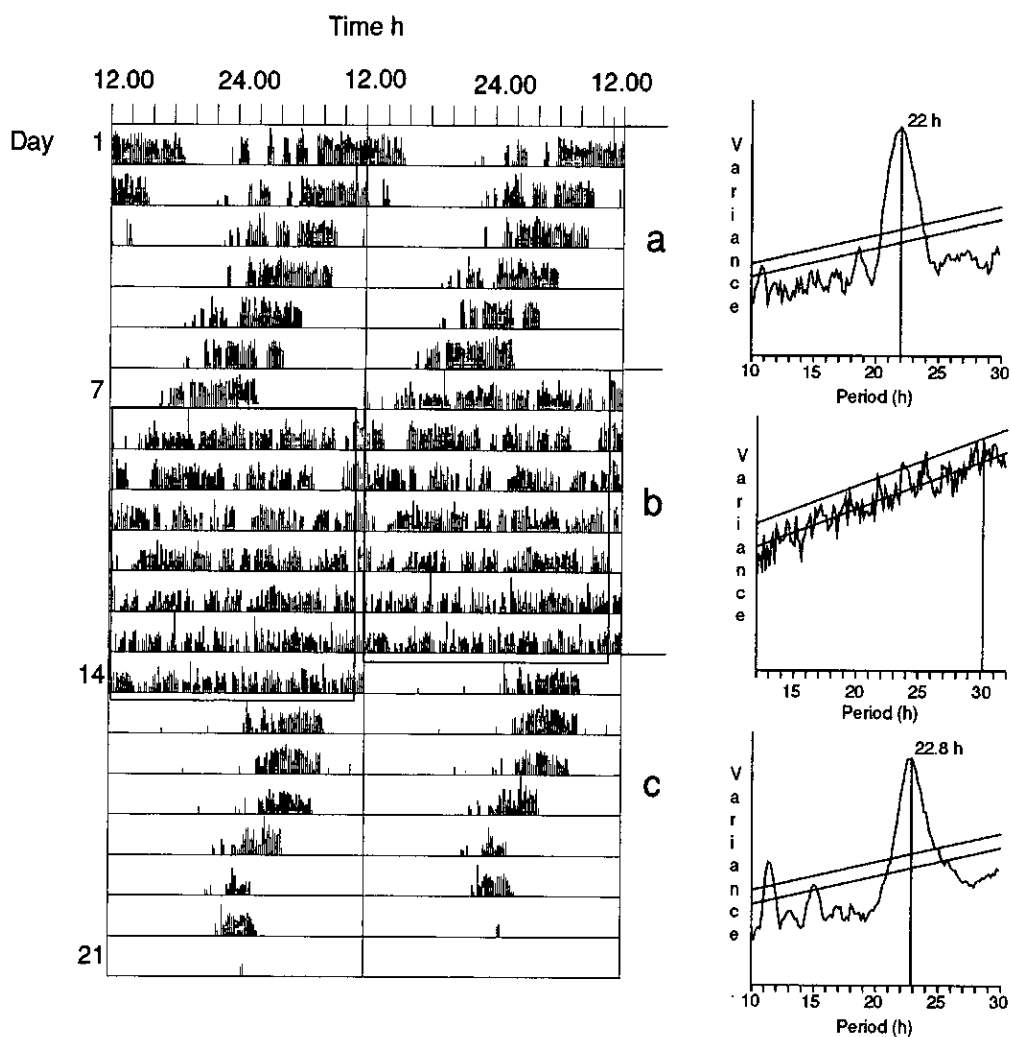
Figure 2.4.5.6. Actogram of a fly failing to entrain to LD 23:1. The fly displayed a rhythmic pattern with a τ value shorter than 24 h during its initial free-run (a). Arrhythmicity was observed upon transfer to LD 23:1 (b). Periodogram analysis failed to detect any real rhythmicity during this period. Rhythmicity was resumed as soon as the fly was returned to DD for the final free-run (c).

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rhythm appeared to be arrhythmic, it may be misleading to conclude that the fly failed to entrain to LD 23:1. The arrhythmicity observed may due to the light rebound induced by the light-off signal, which happened to extend into the dark phase and produced the masking effect seen as arrhythmicity.

Despite the differences observed between light/dark cycles with short or long photo phase, some general features were observed when entrainment occurred:

(1) flies entrained firmly to 24 h light/dark cycles with light phases ranging from 4 - 20 hours;

(2) maximum of activity occurred in the centre of light phase, except entrainment to a light/dark cycle with very short photoperiod (LD 1:23 or 2:22);

(3) the increase or decrease of activity phase (α) also occurred centring the middle of the light phase, which suggested that *C. vicina* has a clearly diurnal locomotor behaviour pattern (Kenny, 1989);

(4) entrainment broke down and the activity rhythm resumed its free-running state upon transfer from LD back to DD, which is strong evidence for the endogeneity of the oscillatory system in *C. vicina*.

(5) after entrainment to a light/dark cycle with a light phase shorter than 14 h, on transfer back to DD, activity always started approximately where the activity of the last cycle began (Fig. 2.4.5.2, 2.4.5.3 & 2.4.5.4), whereas, if the light phase of the light/dark exceeded 14 h, activity always began several hours later (Fig. 2.4.5.5 & 2.4.5.6). This is possibly because the long photoperiod resulted in the increase of α which was then shortened on transfer back to DD.

2.4.6. Entrainment to LD1:23 Cycles

Records from the previous experiment showed that not all flies entrained to LD1:23. Flies failing to entrain either free-ran as in constant conditions, or, more interestingly, displayed splitting of the activity rhythm. Such observations led to an assumption that entrainment to this particular light/dark cycle may depend on the circadian phase where the light pulses first perturbed. Therefore, to test this assumption, a further investigation applying only LD1:23 was carried out.

Flies were exposed to LD 1:23 for 14 days (day 8 - 21) after the initial free-run in DD (7 days). On day 8, lights were arranged to come on at 2-hour intervals so as to perturb the activity rhythm at various times. After day 21, flies were returned to DD for a final free-run. Results were pooled with data obtained from the previous entrainment experiments (2.4.5). The results were divided in four groups, depending on the activity patterns during the light/dark cycle. The majority of flies (72.3%, N=47) entrained to LD 1:23 firmly (Fig. 2.4.6.1), although some went through several cycles of transients before entrainment was eventually achieved (Fig. 2.4.6.2). Two cases (4.3%) were considered as doubtful entrainment (Fig. 2.4.6.3). Flies failing to entrain displayed either a free-running pattern (19.1%) similar to the one in DD with bouts of activity occurring exactly where the light pulses fell (Fig. 2.4.6.4); or a splitting of the activity rhythm (4.3%) (Fig. 2.4.6.5 & Fig. 2.4.6.6).

Splitting

When exposed to LD 1:23, some flies showed splitting of the activity rhythm, providing evidence in favour of the multioscillatory hypothesis (see general
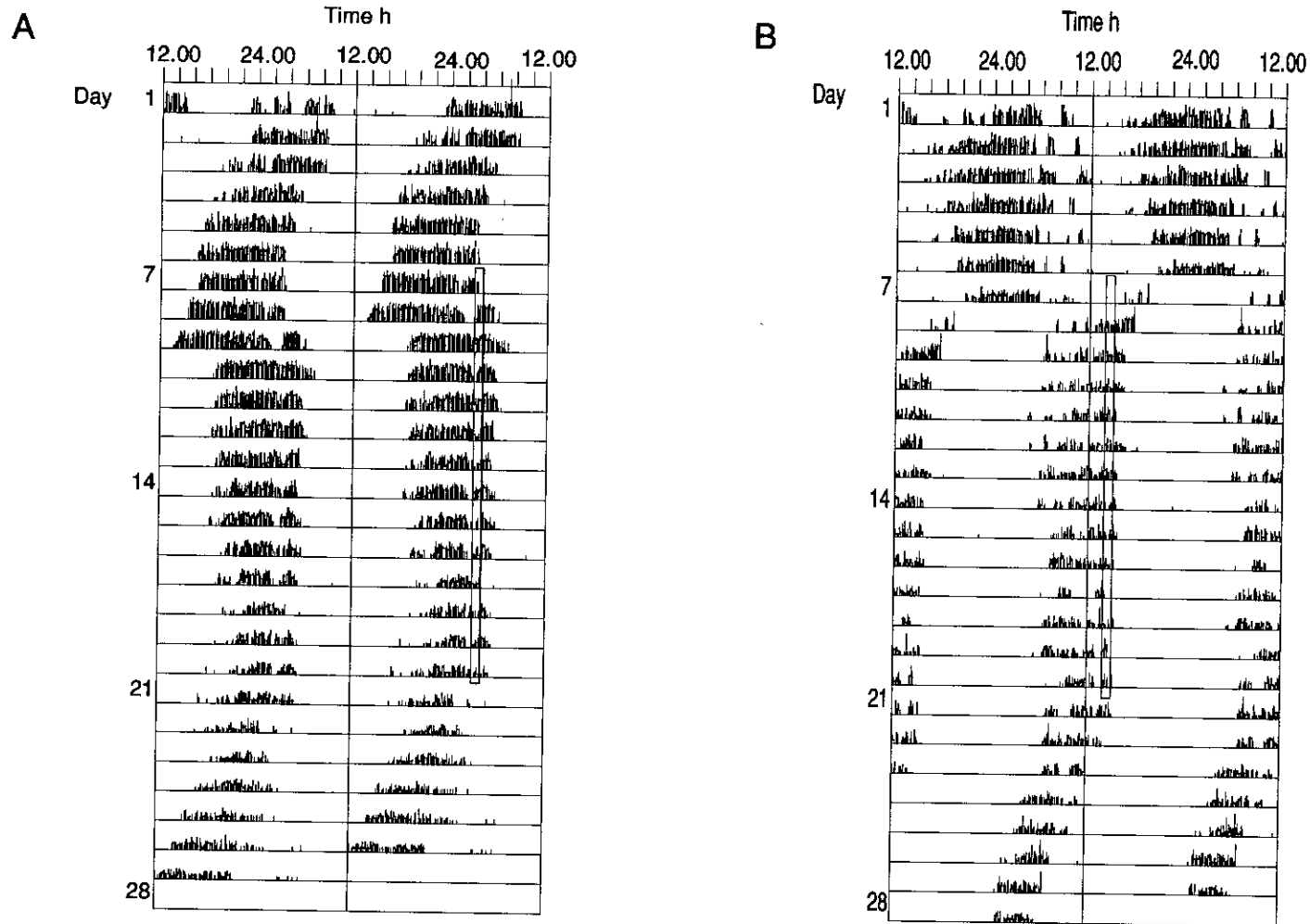
Figure 2.4.6.1. Actograms showing firm entrainment to LD 1:23. In A, the first light pulse provoked delayed phase-shifts which led to the entrainment, whereas, in B, this was achieved by advanced phase-shifts.
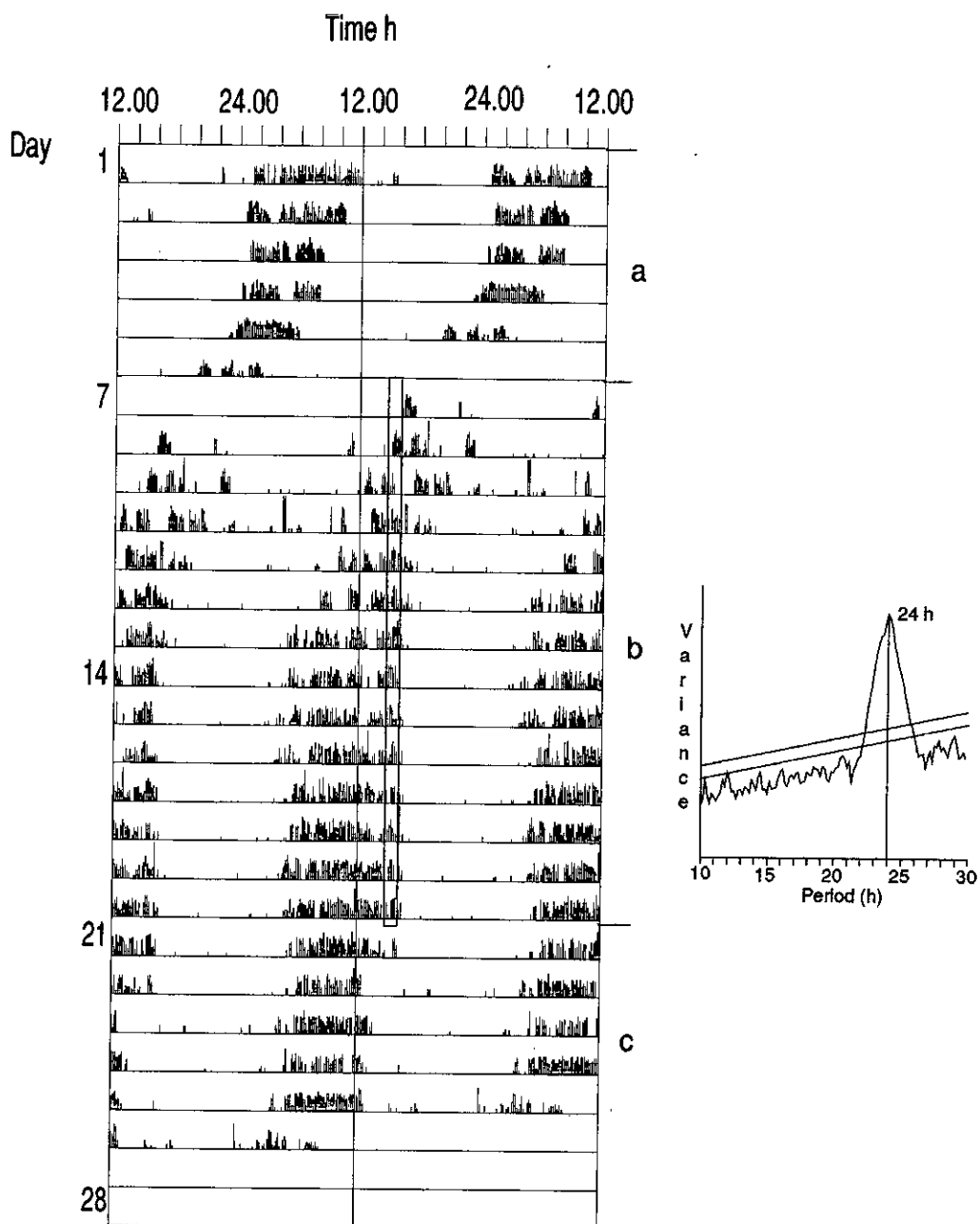
Figure 2.4.6.2. An example of an activity rhythm undergoing transients before achieving entrainment to LD 1:23. The light pulses of the first couple of light/dark cycles perturbed the early subjective day (the insensitive area) and caused no phase-shift (day 7 - 13). Therefore, the activity rhythm went through several cycles of transients before it was eventually entrained firmly to LD 1:23 (b).
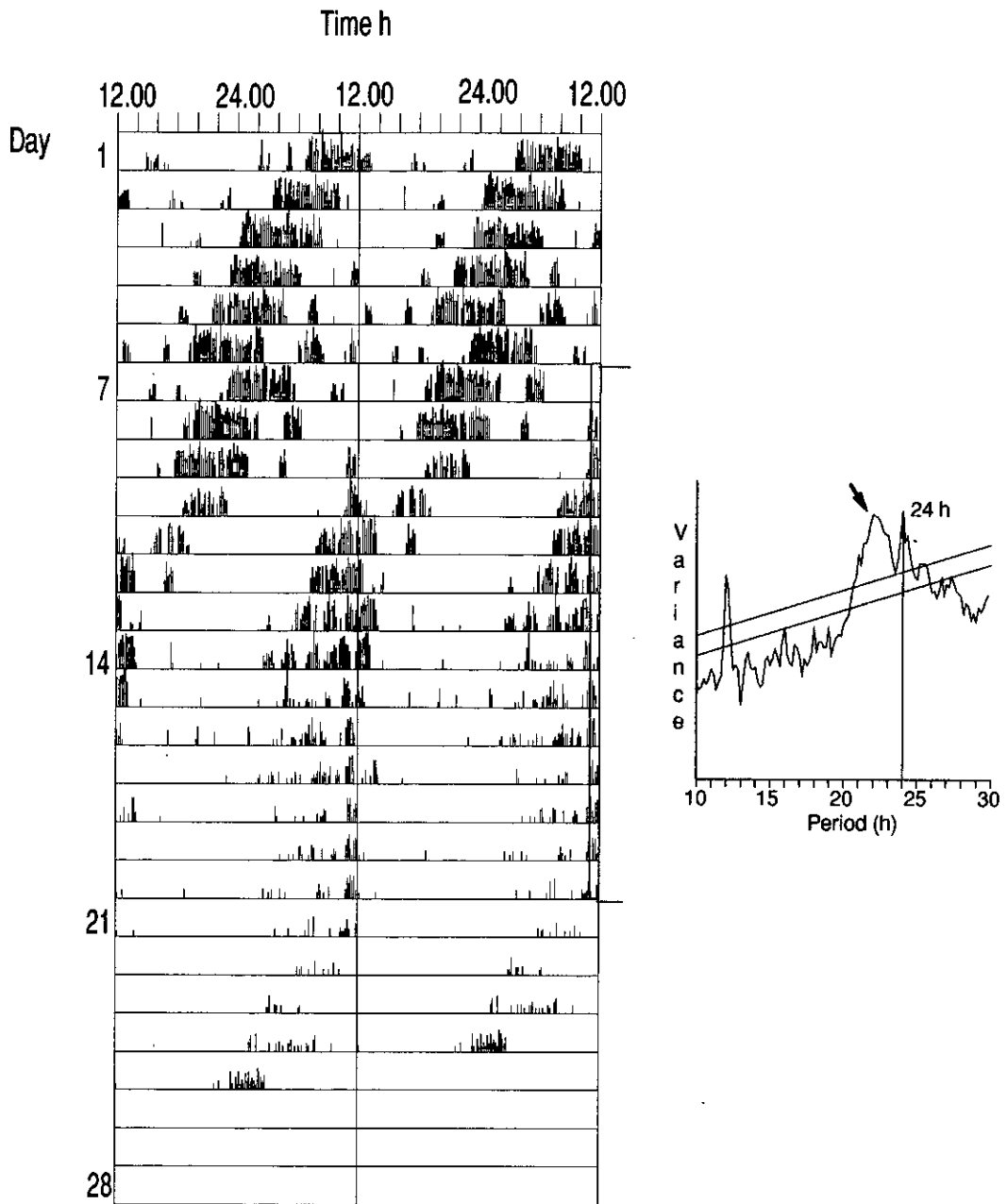
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Figure 2.4.6.3. An example of doubtful entrainment. The activity rhythm free-ran in LD 1:23 for several days (day 7 - 14) before showing a tendency to settle for an entrainment (day 14 - 21). Result of periodogram analysis showed two significant peaks, one around 23 h (arrow), the other exactly 24 h.
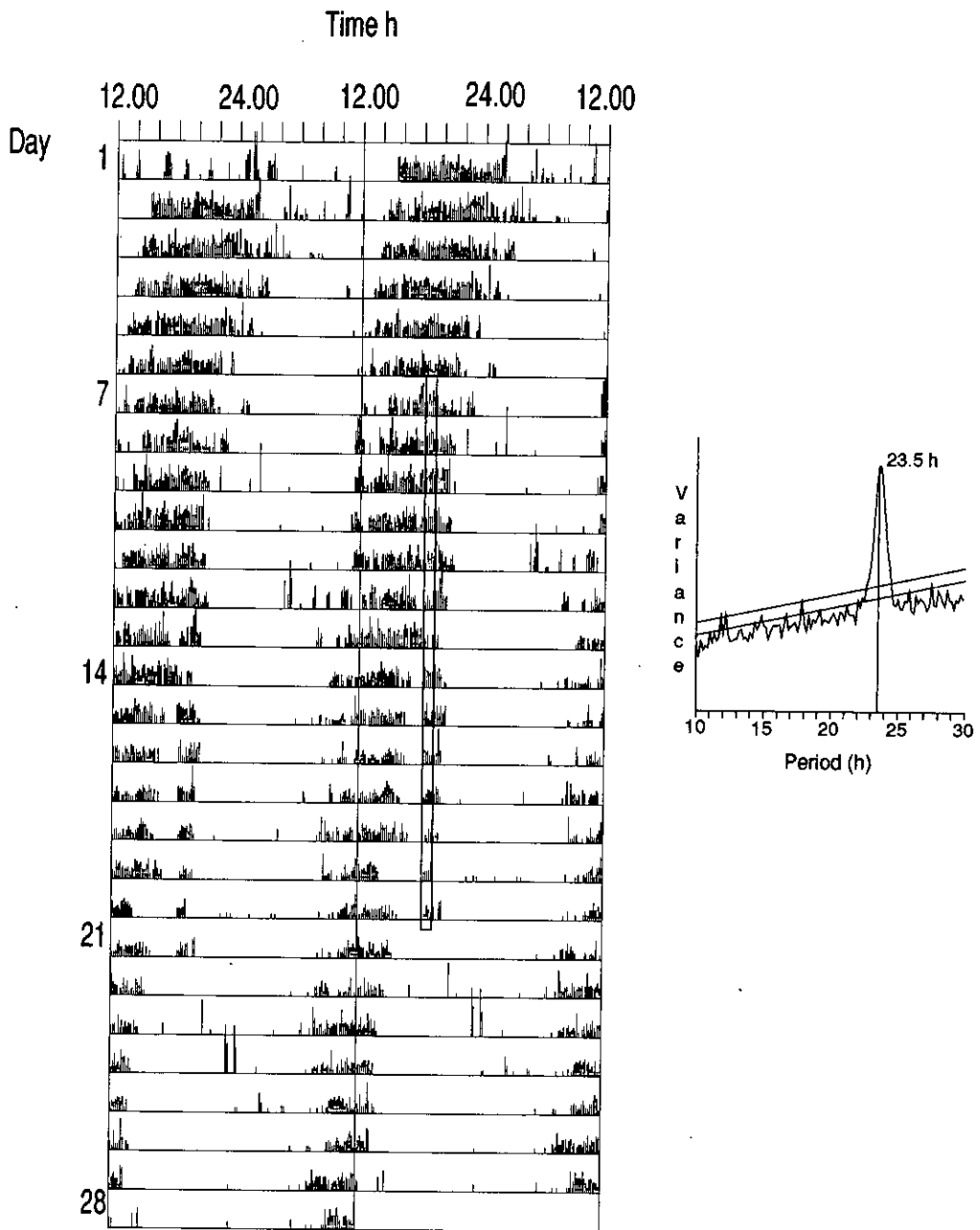
Figure 2.4.6.4. An example of the activity rhythm free-running in LD 1:23. The actogram showed that the light pulses provoked no phase-shifts but exogenous effects - small bouts of activity occurred only where the light stimuli fell.
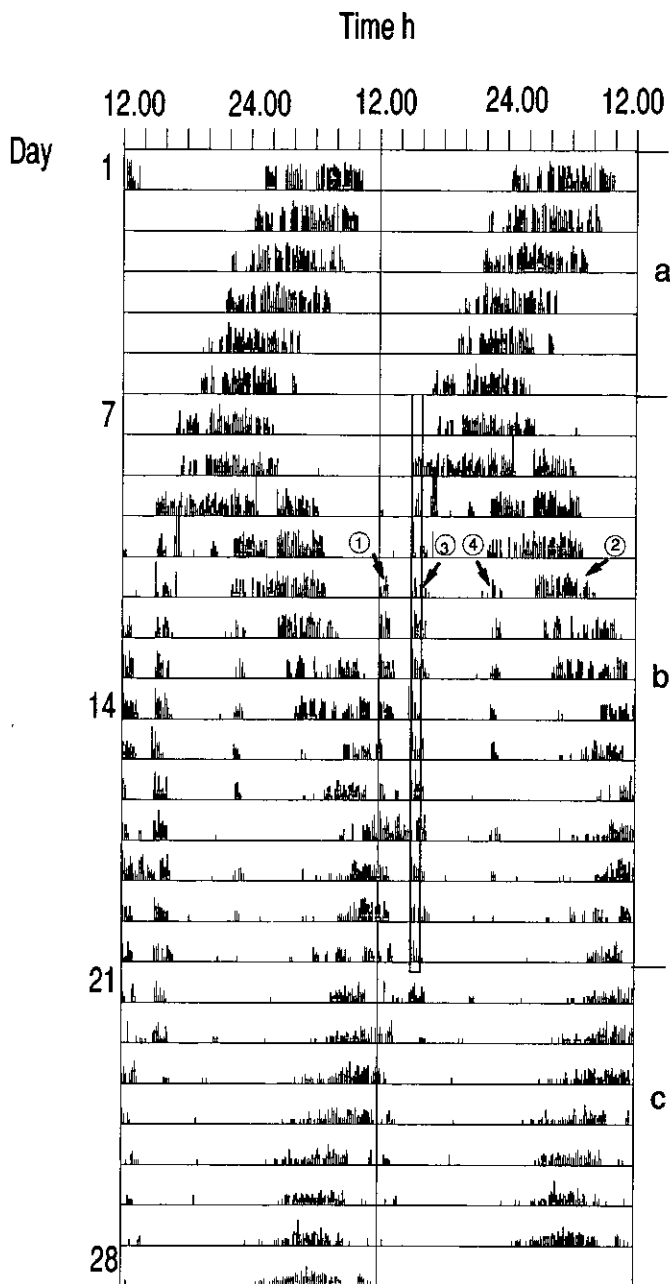
Figure 2.4.6.5. An example of splitting induced by LD 1:23. The first light pulse perturbed the late subjective night and loosened the coupling of the oscillation, which was seen as a gradual split of the activity rhythm into four components, only three of which were endogenous (①,③ & ④). The main activity band (②) free-ran with a period greater than 24h, whereas two small bouts of activity, one occurring before light-on (①) and the other several hours after light-off (④), appeared to be entrained to the light/dark cycle. A fourth component (③), occurring only where the light pulse perturbed, was regarded as the direct response to the light stimuli. Upon transfer to DD, the exogenous component disappeared promptly, whereas the endogenous components resumed the free-running status and merged together after several cycles. This result provided strong evidence in favour of the multioscillatory hypothesis.

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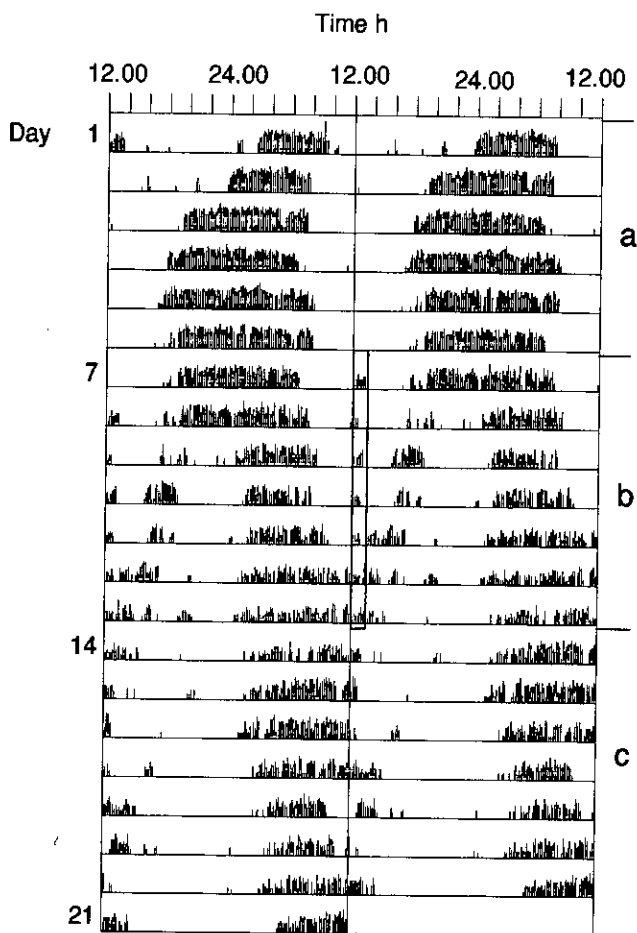
Figure 2.4.6.6. Another example of splitting induced by LD 1:23. The first light pulse perturbed the middle of the subjective night (Ct18), thereby, causing a direct response and splitting as seen in Fig 2.3.6.5. The split components free-ran with their own periods, one shorter than 24 h (fast component) while the other greater than 24 h (slow component). Owing to an insufficient number of light/dark cycles, no firm entrainment was achieved before the final free-run.

discussion). When referring to the light PRC (Fig. 2.4.4.1), such splitting was observed most frequently when the initial light pulse fell close to the middle of the subjective night (i.e. Ct18 - Ct20) where delay phase-shifts give way to advance phase-shifts. The activity rhythm then gradually split into several components, each responding differently to the light pulse. In Fig. 2.4.6.5, the activity rhythm showed three independent components (①, ② & ④ as indicated in the figure). The main activity band (②) free-ran with a period greater than 24 h, whereas two small bouts of activity, one occurring before light-on (①) and the other several hours after light-off (④), appeared to be entrained to the light/dark cycle. A fourth component of the activity rhythm appeared only where the light pulse occurred (③ as indicated in the figure) was regarded as an exogenous response. Similar exogenous responses were also observed in Fig. 2.4.6.6 while the endogenous rhythm split into two free-running components - the 'slow' ($\tau > 24$ h) and the 'fast' ($\tau < 24$ h). Despite the slightly different response to the light stimuli, the return to DD appeared to have the same effect on both flies - the exogenous components disappeared promptly upon transfer to DD, while the endogenous components gradually joined together and resumed the free-running state. The fact that different components of the activity rhythm free-ran or entrained to the light/dark cycle simultaneously (Fig. 2.4.6.5) indicated an interference with the coupling mechanism, which resulted in the splitting of the activity rhythm. According to these results, such a phenomenon is likely to occur when the first light pulse falls in the middle of the subjective night.

Entrainment

Owing to the very short length of the light phase, most of the activity occurred outside the light phase, before light-on in almost all cases, regardless of the direction of phase-shifts (Fig. 2.4.6.1.A, B). Furthermore, some flies were even entrained to the light/dark cycle with the entire activity occurring outside the light phase, if the small bouts of activity directly provoked by the light stimuli were excluded (Fig. 2.4.6.7). When the light pulse perturbed the insensitive area, for example Ct0 in Fig. 2.4.6.2, the activity rhythm was observed to go through several cycles of transients before entrainment was finally achieved. Such an observation can be interpreted as that the rhythm kept free-running while the insensitive area was perturbed until eventually the light pulse fell in a circadian phase which caused the phase-shift needed for entrainment.

One particular interesting example was shown in Fig. 2.4.6.8. Firstly, the fly displayed spontaneous splitting during its initial free-run. Then, when exposed to LD 1:23, the split components gradually joined into one main activity band which was firmly entrained to the light/dark cycle. Finally, splitting occurred again when the fly was returned to DD. This suggested that a light/dark cycle may act to strengthen a rather loose coupling between constituent oscillators.

The frequent occurrence of entrainment to LD 1:23 led to the conclusion that a light/dark cycle with light phase as short as 1 hour is able to entrain the activity rhythm of *C. vicina* provided that the number of light/dark cycles is sufficient and that the first light pulse falls outside the area where splitting frequently occurs. Moreover, LD 1:23 appeared to be a useful tool to provoke splitting of the activity rhythm, thereby providing a possibility to explore the circadian oscillation beyond the
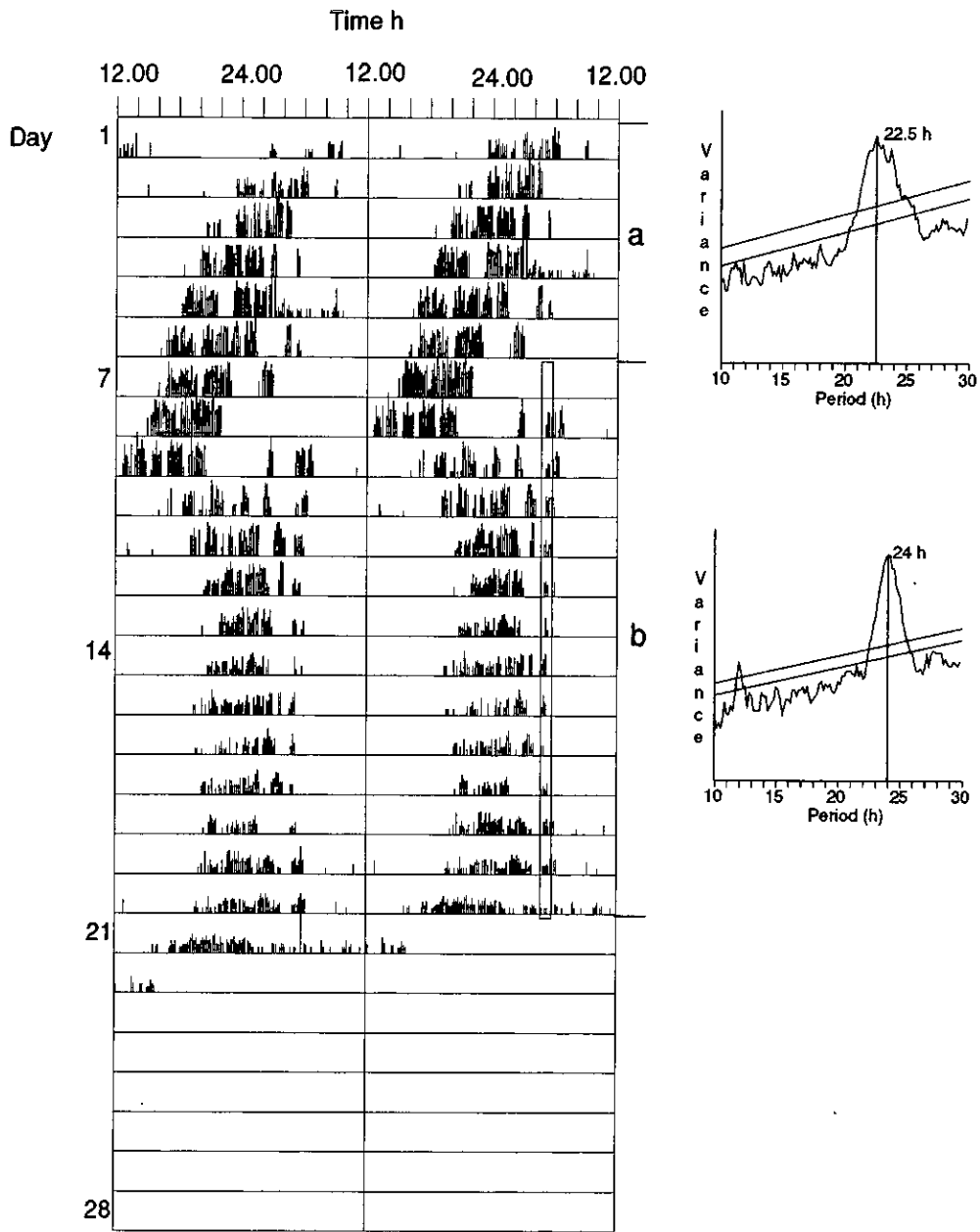
Figure 2.4.6.7. The activity rhythm appeared to entrain firmly to LD 1:23 with all activity occurring outside the light phase. The small bouts of activity occurring only where the light pulses fell were regarded as the direct response to the light stimuli. Such result suggests that the occurrence of activity within the light phase of the light/dark cycle is not necessary for entrainment to a light/dark cycle with light phase as short as 1 hour.
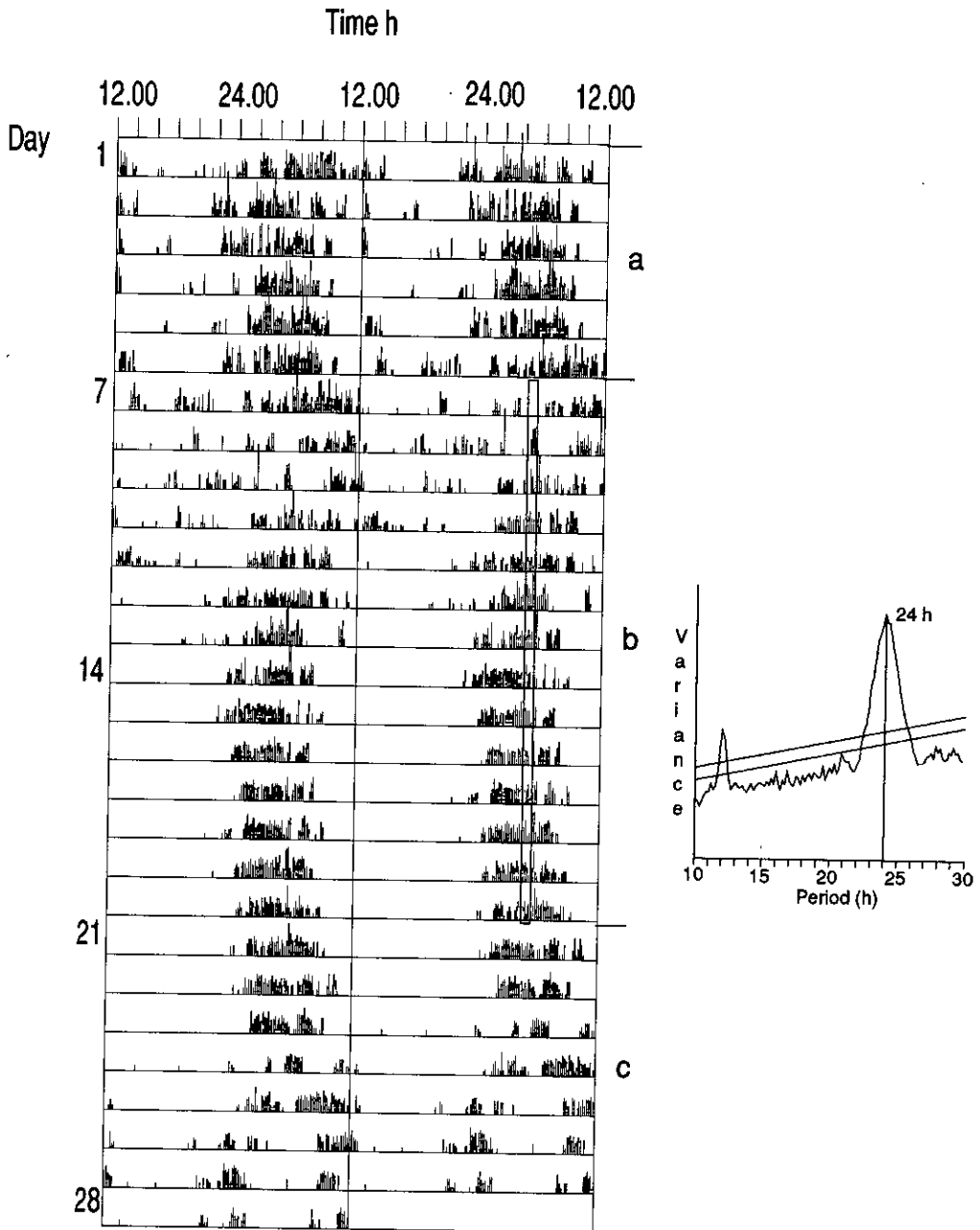
Figure 2.4.6.8. In this actogram, an intrinsically loose coupling was tightened by LD 1:23. The activity rhythm split spontaneously during the initial free-run (a) and was tightened into a main activity band which entrained firmly to LD 1:23 (b). The spontaneous split reoccurred after the fly was transferred back to DD for the final free-run (c).

peripheral aspect which is normally limited to observations on changes of the overall overt rhythm.

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2.4.7. Entrainment to LD 12:12 with Reduced Light Intensities

In previous experiments (section 2.4.3) it was shown that flies exposed to dim LL (< 0.03 Wm^{-2}) free-ran with a lengthened τ, whereas flies exposed to LL of a brighter intensity became arrhythmic. Such apparently different responses to the two photic conditions raised the question - whether a 'weaker' signal, e.g. a light/dark cycle with reduced light intensity, can act as an efficient entraining agent? The present investigation was carried out to answer the question in the hope that the result may also shed some light on the photoreception mechanism for entrainment.

Experimental flies were exposed to LD 12:12 of reduced intensities ranging from 0.03 to 0.009 Wm^{-2} for 11 days after the initial free-run in DD (7 days). On day 19, for flies that showed firm entrainment, the light/dark cycle was shifted 7 hours later with other conditions unchanged; otherwise, flies remained in the unchanged conditions. The thresholds applied here for the definition of 'dim' and 'bright' light intensity were the result of the previous experiments.

Despite the intensity, all flies (N=45) were entrained to the light/dark cycles they were exposed to, some entrained immediately while some went through a few cycles of transients. Not surprisingly, firm entrainment was observed more frequently in flies exposed to brighter light intensities. Therefore, flies selected to receive the shift of light/dark cycle were mostly those exposed to bright light intensity (> 0.02 Wm^{-2}).

An instant delay of the activity rhythm was observed after a shift in the light/dark cycle, and entrainment was achieved after several transient cycles (Fig. 2.4.7.1). Working on the perch-hopping rhythm of the house finch, Enright (1965) applied a 6-hour shift of the light/dark cycle (intensity 170 lux) which also caused delayed phase-shifts and led to transients before the rhythm was firmly re-entrained. As shown in Fig. 2.4.7.1, the activity rhythm of *C.vicina* was displayed in two components, both entrained to the light/dark cycle (intensity = 0.02 Wm^{-2}) applied during day 8 - 18. The main activity band was restricted within the light phase while a weak but clear bout of activity occurred several hours after light-off. Evidently, the latter was not provoked by any photic stimuli, thereby, appeared to be an endogenous component. The main activity band also appeared to be endogenous since it went through transients rather than abrupt phase jump responding directly to the shifted light/dark cycle. This, again, provided supportive evidence in favour of the multioscillatory hypothesis.

A distinct phenomenon was observed in flies exposed to light intensities less than 0.012 Wm^{-2}. Flies exposed to such dim light intensities restricted their activity to the light phase and entrained to the light/dark cycles at the younger stage (day 8 to 14). As the flies became older, although light/dark ratio and light intensity remained the same, the onset of activity occurred several (2 - 6) hours before light-on and the entrainment appeared to be more unstable compared to the period when the flies were younger (Fig. 2.4.7.2). This phenomenon was not observed in flies exposed to brighter light intensities (Fig. 2.4.7.3). The explanation could be that as flies get
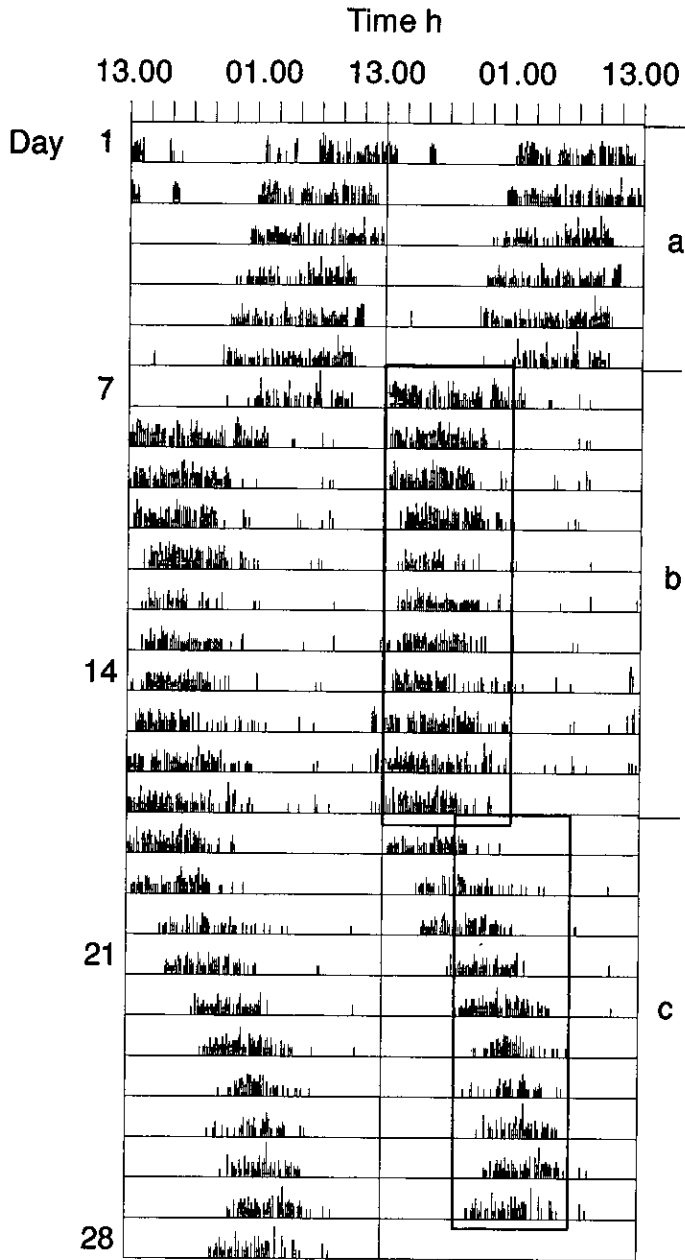
Figure 2.4.7.1. An example of re-entrainment after shifting the light/dark cycle. The experimental photic conditions were: (a) day 1-7 in DD; (b) day 8 - 18 in LD 12:12 at 0.02 Wm⁻² and (c) on day 19, LD 12:12 was shifted 7 hours later, other photic conditions remained the same as b. The activity rhythm free-ran in DD (a), entrained firmly to LD 12:12 (b), and, after a few cycles of transients, gradually entrained to the shifted LD 12:12 (c).

Figure 2.4.7.2. An example of entrainment to LD 12:12 of dim light intensity. After the initial free-run in DD (a), the fly was exposed to LD 12:12 at 0.01 Wm⁻² throughout the rest of the experimental period (b). After several cycles of transients, the activity was restricted to the light phase of the LD cycle when the fly was young (up to day 19). Although still entrained weakly to the LD cycle, the activity rhythm started several hours before light-on when the fly became older.

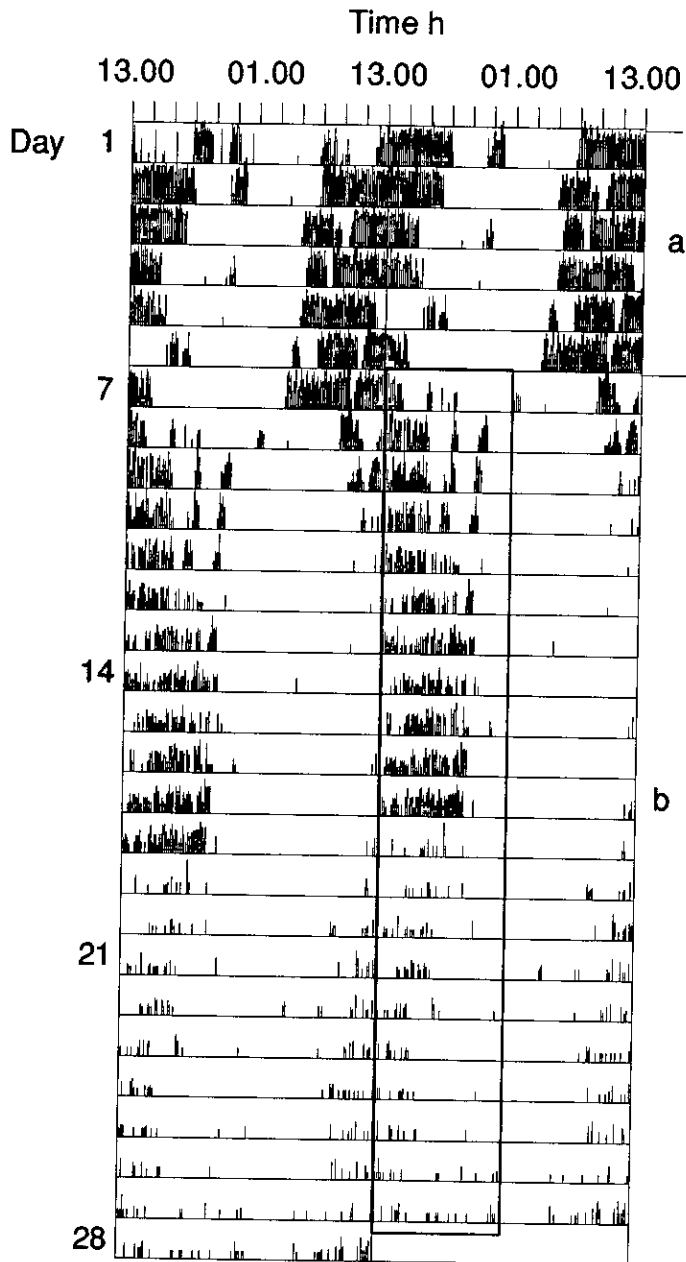**Figure 2.4.7.3.** An example of entrainment to LD 12:12 of bright light intensity. After the initial free-run (a), the fly was exposed to LD 12:12 at 0.03 Wm^{-2} throughout the rest of the experimental period (b). Although the active phase (α) appeared to shorten as the fly became older, the activity rhythm was restricted within the light phase of the LD cycle.

older, they become less sensitive to light, but the decrease is so slight that it only effects photoreception of very dim light.

When comparing the entrainment to LD 12:12 at normal light intensity and reduced light intensity, one obvious difference was noted. At normal light intensity $(0.7 \ Wm^{-2})$, the activity was restricted to the light phase, starting at light-on and ceasing at light-off (Fig. 2.4.7.4), whereas, at reduced light intensities, the activity often ceased several hours before light-off (Fig. 2.4.7.2 & Fig. 2.4.7.3). This suggested that the direct response to exogenous light stimuli was partially eliminated by the decrease of light intensity below a certain threshold. Further experiments exposing bilobectomized flies to a light/dark cycle with reduced light intensity should provide more information to aid confirmation of this conclusion.

No obvious suppression of activity level was observed at intensities (0.03 - 0.009 Wm^{-2}) applied in the present study. In comparison, a more obvious suppression of the activity level in brighter LL which led to arrhythmicity was observed (Fig. 2.4.3.1). This suggested that the suppression might be caused by the spreading of activity over the long period, which somehow reduced the activity level in balance.
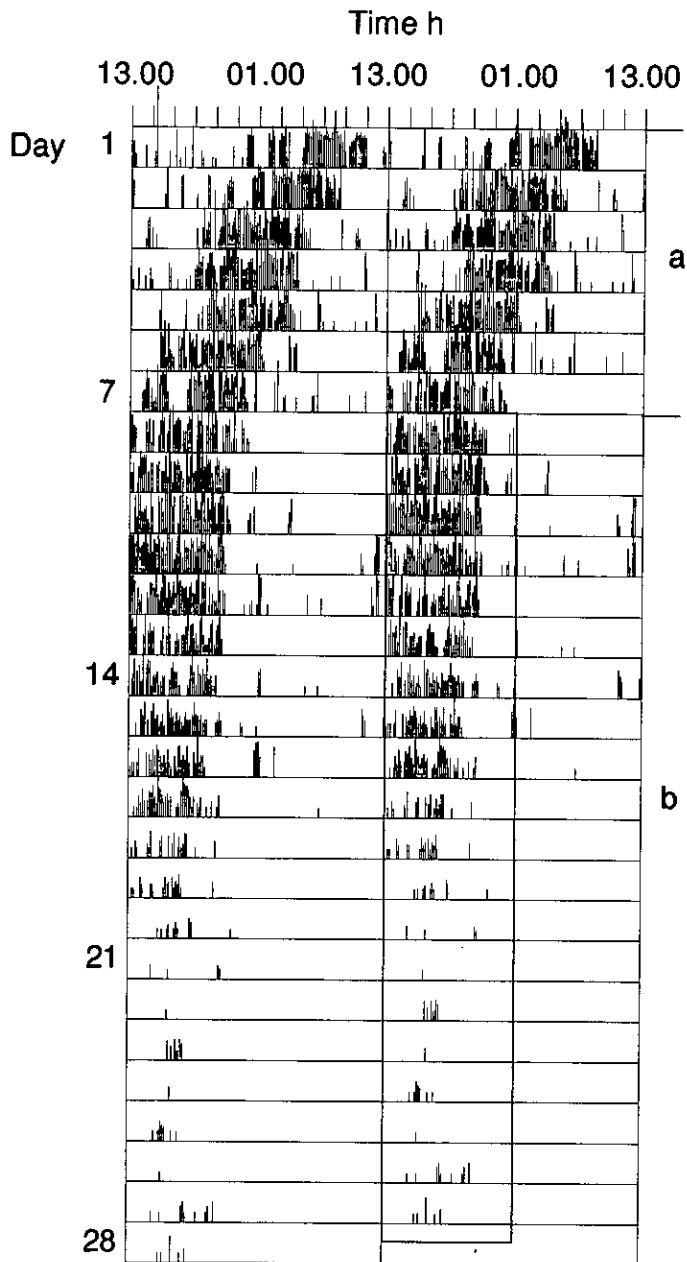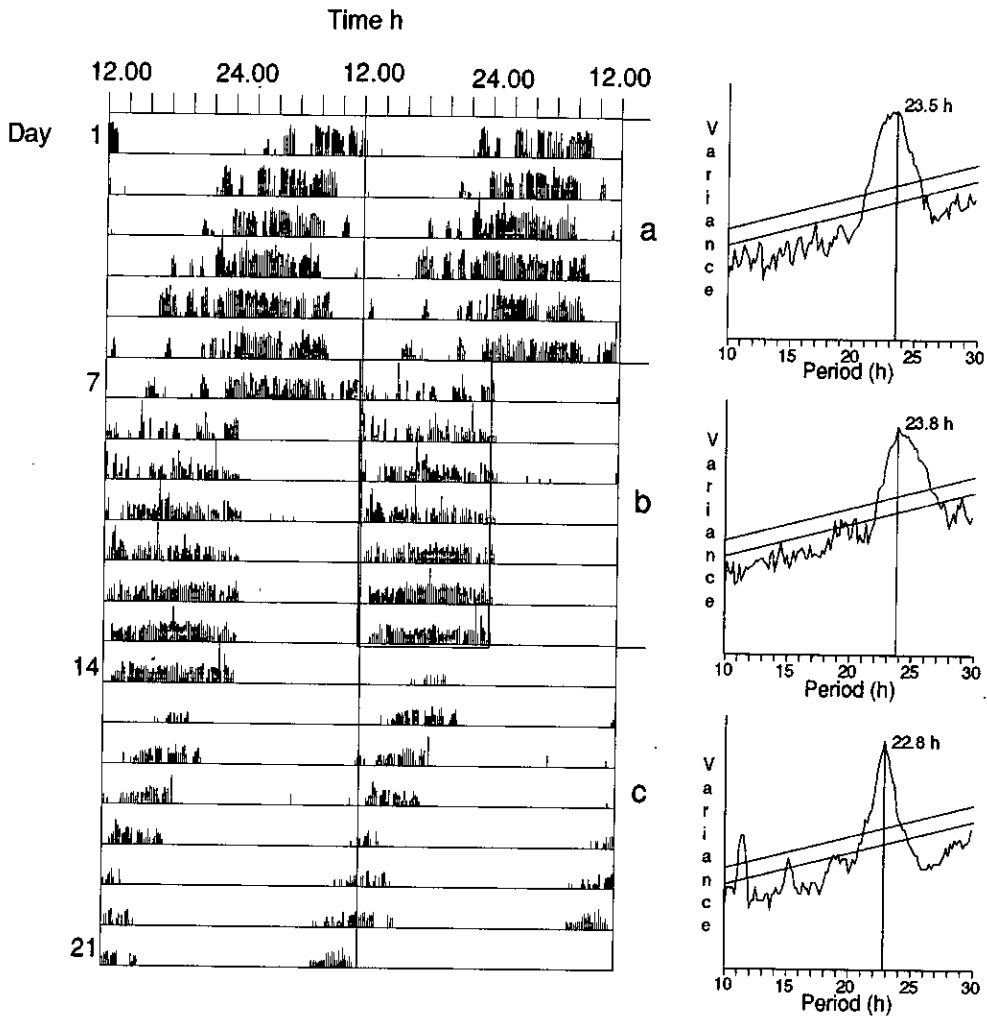
Figure 2.4.7.4. An example of entrainment to LD 12:12 of normal light intensity. After the initial free-run (a), the fly was exposed to LD 12:12 at 0.7 Wm^{-2} (b), then was returned to DD for the final free-run (c). Unlike the entrainment observed at dimmer intensities (Fig. 2.4.7.2 & 2.4.7.3), the fly did not cease activity until light-off. Moreover, the exogenous effect of light-off signal (light-rebound) was never observed in flies exposed to dimmer intensities.

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2.5. Summary

1. The observation that the locomotor activity rhythm of *C. vicina* persisted in constant conditions, DD or dim LL, provided evidence to support the existence of a **self-sustained oscillatory system**. Such a persisting rhythm free-ran in DD with a mean period length 22.68 ± 0.596 h (N=200), which deviates from that of the solar day (24 h), thereby confirming the **endogeneity** of the underlying oscillatory system.

2. The result that Q_{10}s obtained in both temperature step-up and -down experiments were all very close to 1 indicated that the clock system is highly **temperature-compensated**, within the temperature range at which organisms function normally. As with the light PRC, the temperature PRCs for temperature steps-up and -down consist of both delay and advance portions, thereby suggesting the entrainability of the activity rhythm to temperature cycles.

3. The PRC obtained for 1 h light pulses proved to be reliable for predicting the phase-shifts required for entrainment to 24 h light/dark cycles, i.e. it is consistent with the equation for entrainment: $\Delta\phi = \mathbf{T} - \tau$.

4. Upon transfer from DD to dim LL, the activity rhythm was observed to change its free-running direction and free-run with a lengthened τ, i.e. $\tau_{LL} > \tau_{DD}$. Such a conclusion **violates Aschoff's rule** which predicts the shortening of τ upon transfer to LL, for an diurnal animal like *C. vicina*.

5. The persistence of the activity rhythm in dim constant light (<0.02 Wm^{-2}) suggested that the direct response to light, such as the arrhythmicity seen in bright

LL, can be eliminated by the reduction of light intensity to a certain threshold. This is supported by the intermediate behaviour (Fig. 2.4.3.2) observed in continuous light at an irradiance between 0.03 and 0.02 Wm^{-2}. Therefore, in addition to constant darkness (DD), dim LL may provide a useful tool in revealing the nature of the circadian rhythmicity in *C. vicina*. Moreover, the occurrence of entrainment to light/dark cycles of such dim light intensity suggested that such dim light can also act an efficient signal of light-dark transition.

6. The splitting of the activity rhythm into two or more components, occurring spontaneously (type 2 free-running activity pattern) or induced by an 1 hour light pulse, provided strong evidence that the circadian rhythm of *C. vicina* is under the control of a coupled multioscillatory system, thereby supporting the **multioscillatory hypothesis**.

7. The frequent occurrence of splitting of the activity rhythm when applying LD 1:23 at circadian times within the area where delayed phase-shifts give way to the advanced phase-shifts (Ct18-Ct20) suggested that it provides a convenient tool to test the multioscillatory hypothesis.

8. The observation that the components of a split rhythm demonstrated free-run or entrainment simultaneously implied that synchronisation is not necessarily the result of coupling, individual oscillators appeared to entrain to the light/dark cycle while their counterparts responded differently.

9. The role that light plays in the locomotor activity rhythm of *C. vicina* appeared to be more complicated than resetting and entrainment. When the light is dim and continuous, the rhythm free-runs with a lengthened period, whereas, when it is bright

and short (1 hour light pulse) and perturbs a certain circadian phase, the coupling loosens and the rhythm splits into several components (Fig. 2.4.6.5 & 2.4.6.6). Nevertheless, a light/dark cycle was also observed to tighten an intrinsically loosened coupling (Fig. 2.4.6.8). These observations suggested that the coupling between different oscillators may depend, at least partly, on the light intensity, which, is consistent with the multioscillatory hypothesis.

The direct and indirect evidence obtained in the present investigation provides the basis on which a heuristic model was developed to describe the circadian oscillatory system in *C. vicina* (Fig. 2.5.1), which is in favour of the multioscillatory hypothesis. In this model, a number of oscillators, each functioning independently with its own period, couple to generate an overall output - the overt rhythm. In theory, when the coupling is tight, the system displays a unified response to a light stimulus (pulse), which accordingly yields only one PRC; whereas in the case of a loosened coupling, PRCs for individual oscillators may be revealed. Such loosening can be intrinsic or, as reported in the present study, induced by repeated 1 hour light pulses (LD 1:23). This, therefore, provides the most persuasive evidence for the multioscillatory hypothesis.
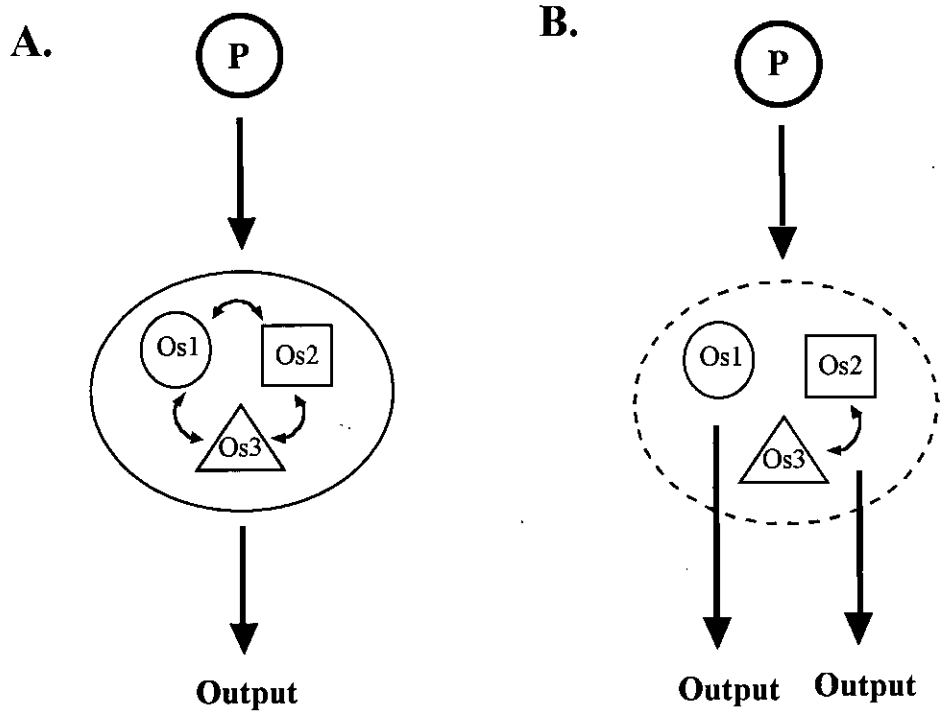
Figure 2.5.1. A heuristic model for the circadian system in *C. vicina* (A). The coupling can be loosened, completely or partially, by light stimuli which result in the splitting of the activity rhythm into several components (B). P = photoreception pathway, Os = oscillators, the small arrows pointing at both directions indicate the coupling between oscillators.

Chapter 3

Clock and Photoreceptor Location

3.1. Introduction

Efforts devoted to studies of the circadian system have been partially directed to a search for the anatomical locations of the clock and photoreceptor. Results of such studies are prerequisites for understanding local mechanisms underlying clock components, such as the coupling between pacemakers and the pathway of photoreception, thereby aiding our understanding of the entire circadian 'network'. However, despite the efforts, progress has been slow due to the diversity of physical structure among organisms and the complication of the clock system.

Approaches interfering with the clock mechanism have provided researchers with evidence to develop conceptual models (Fig. 3.1.1) aimed to describe the circadian clock system. Although minor differences exist between models, the orthodox proposition is that the circadian system consists of three prime components: circadian pacemaker(s) which generates the near 24 h oscillation, photoreceptor(s) which mediate synchronisation or entrainment to the environmental light/dark cycle, and a regulated system which is driven by the pacemaker(s) to express the overt rhythms. Effects on the performance of the clock after surgical treatments can be hypothesised

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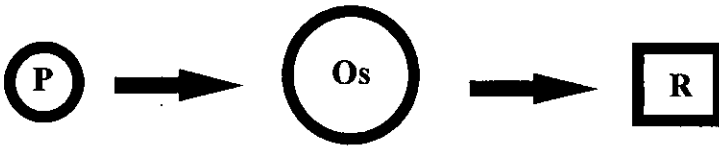
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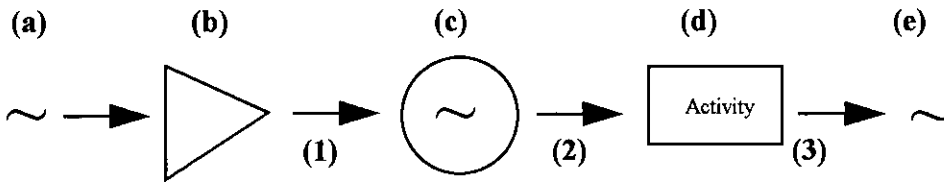
Fig. 3.1.1. A: An oscillatory model of the circadian system (Pittendrigh and Bruce, 1957). Os: oscillator; P: photoreceptor; R: overt rhythm. Coupling pathways are designated by arrows. B: A conceptual model of the circadian system (Eskin, 1979). (a): input; (b): photoreceptor; (c): circadian pacemaker; (d): regulatory system; (e): output; the arrows indicate pathways between circadian components; disconnection of pathway (1) or destroying (b) results in freerun in a light/dark cycle; while interfering pathways (2)/(3) or destroying (c)/(d) results in arrhythmicity.

according to these models. For example, removal of tissue which includes the pacemaker should cause arrhythmicity and the tissue itself should show circadian function in complete isolation from the rest of the body. Furthermore, transplantation of this tissue to a new host should reintroduce a rhythm to the recipient which was deprived of rhythmicity. On the other hand, destroying or disconnecting the photoreceptor should isolate the oscillator from photic information and cause the rhythm to free-run in a light/dark cycle.

The importance of the nervous and neuroendocrine system in controlling invertebrate circadian rhythms was noted as early as 1911 by Demoll (from Brady, 1982b), who suggested that periodic colour changes in arthropods were so controlled. In the past few decades, increasing evidence has been obtained to support the proposition that the central nervous and neuroendocrine systems are responsible for the generation and coordination of circadian rhythmicity. Following this direction, search for the insect circadian clock in the 1960s, was focused on cockroaches. This was partly because of their large size and ease of handling and partly because of the series of reports published by Harker (1954; 1955; 1956; 1960), in which she suggested that the locomotor activity rhythms of the cockroach *Periplaneta americana* were under the control of an autonomous endocrine clock located in the suboesophageal ganglion and that the ocelli were the photoreceptors for light entrainment. She further suggested that the hormonal rhythm in the subesophageal ganglion was maintained by neurosecretion from the corpora cardiaca and that phase shifting was controlled by a second clock responding to photic information mediated by the ocelli (Harker, 1954; 1956; 1960). Her novel claims aroused great interest but

could not be corroborated by other researchers (Brady, 1967; Nishiitsutsuji-Uwo and Pittendrigh, 1968a, b; Roberts, 1965; 1966). The series of reports published by Nishiitsutsuji-Uwo and Pittendrigh (1968a, b) is a representative example of the studies carried out in attempts to test Harker's work. Their studies provided several lines of contrasting evidence against Harker's conclusions, among which the most provoking suggestion was that the optic lobes were the crucial elements for the control of circadian rhythmicity. Moreover, they established two distinct facts from cockroaches: the compound eyes were the sole photoreceptors for entrainment (Nishiitsutsuji-Uwo and Pittendrigh, 1968a), and ablation of the optic lobes abolished rhythmicity (Nishiitsutsuji-Uwo, and Pittendrigh, 1968b). Successive studies applying surgical approaches such as transplantations, lesions and ablations resulted in the accumulation of supportive evidence for such conclusions (Colwell and Page, 1990; Page, 1978; 1982; 1983a, b; Roberts, 1974; Sokolove and Loher, 1975). It is now firmly established that, in cockroaches, the only operations which abolish the locomotor activity rhythm are those that interrupt the neural pathways between the optic lobes and the brain and between the brain and the thorax.

Although surgical techniques have been the most commonly applied approaches when investigating the anatomical location of circadian pacemakers, the results so produced have suffered from a major difficulty in interpretation. As shown in many models, the expression of an overt rhythm involves both the pacemakers and a regulated system driven by them. When 'after effects', such as disappearance, suppression or modification of the activity rhythm are observed after surgical treatments, it is difficult to prove whether the parts affected contain the pacemakers,

or simply components necessary for the exhibition of overt rhythms. Pittendrigh (1976) suggested that the only parameters which can be considered to reflect the state of the pacemaker are its phase and its free-running period in steady state. According to this theory, transplantation has been utilised to resolve the doubts. It is only when transplantation of a putative tissue reintroduces the rhythm of a donor to a recipient, deprived of rhythm, that a conclusive statement can be made.

3.1.1. The Role that Optic Lobes Play as the Circadian Oscillators in Insects

Ever since Nishiitsutsuji-Uwo and Pittendrigh (1968a, b) proposed that optic lobes play an important role in controlling cockroach locomotor activity rhythms, the search for insect circadian pacemakers has been focused on this bilaterally paired tissue. Reports have been published on a number of species, including cockroaches, crickets, two beetles, the New Zealand tree weta, moths and flies. Results of these studies classified the experimental insects into two groups, depending on whether or not circadian rhythmicity persisted after optic lobe ablation (bilobectomy). Species classified into the first group, i.e. with their circadian pacemakers located in the optic lobes, are hemimetabolous mainly orthopteroid insects which include the cockroaches, *Periplaneta americana* (Nishiitsutsuji-Uwo and Pittendrigh, 1968a, b) and *Leucophaea maderae* (Colwell and Page, 1990; Page, 1982; 1983a, b; Page *et al.*, 1977; Roberts, 1974), the crickets, *Teleogryllus commodus* (Loher, 1972; Sokolove and Loher, 1975; Wiedenmann, 1983) and *Gryllus bimaculatus* (Tomioka, 1993; Tomioka and Chiba, 1986; Tomioka and Chiba, 1989), and the New Zealand tree weta, *Hemideina thoracica* (Waddell *et al.*, 1990). Although a member of the

holometabola, the beetle, *Anthia sexguttata* (Fleissner, 1982) has also been placed in this group. On the other hand, species that demonstrate no effect on the persistence of the circadian rhythm after bilobectomy are classified into the second group (holometabola) which includes moths and flies, such as the giant silkmoths, *Hyalophora cecropia* and *Antheraea pernyi* (Truman, 1972), the mosquito *Culex pipiens* (Kasai and Chiba, 1987), the house fly, *Musca domestica* (Helfrich *et al.*, 1985), the fruit fly *Drosophila melanogaster* (Helfrich, 1987; Helfrich and Engelmann, 1983; Konopka and Benzer, 1971), and the blowfly, *Calliphora vicina* (Cymborowski *et al.*, 1994). Except the beetle, such classification coincides with the method of metamorphosis, which deserves further investigation and may provide useful information in the developmental aspect. Although little evidence has been produced to suggest the involvement of optic lobes in the circadian pacemaking in holometabolous species, such possibility should not be completely ruled out. Investigating the locomotor activity patterns of *Drosophila* brain mutants with reductions in the optic lobes, Helfrich (1987) suggested that optic lobes could act as a form of coupling device on oscillators located in the central brain, reduction of the optic lobes reducing the coupling strength between these oscillators.

Since the involvement of the optic lobes in the circadian pacemaking in some insects has been confirmed, it is appropriate to carry out a dissection in order to localise the pacemaker(s) more precisely. As illustrated in Fig. 3.1.2, a typical insect optic lobe has three neuropiles, the lamina, medulla and lobula. Further studies in search of the circadian pacemakers have been focused on these neuropiles and led to diverse conclusions. Results of surgical experiments on several insects, such as the
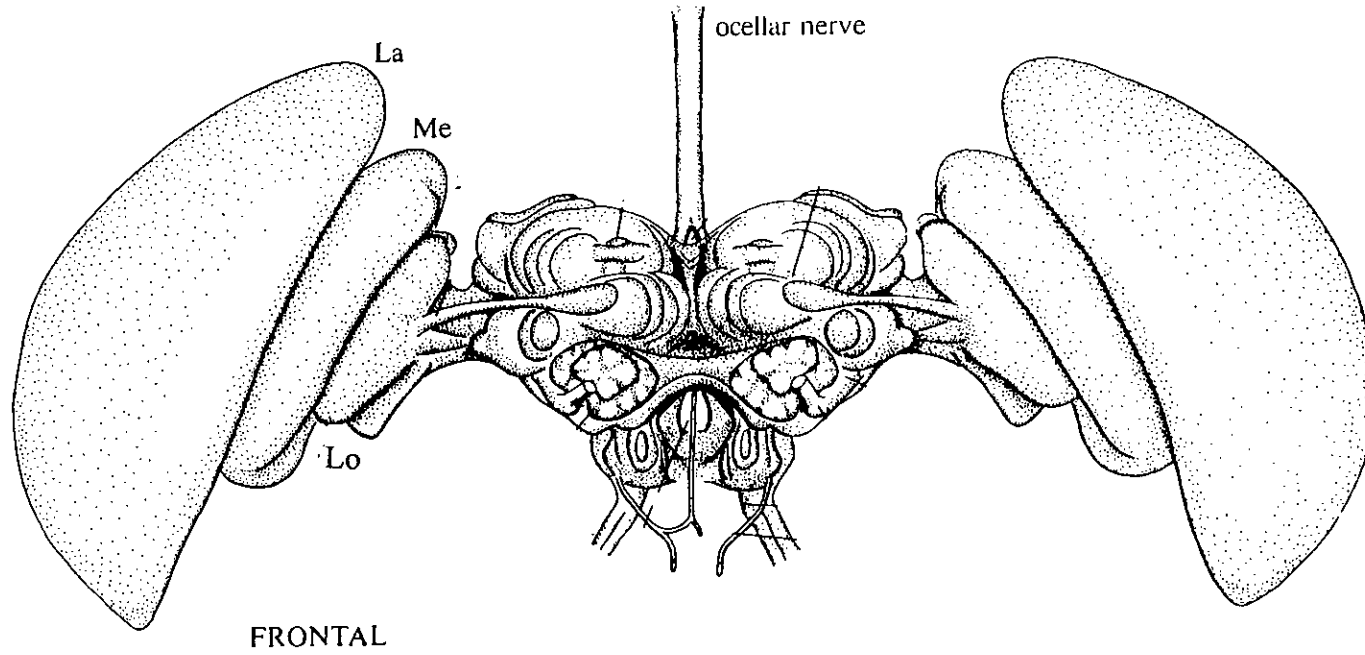
Figure 3.1.2. Frontal view of an insect brain. Each of the symmetrical optic lobes consists of three main neuropiles, the lamina (La), the medulla (Me) and the lobula (Lo) (from Strausfeld, 1976).

cockroaches (Page, 1978; 1982; Roberts, 1974), the cricket *T. commodus* (Sokolove and Loher, 1975) and the beetle *A. sexguttata* (Fleissner, 1982), have confirmed the importance of the lobula in controlling the rhythm. On the other hand, the lamina-medulla complex of the cricket *G. bimaculatus* exhibited clear circadian rhythms when isolated from the rest of the brain (Tomioka and Chiba, 1986), thereby indicating the importance of these neuropiles. Helfrich (1987) found no significant difference in the locomotor activity patterns of *Drosophila* brain mutants, even when all three neuropiles were reduced to various extents, as compared with the wildtype flies.

Efforts devoted to the search of the insect circadian clock, however, led to the consensus that the circadian pacemakers reside in bilaterally paired structures in the central nervous system. Apart from insects, circadian pacemakers have also been localised in the nervous system of several other bilaterally symmetric animals including molluscs (each of the eyes) (Jacklet, 1969; Page and Nalovic, 1992), mammals (the suprachiasmatic nucleus, SCN) (Hakim *et al.*, 1991; Rusak, 1989) and birds (pineal gland) (Gaston and Menaker, 1968; Menaker and Zimmerman, 1976; Turek *et al.*, 1976; Zimmerman and Menaker, 1975). Nevertheless, some experimental results indicated that the pars intercerebralis (midbrain) served as a site of coupling between a circadian pacemaker and various overt behaviours in the cricket *T. commodus* (Sokolove and Loher, 1975) and the cockroach *L. maderae* (Page, 1978). Tomioka (1985) failed to abolish rhythmicity after bilateral optic tract severance or removal of lamina-medulla and suggested the involvement of an oscillatory structure outside the optic lobes. Therefore, these results suggested the

possibility that part of the oscillatory system might be located outside optic lobes for some of the species classified in group 1.

3.1.2. Photoreception Pathways and Locations of Photoreceptors

Despite being one of the prime elements that constitute the circadian clock, photoreceptors, known to be responsible for photic entrainment in insects, attract little attention, compared to efforts devoted to studies of circadian oscillators/ pacemakers. Concerning their functions, photoreceptors should be located more peripherally than the central role of the circadian system - the pacemaker - for the convenience of receiving/transferring environmental stimuli. This presumption, therefore, suggested a more convenient access to the components involved in photoreception.

It is natural to presume that the visual system is involved in photic entrainment. Therefore, in the search for the cockroach photoreceptors, attention was firstly directed at ocelli (Harker, 1956). Failure to corroborate Harker's result (Roberts, 1965; Nishiitsutsuji-Uwo and Pittendrigh, 1968b; Page, 1978), however, confirmed the involvement of compound eyes in the photoreception in cockroaches. Results of these studies have established firmly that the compound eyes are the only photoreceptors involved in the entrainment of cockroaches. Similar results were obtained in the cricket *T. commodus* (Loher, 1972; Sokolove and Loher, 1975). On the other hand, results obtained from most holometabolous insects indicated that the central brain almost certainly contains the relevant structures for the circadian photoreception. Supportive results were obtained in *Musca domestica* (Helfrich *et*

al., 1985), *Drosophila* brain mutants (Dushay *et al.*, 1989; Helfrich, 1987) and the beetles, *Pachymorpha sexguttata* (formerly *Anthia sexguttata*) (Fleissner, 1982) and *Zophobas morio* (Fleissner *et al.*, 1993). A more recent report (Cymborowski *et al.*, 1994) also supported the existence of the extra-retinal photoreceptors but did not exclude the involvement of compound eyes in photoreception in the blow fly *C. vicina.*

3.1.3. The Role that an Arrestin-like Protein Plays in the Circadian System

Both vertebrates and invertebrates have two functionally distinct types of photoreceptor: visual and non-visual, the latter being involved in the synchronisation of circadian rhythmicity with the environmental light/dark cycles. In some insect species, results of surgical studies have confirmed that such non-visual photoreceptors are situated outside the retina (see section 3.1.2). More recently, the application of electrophysiological, biochemical and microsequencing techniques in the search of insect extra-retinal photoreceptors has produced interesting results (Bentrop *et al.*, 1993; Cymborowski *et al.*, 1996; Cymborowski and Korf, 1995; Plangger *et al.*, 1994; Yamada *et al.*, 1990) in which attention has been directed to arrestin, an important element in the phototransduction cascade in both vertebrate and invertebrate photoreceptors. Applying a radioactive label, Matsumoto and Pak (1984) identified three retina-specific polypeptides among which a 49-kD polypeptide was suggested to be involved in the photoreceptor excitation/modulation in *Drosophila melanogaster*. Later, with more advanced techniques, such a protein has been also identified as the homologue of vertebrate arrestin (Yamada *et al.*, 1990).

Reports on the identification of such a homologue (arrestin-like protein) have been limited to very few insect species, including the blow fly *C. vicina* (Bentrop *et al.*, 1993; Plangger *et al.*, 1994). One of the two arrestins identified in *C. vicina*, arrestin 2 (Arr2), was reported to be the homologue of the *Drosophila* arrestin-like protein (Plangger *et al.*, 1994). Bentrop *et al* (1993) confirmed the involvement of Arr2 in the photoreception mechanism. These results inspired the application of the S-antigen-immunoreaction as a marker for extra-retinal photoreceptors in *C. vicina*. Applying such a technique, Cymborowski and Korf (1995) demonstrated the distribution of S-antigen-immunoreactive neurons in the blow fly brain, which suggested candidate sites for extra-retinal photoreceptors. Their results provided a basis for a very recent study (Cymborowski *et al.*, 1996) which investigated locomotor activity rhythms of *C. vicina* injected with S-antigen antibody. Results of this study (detailed account see 3.3.2) suggested that the four groups of arrestin-positive neurons identified in the brain in *C. vicina* were the likely site for extra-optic photoreceptors.

3.1.4. Aims of the Present Investigation

I. Application of Microsurgery as a Means to Localise the Circadian Pacemaker and the Photoreceptor(s)

The search for the anatomical location of clock components has attracted as much attention as other attempts to study the circadian system but with less success, mainly because of the small body size of insects. However, results of such studies have led to

a consensus that the circadian oscillator(s) and the photoreceptor(s) may both be located within bilaterally paired structures. Working with cockroaches, early researchers noted that the optic lobes were the crucial elements for the control of insect circadian rhythmicity (see 3.1.1). Since then, particular attention has been directed to the bilaterally paired optic lobes, which confirmed their role as the circadian pacemaker in many insect species, especially cockroaches, crickets and a beetle (detailed account see chapter 3 introduction). Although contrasting evidence has been obtained in some Diptera (see introduction), the possibility that optic lobes house the pacemaker have not been excluded completely. Moreover, evidence has been produced to support the existence of extra-retinal photoreceptors in higher flies, such as the house fly *Musca domestica* (Helfrich *et al.*, 1985), the fruit fly *Drosophila* (Helfrich, 1987; Dushay *et al.*, 1989) and the blow fly *Calliphora vicina* (Cymborowski *et al.*, 1994). Therefore, the present investigation, designed to search for the anatomical location of the pacemaker and photoreceptor(s)in *C. vicina* , was focused on the optic lobes.

II. Injection of S-antigen Antibody as a Means to Explore the Involvement of the Brain in the Photoreception for Entrainment

Since both the free-running activity rhythm and its entrainment to a light/dark cycle remained virtually unaltered after optic tract severance or bilobectomy (Cymborowski *et al.*, 1994), the brain was therefore assumed to be the most likely site for both extra-retinal photoreceptor(s) and the circadian pacemaker. Candidate extra-retinal photoreceptor neurons have been identified in *C. vicina* by an immunocytological

technique, applying a polyclonal antibody against bovine S-antigen (arrestin) raised in rabbits (Cymborowski and Korf, 1995). Their results demonstrated a wide distribution of immunoreactively labelled neurons throughout the visual system, including the compound eyes, the optic lobes and the brain. In the fly's brain, a positive S-antigen immunoreactivity were observed in four bilaterally arranged groups of neurons which were suggested to be the candidate sites for the extra-retina photoreceptors. These results provided concrete bases for the present investigation designed to reveal the involvement of the brain (neurons) in the photoreception for entrainment of the locomotor activity rhythm in C. vicina. The pre-experimental expectation was therefore that the injected S-antigen antibody should bind to the arrestin homologue contained in the brain (neurons), thereby blocking the photoreception pathway, partially or completely, and interfering with entrainment to a light/dark cycle. If so, such results could then provide evidence to support the presumption that these brain neurons are involved in the photoreception for entrainment in C. vicina.

3.2. Materials and Methods

3.2.1. Surgical Treatments (Fig. 3.2.1.1)

All experimental flies were selected from the stock cultures maintained under conditions as described in section 2.2.1. Locomotor activity rhythms were recorded as described in 2.2.2. Maintenance of larval cultures was also described in 2.2.1. Experimental flies were placed in free-running conditions (DD, 20°C) for 4-7 days
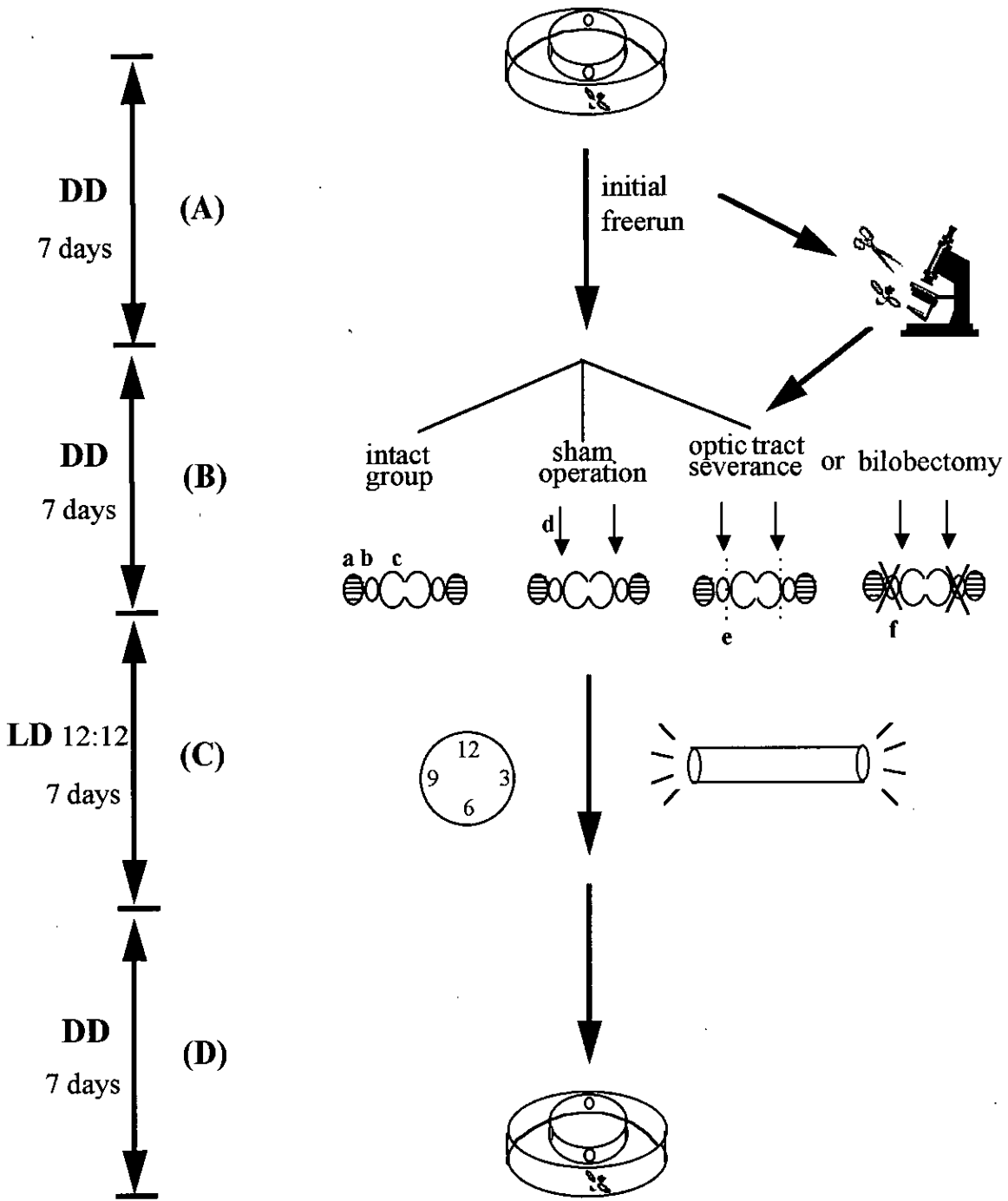
Fig. 3.2.1.1 The experimental protocol to test whether compound eyes and optic lobes are involved in the photic entrainment of the locomotor activity rhythm in *C. vicina*. (A): initial free-run; (B): flies were put back to DD after surgery; (C): all flies were exposed to LD 12:12; (D): final free-run. (a): compound eye; (b): optical lobe; (c): brain; (d): cuts along cuticular landmarks (sham operation); (e): cuts between optic lobe and brain (optic lobe severance); (f): removal of both optic lobes (bilobectomy).

prior to the operation. The duration of the operation performed on each fly was limited to between 1-2 minutes, so as to limit exposure to the high light intensity required during surgery. Surgery was performed under a dissecting microscope (Wild Heerbrugg). Instruments used were: a fine razor blade (self-made), a pair of fine scissors (Dixey) and a pair of fine forceps (No. 5). Pieces of flattened plasticine were used as a 'bed' for the operation as well as to hold the fly to a convenient position. Low melting point (30°C) wax was applied for sealing the cuts. No anaesthesia was applied.

Optic tract severance

(1) A female fly was taken out of the recording device and mounted, with head facing downwards, onto a piece of plasticine, flattened and shaped to hold the fly. The fly was held still by covering a piece of thin plasticine over the body and pressing down firmly on both sides. To expose the site for operation, the fly's head was pushed gently downwards and secured with another piece of plasticine. This position facilitated access to the rear of the head capsule where two distinct symmetrical sutures were situated and used as 'landmarks' for incisions.

(2) The operation was performed on one side at a time. Incisions were made with a piece of fine razor blade. The first cut was made along one of the cuticular 'landmarks' followed by another straight cut around the middle line of the cuticle; finally a parallel cut was made to join the two straight cuts. The cuticular area encompassed by the cuts was then lifted and tagged to expose the optic lobe and tracts.

(3) With the blade, a cut between the optic lobe and the brain was made to disconnect them completely. A drop of penicillin was added to the body fluid to prevent infection. The flap of cuticle was then replaced and sealed with low melting point wax. In the case of bilateral cuts, the same surgical procedure was then repeated on the other side.

(4) Finally, the surgical treatment was completed by covering all three ocelli with black wax. The operated fly was then released from the plasticine and placed in the recording device (2.2.2). Care was taken while handling flies during the operation because any slight damage might have affected the exhibition of locomotor activity, thereby leading to inappropriate conclusions.

Bilobectomy

Since prior study of optic tracts severance showed no interruption to the exhibition of circadian rhythm and pathway for light entrainment (Cymborowski *et al.*, 1994), complete removal of the optic lobe (lobectomy) was then performed. Flies were mounted and heads cut open as described previously (see optic tract severance). For lobectomy, however, a pair of fine scissors was used to disconnect the optic lobe from the surrounding tissues. The disconnected optic lobe was then removed with a pair of fine forceps. The operation was then repeated on the other side. Cuts were sealed and ocelli covered as described previously (see optic tract severance).

Sham operation

The head of a sham operated fly was cut, opened and sealed as the operated groups, but with optic tracts and lobes left intact.

Intact group

After the initial free-run, flies in this group were taken out of the recording chamber and exposed to the same light conditions needed for surgical treatment, but then returned to the recording device without operation.

Postmortem histological examination

At the end of each experiment, histological examination was made of representative flies to determine the precision of the cuts between optic lobes and brain, or whether complete lobectomy had been achieved. The following is a brief description of the procedure:

1. Fly heads were fixed in Bouin's for at least 24 hours.

2. Samples were then dipped in 50% alcohol to wash away the excessive Bouin.

3. Samples were mounted in low-melting-point paraffin wax for sectioning.

4. Sections were cut at 10 μm, mounted onto a glass slide and stained with paraldehyde fuchsin.

3.2.2. Injections of S-antigen Antibody (Fig. 3.2.2.1)

Experimental flies were collected from the stock culture (see 2.2.1) and released in free-running conditions (DD, 20°C) for at least 5 days, then were subjected to one of the following treatments:

(1) flies were transferred to LD 12:12 after the injection of S-antigen antibody; or

(2) flies were returned to DD after the injection of S-antigen antibody; or

(3) flies were transferred to LD 12:12 after the injection of vehicle; or

(4) flies were returned to DD after the injection of vehicle; or
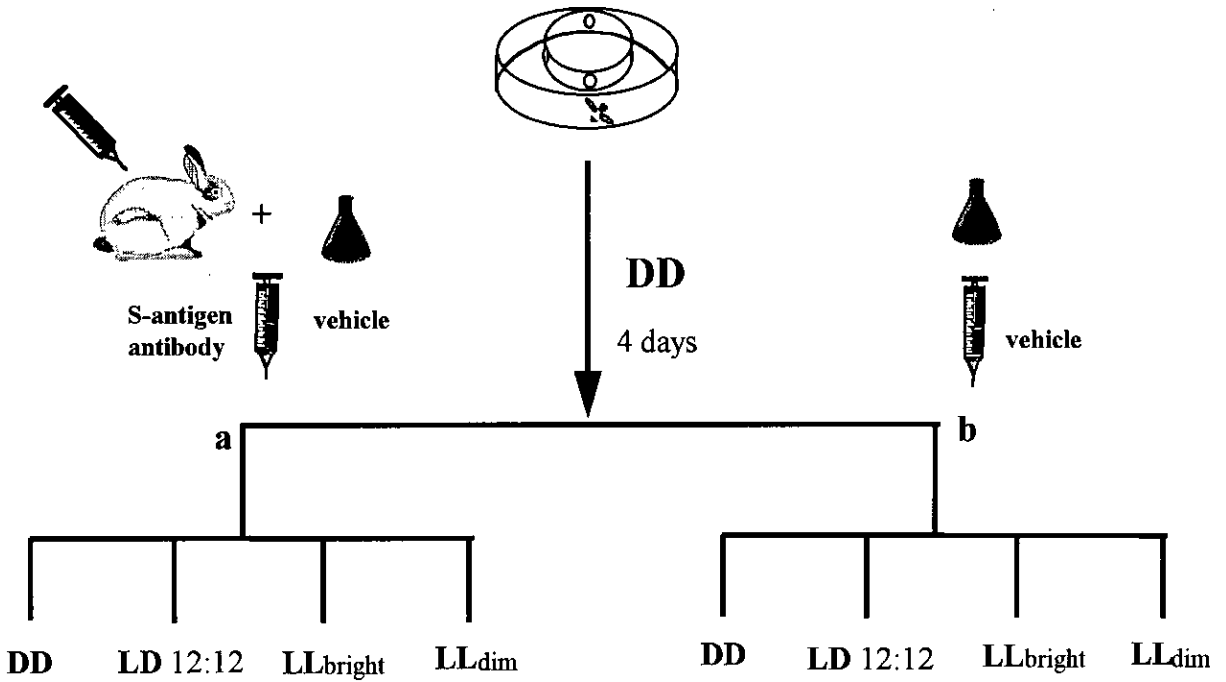
Fig. 3.2.2.1 Protocol for S-antigen antibody injection experiment. After the initial free-run in DD (normally 4 days), experimental flies were treated in two groups: (a) injection of S-antigen antibody and vehicle mixture, and (b) injection of vehicle alone. The injected flies were then transfer to one of the following light conditions: DD; or LD 12:12; or LLbright: continuous light of a reduced bright light intensity (> 0.04 Wm^{-2}); or LLdim: continuous light of a reduced dim light intensity (~ 0.018 Wm^{-2}).

(5) flies were transferred to continuous light of a reduced bright light intensity ($>$ 0.04 Wm^{-2}) after the injection of S-antigen antibody; or

(6) flies were transferred to light conditions as in (5) after the injection of vehicle; or

(7) flies were transferred to continuous light of a reduced dim light intensity ($<$ 0.02 Wm^{-2}) after the injection of S-antigen antibody; and

(8) flies were transferred to light conditions as in (7) after the injection of vehicle.

The antibody used in injection was raised in rabbits against bovine S-antigen (arrestin) (NEI 04111083; dilution 1:4000) (special thanks for Professor Horst-W Korf of Johann Wolfgang Goethe-University, Frankfürt/Main Germany). The vehicle injected to control flies was a mixture of PBS and 1% BSA. For each injection, 0.5 µl of antibody or vehicle was injected through the right compound eye into the optic lobe using a very fine glass capillary. All injections were performed shortly after lights on (for the flies exposed to LD 12:12 or those transferred to continuous light). Transfer to various light conditions after injection was within minutes. Arrhythmicity was observed in flies exposed to continuous light at an irradiance greater than 0.03 Wm^{-2} in a previous experiment (2.4.3), whereas, below 0.02 Wm^{-2}, rhythmicity was displayed with a lengthened period. The irradiances applied in the present experiment were chosen according to such a result. Methods for the reduction of irradiance were described in 2.3.3.

3.3.1. Effects of Bilateral Optic Tract Severance and Bilobectomy on Locomotor Activity Rhythms

Results of the surgical treatments were examined and discussed according to Eskin's conceptual model (Fig. 3.1.1.B) which predicts that disconnection made between the photoreceptor and the oscillator should result in free-run in a light/dark cycle, and that destroying the oscillator/pacemaker should result in arrhythmicity. In addition, some of the parameters, such as the free-running period (τ), the active phase (α) and the daily amount of activity, were analysed to investigate further the effects of surgeries on the circadian locomotor activity.

Data obtained in both optic tract severance and bilobectomy experiments were shown in Table 3.3.1.1. The majority of normal control (intact) flies (94.1%, N=17) showed clear rhythms (Fig. 3.3.1.1) with a mean free-running period of 22.43 $\pm$ 0.83 h during the initial free-run in DD (day 1-14). These intact flies showed firm entrainment upon transfer to LD 12:12 (day 15-21) and resumed their free-running behaviour when returned to DD for the final free-run (day 22-28). A similar result was obtained with sham-operated flies. After the operation only one fly was observed to display the 'doubtful' category of post-operative behaviour, the rest of the group (96.2%, N=26) showed an undisturbed free-running rhythm in DD (day 8-14) with the mean period of 23.42 $\pm$ 0.79 h which was not significantly different from that of the initial free-run prior to the operation (22.84 $\pm$ 0.61 h) (Wilcoxon,

	N	Rhythmic % (n)	Arrhythmic % (n)	Doubtful % (n)
Intact control	17	94.1 (16)	0	5.9 (1)
Sham-operated control	26	96.2 (25)	0	3.8 (1)
Optic tract severance	23	82.6 (19)	4.3 (1)	13.0 (3)
Bilobectomy	37	81.1 (30)	5.4 (2)	13.5 (5)

Table 3.3.1.1. Effects of bilateral optic tract severance and bilobectomy on the free-running locomotor activity rhythm in the blow fly *Calliphora vicina*.
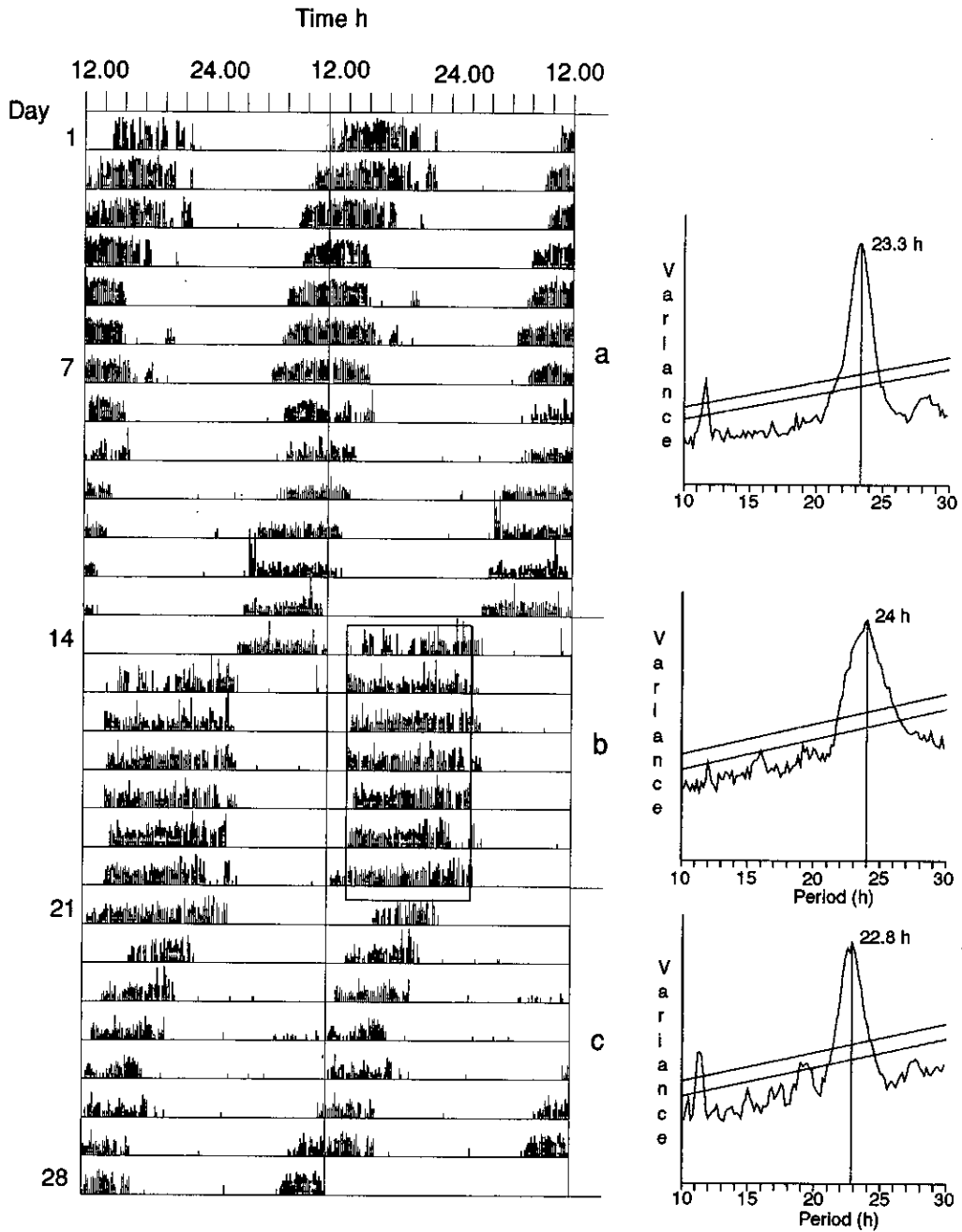
Figure 3.3.1.1. Actogram showing the activity rhythm of an intact control fly firstly free-ran in DD (a), then entrained to LD 12:12 (b), and resumed its free-running behaviour in the final free-run (c).

T=32.5, N=16, not significant). Moreover, the mean active phase (α) after operation (11.42 ± 2.5273 h) was also not significantly different from that prior to the operation (10.12 ± 2.7327 h) (T=33.5). However, the mean amount of daily activity was significantly reduced (T=16, P<0.01). These operated flies were then entrained to LD 12:12 (day 15-21) and resumed their free-running behaviour in the final free-run as the intact flies. A representative actogram of a sham-operated fly is shown in Fig. 3.3.1.2.

Optic tract severance had little effect on the rhythm of locomotor activity in all aspects examined in this study. Most operated flies (82.6%, N=23) showed clear rhythms with only a few doubtful cases (13%) and one arrhythmic case (4.3%). Concerning only the proportion of the rhythmic and arrhythmic cases, this result was not significantly different from the sham-operated group (χ^2 = 0.2653, d.f. = 1).

Clear post-operative rhythmicity was observed in most bilobectomized flies (81.1%, N=37), which was not significantly different from that of the sham-operated group (χ^2 = 0.3968, d.f. = 1). The activity rhythms of these bilobectomized flies free-ran in DD with both the mean period (τ_{DD} = 23.53 ± 1.1967 h) and the mean active phase (α = 11.57 ± 2.7597 h) not significantly different from those prior to the operation (τ_{DD} = 23.13 ± 0.7430 h, α = 10.84 ± 1.8975 h) (Wilcoxon, T=16.5; T=17.0). Nevertheless, as with the sham-operated group, statistical analysis showed a significant post-operative reduction in the daily amount of activity (T=20, P < 0.01). A representative actogram of a bilobectomized fly was shown in Fig. 3.3.1.3.
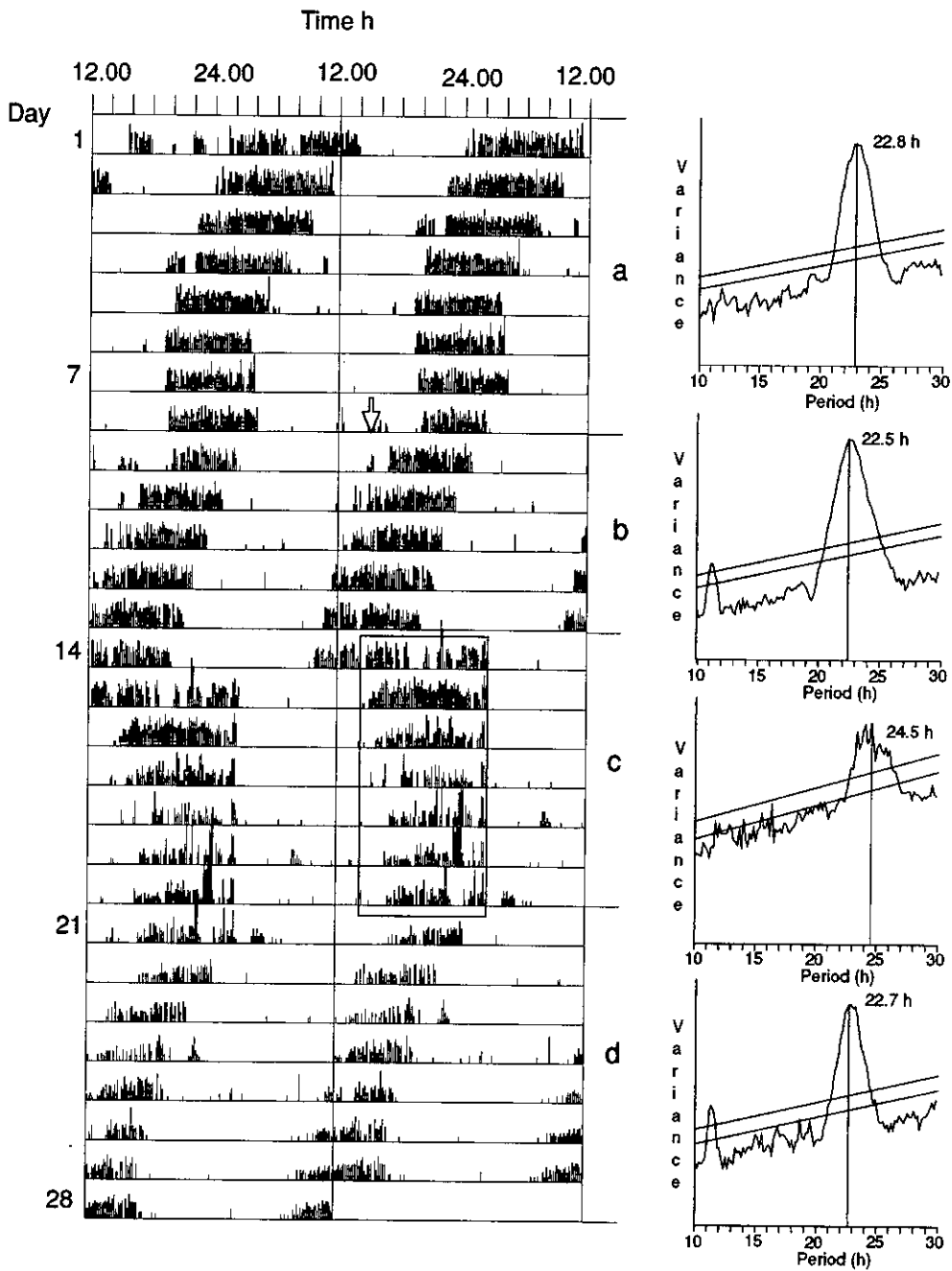
Figure 3.3.1.2. Actogram of an sham-operated fly. After the initial free-run in DD (a), the fly was operated on day 9 (arrow) and allowed to free-run in DD for 6 more days (b). This was then followed by exposure to LD 12:12 (c) and the final free-run in DD (d). No difference was observed on the free-running and entraining behaviour, comparing to the intact control fly (Fig. 3.3.1.1).

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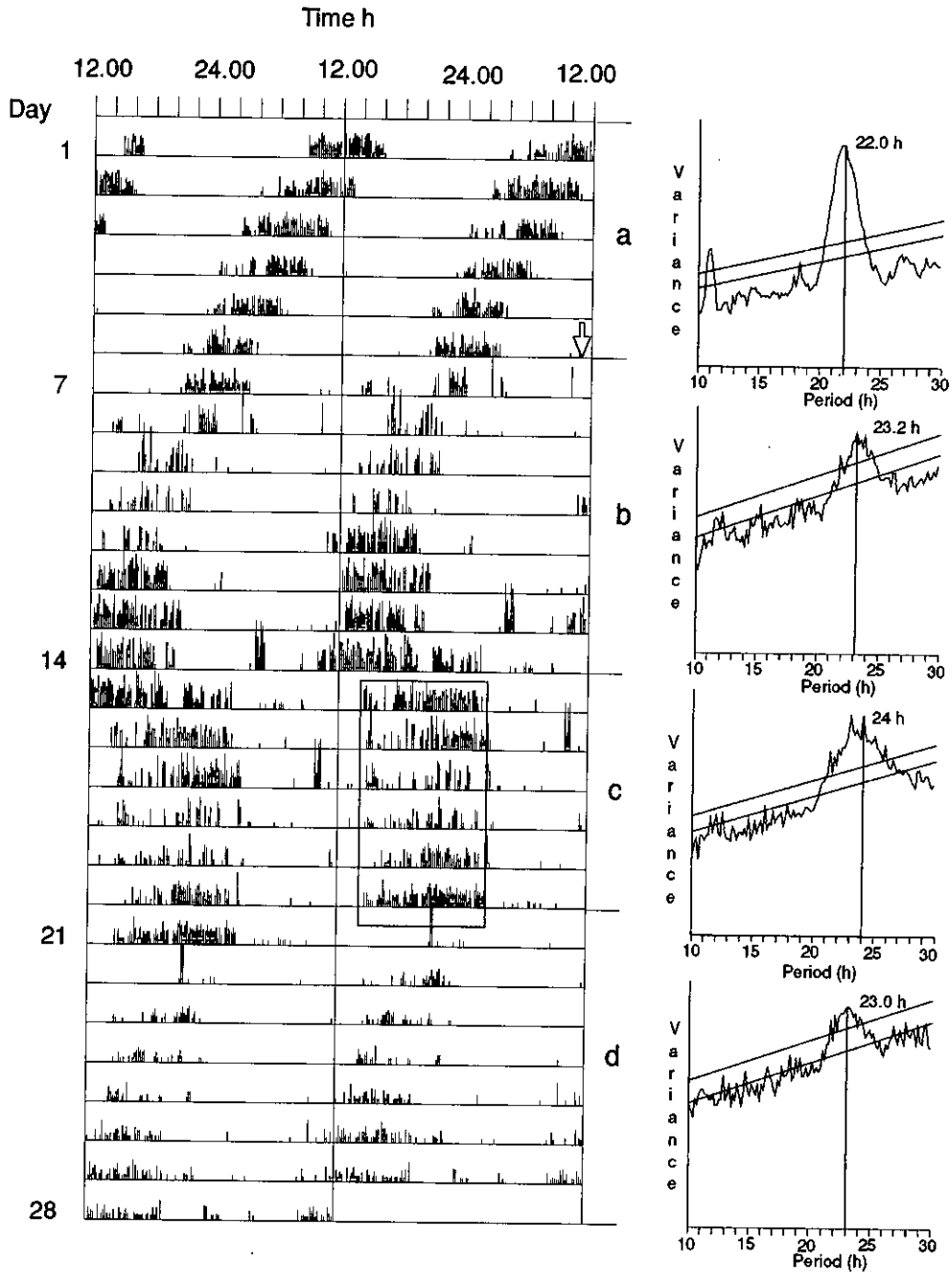
Figure 3.3.1.3. Actogram of a bilobectomized fly showing virtually unaltered free-running and entraining behaviour. After the operation on day 7 (arrow), the fly was transferred to DD (b), exposed to LD 12:12 (c), and returned to DD for the final free-run (d). Clear rhythmicity observed throughout the experiment indicated that complete removal of the optic lobes did not interfere with the circadian pacemaking. Entrainment was achieved upon exposure to the light/dark cycle, therefore suggested that both the optic lobes and the compound eyes are not necessary for the entrainment to a light/dark cycle in *C. vicina*.

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In one case, a post-operative splitting of the activity rhythm was observed when the fly was operated in the middle of the subjective day (Fig. 3.3.1.4). The effect of light stimuli during the operation was excluded, according to the result of a previous experiment - a light stimulus perturbing the middle of the subjective day often caused a delay phase-shift, but not splitting of the activity rhythm (2.3.6). Therefore, the splitting may be attributed to removal of the optic lobes, which may have resulted in a loosening of the coupling. Helfrich (1987), investigating the locomotor activity patterns of *Drosophila* brain mutants with reductions in the optic lobes, suggested that optic lobes could act as a form of coupling device on oscillators located in the central brain, reduction of the optic lobes reducing the coupling strength between these oscillators. However, in the present study, no conclusive suggestion could be developed from such an observation, due to the infrequent occurrence of such splitting.

The pathway (optic track and lobes) between the compound eyes and the brain of a control or sham-operated fly was intact, whereas, in a bilobectomized fly, such a pathway was disconnected by the removal of the optic lobes. Although the disconnection did not appear to disrupt entrainment to a light/dark cycle, an interesting phenomenon was observed. A noticeable proportion of the control (37.5%, N=8) and sham-operated (36.4%, N=11) flies showed an increase in activity near light-off (light rebound effect), whereas none of the bilobecotomized flies displayed such a response (Fig. 3.3.1.5). This result suggested that the compound eyes are not necessary for entrainment to a light/dark cycle in *C. vicina*; however,
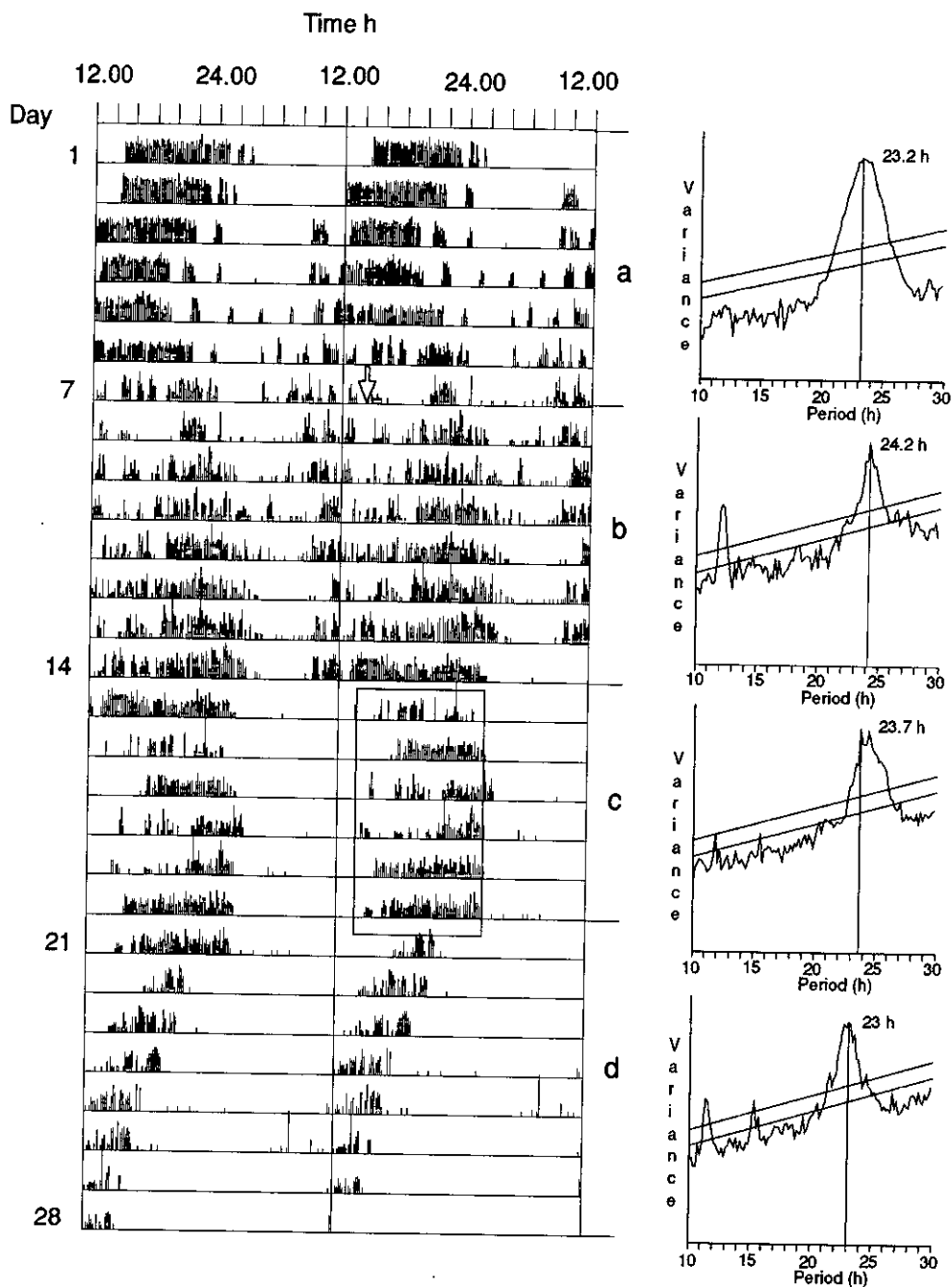
Figure 3.3.1.4. An actogram showing a post-operative split after the complete removal of the optic lobes (bilobectomy). Although the coupling was intrinsically loose in the initial free-run (a), the operation (arrow), however, appeared to decoupled the oscillation further, which resulted in a split of the activity (b). The loosened coupling was tightened by the light/dark cycle (c) and did not reoccur in the final free-run (d). The result suggested that the optic lobes may be involved in the coupling mechanism of circadian rhythmicity in *C. vicina*.

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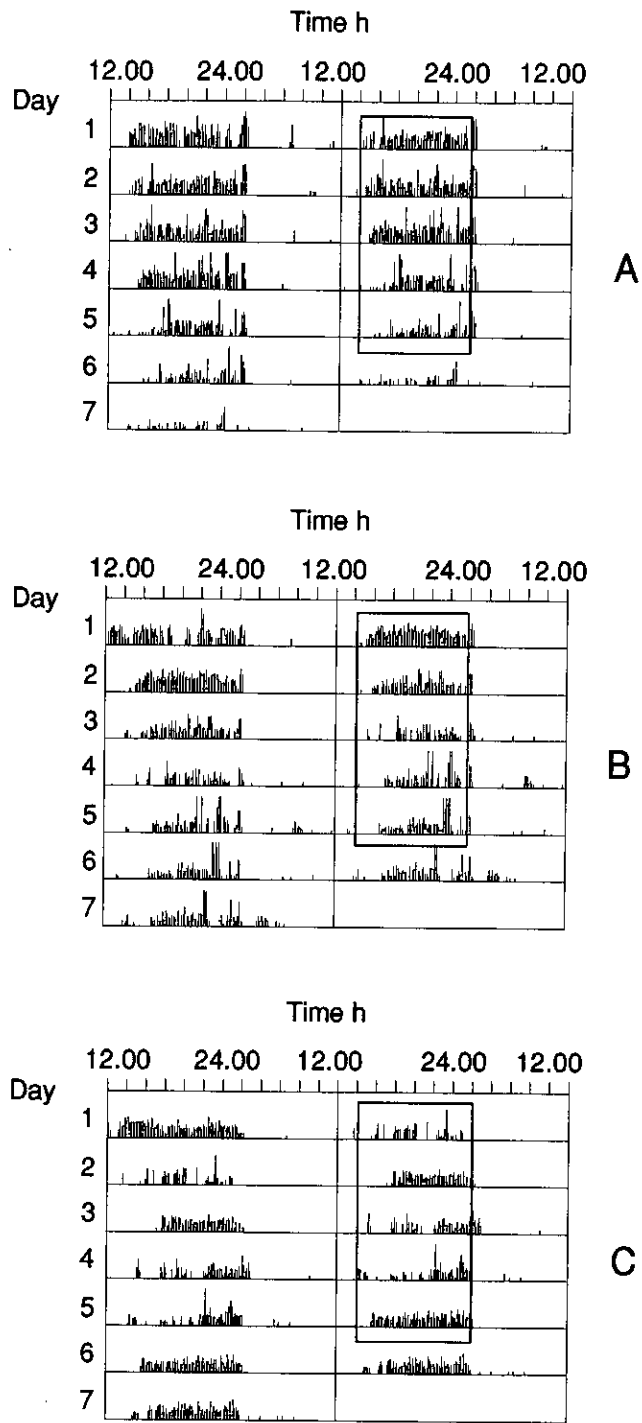
Figure 3.3.1.5. When exposed to LD 12:12, an increase in the activity near light-off (light rebound effect) was observed in intact control (A) and sham-operated (B) flies; whereas such an exogenous effect appeared to be eliminated in bilobectomized flies (C).

their involvement in photoreception producing exogenous 'masking' effects should not be excluded completely.

To sum up, results of the present investigation showed that circadian locomotor activity and its entrainment to the light/dark cycle continued in optic tract severed or bilobectomized flies. Although such surgeries reduced the daily amount of activity and eliminated the 'light-off effect', both the free-running period in DD (τ_{DD}) and the active phase (α) were virtually unaltered. These observations showed that the optic lobes are not essential for the generation/expression of the locomotor activity rhythm in the blow fly *C vicina*. An integrated conclusion is therefore that the circadian pacemaker is located outside the optic lobes, possibly in the brain itself, and that neither the optic lobes nor the compound eyes are necessary for entrainment to a light/dark cycle.

Post-mortem histological examinations (relevant pictures see appendix I) were made of the optic tract severed and bilobectomized flies which survived the experimental period (28 days). For the optic tract severed flies, examination showed that transection was complete and in the correct position on each side of the optic lobes. During the post-operative period (3 weeks), there was no sign of connections re-generated between the optic lobes and the brain. However, some degeneration was observed at the incision. For bilobectomized flies, examination confirmed the complete removal of the optic lobes. In the very few cases where minor portions of the optic lobes remained, no differences were observed in either the free-running or the entraining behaviour. (All post-mortem histological examinations were carried

out under collaboration with Professor B. Cymborowski of the University of Warsaw, Department of Invertebrate Physiology)

3.3.2. Effects of S-antigen Antibody Injection on Locomotor Activity Rhythms

Although the involvement of arrestin in the vertebrate phototransduction cascade has been well documented, the corresponding role it plays in invertebrate photoreception is yet to be investigated. The identification of homologues of vertebrate arrestin in insects provides a feasible protocol for the search of photoreceptors. Efforts devoted to such a study have resulted in the identification of an arrestin-like protein in *C. vicina* (Bentrop *et al.*, 1993; Plangger *et al.*, 1994), as well as providing a structural basis of candidate sites for extra-retinal photoreceptors (Cymborowski and Korf, 1995). These results, together with an earlier study (Cymborowski *et al.*, 1994) which suggested that both the compound eyes and the optic lobes were not essential for the photic entrainment in *C.vicina*, directed attentions of the present investigation to four groups of brain neurons reported to be immunoreactively labelled against S-antigen (arrestin). In the present investigation, locomotor activity rhythms of flies injected with S-antigen antibody were recorded to test the presumption that the brain (neurons) may be involved in the photoreception for entrainment in *C. vicina*. Experimental flies were collected from a stock culture (see 2.2.1), allowed to free-run in DD for 5 days then subjected to various experimental conditions (see 3.2.2). The effects of antibody injection will be discussed in two groups: its effects on free-running rhythms, in DD or in dim LL, and entrainment to a light/dark cycle.

Two interesting phenomena were only observed in flies injected with S-antigen antibody but not in the untreated or vehicle controls.

(1) Flies started to run in circles immediately after the injection of antibody, which was regarded as a behavioural sign of unilateral blinding;

(2) A decline in the activity or completely loss of the activity was observed shortly after the injection of antibody, which was thought to be caused by the shock of the injection (also referred as 'post-injection recovering' behaviour in Fig. 3.3.2.1.B, 3.3.2.2.B & 3.3.2.3).

Some flies appeared to suffer from a shortened life span after the injection of antibody or vehicle (Fig. 3.3.2.4.A & 3.3.2.5.A, B), this however rarely hampered consequent analysis.

The free-running behaviour of a fly injected with vehicle (Fig. 3.3.2.1.A) or antibody (Fig. 3.3.2.1.B) then returned to DD was virtually unaltered comparing to that of a untreated fly. The injections of vehicle or antibody did not appear to have any effect on parameters such as period length, active phase or activity level. Flies in both groups continued to free-run in DD with a period exactly the same or very close to the one prior to the injection (Fig. 3.3.2.1.A & B).

All flies injected with vehicle shortly after the transfer from DD to LD 12:12 (irradiance >0.04 Wm^{-2}) appeared to entrain to the light/dark cycle (Fig. 3.3.2.2.A) in a manner identical to the untreated controls. On the contrary, only some of the flies injected with antibody appeared to entrain to the light/dark cycle (35.7%, N=28) (Fig. 3.3.2.2.B), others either displayed a free-running (35.7%) or doubtful (28.6%) behaviour (Table 3.3.2.1.A). Statistical analysis showed that the effects of S-antigen

A.

B.
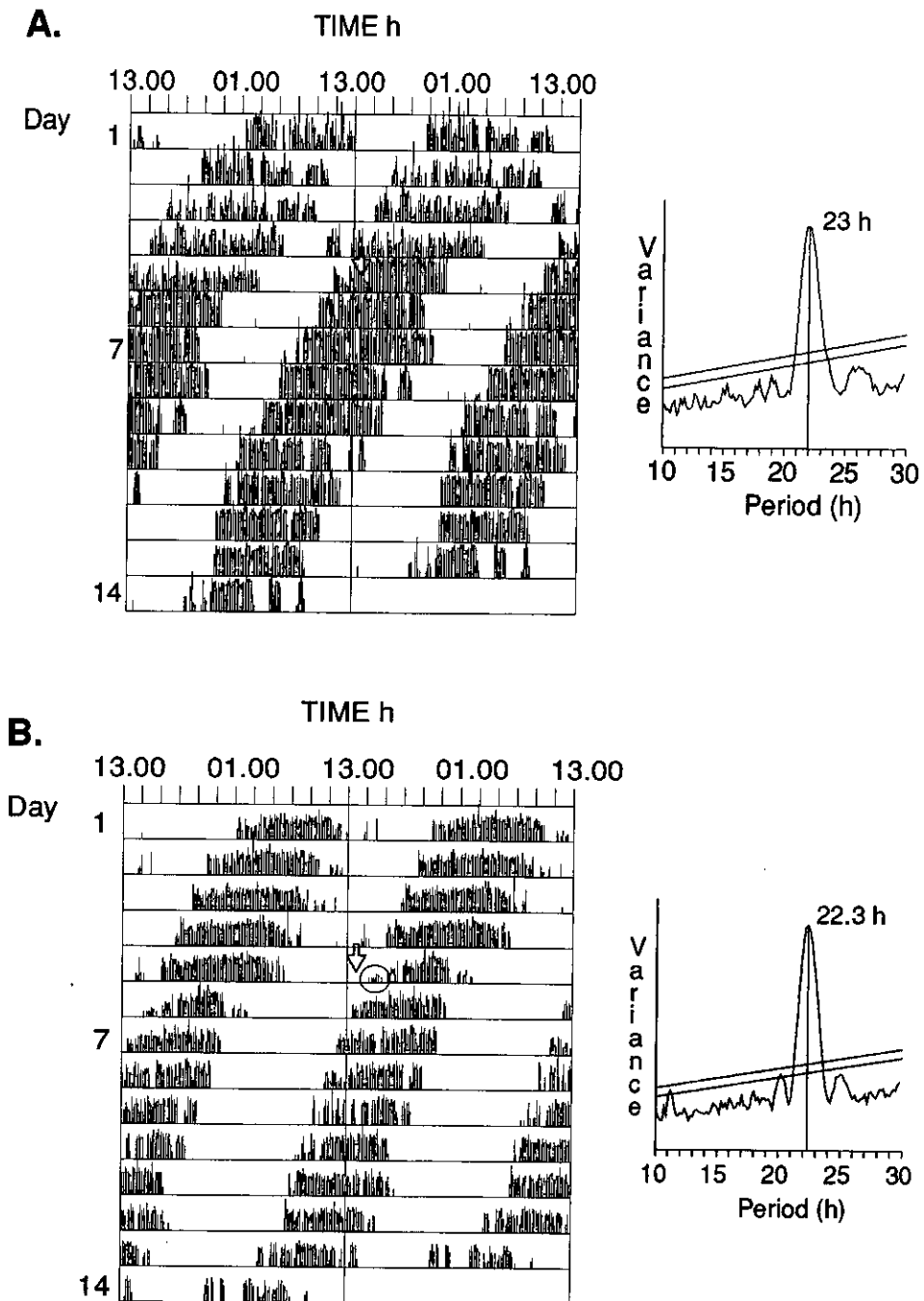
Figure 3.3.2.1. Actograms showing unaltered free-running behaviour of two flies injected with vehicle (A) or S-antigen antibody (B). Flies were kept in DD throughout the experiment except the very short exposure (within 30 sec) to high light intensity required for the injection (arrows). Between these two flies, a slight difference was observed. A decline in activity (circle in B) or a total loss of activity (see Fig. 3.3.2.2.B & 3.3.2.3) observed shortly after the injection (arrow) of antibody was a general phenomenon occurred to antibody-injected flies. Such a 'post-injection recovering' behaviour was never observed in the untreated or vehicle controls. This, however, did not hamper the consequent analysis.
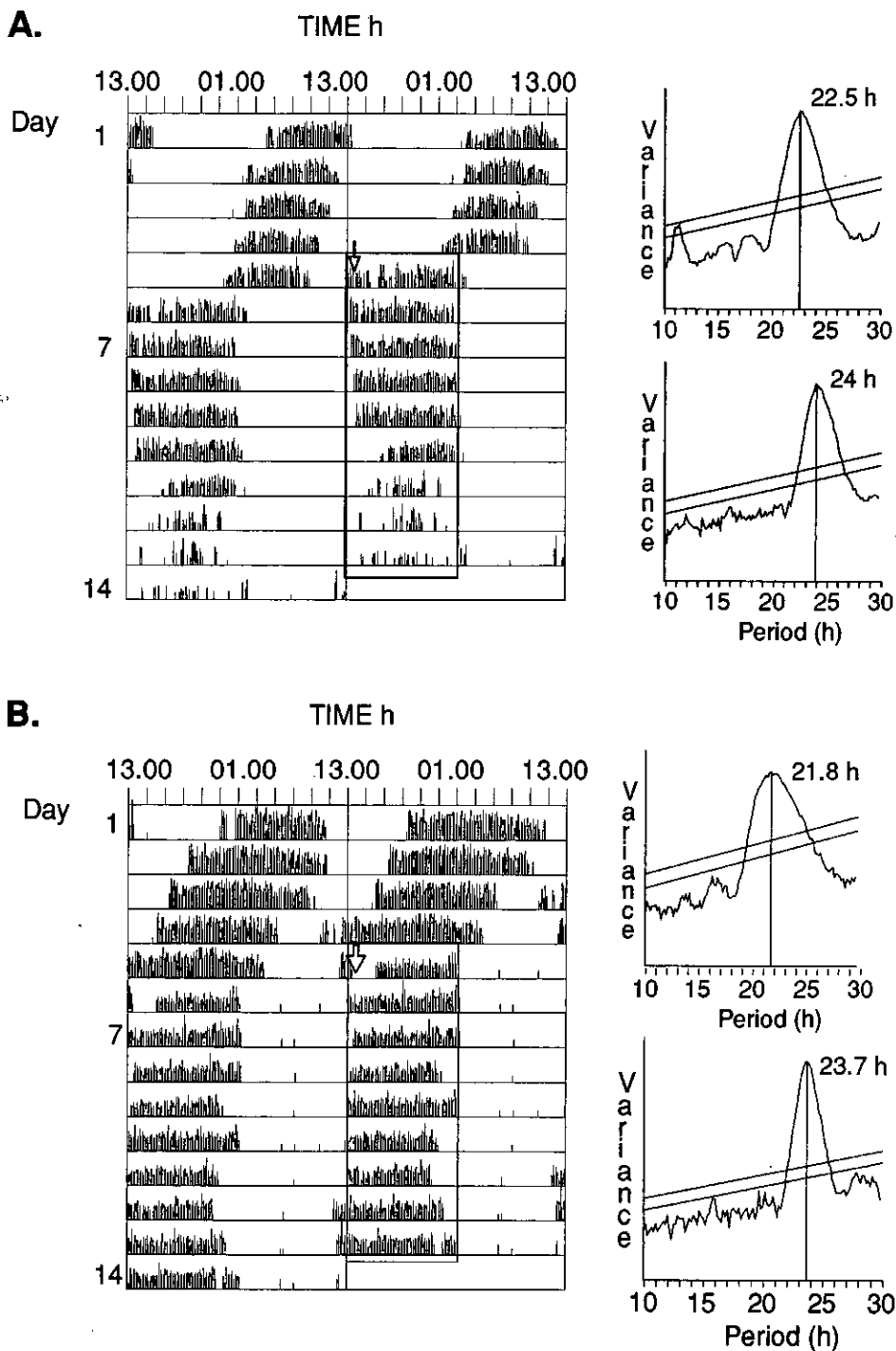
Figure. 3.3.2.2. When exposed to LD 12:12, all flies injected with vehicle entrained to the light/dark cycle (A), whereas only 35.7% (N=28) of flies injected with S-antigen antibody appeared to be entrained (B). After the initial free-run in DD, flies were injected (arrows) shortly after the exposure to LD 12:12 (>0.04 Wm^{-2}) which led to immediate entrainment in both cases. The 'post-injection recovering' behaviour is again observed in the antibody-injected fly (B).
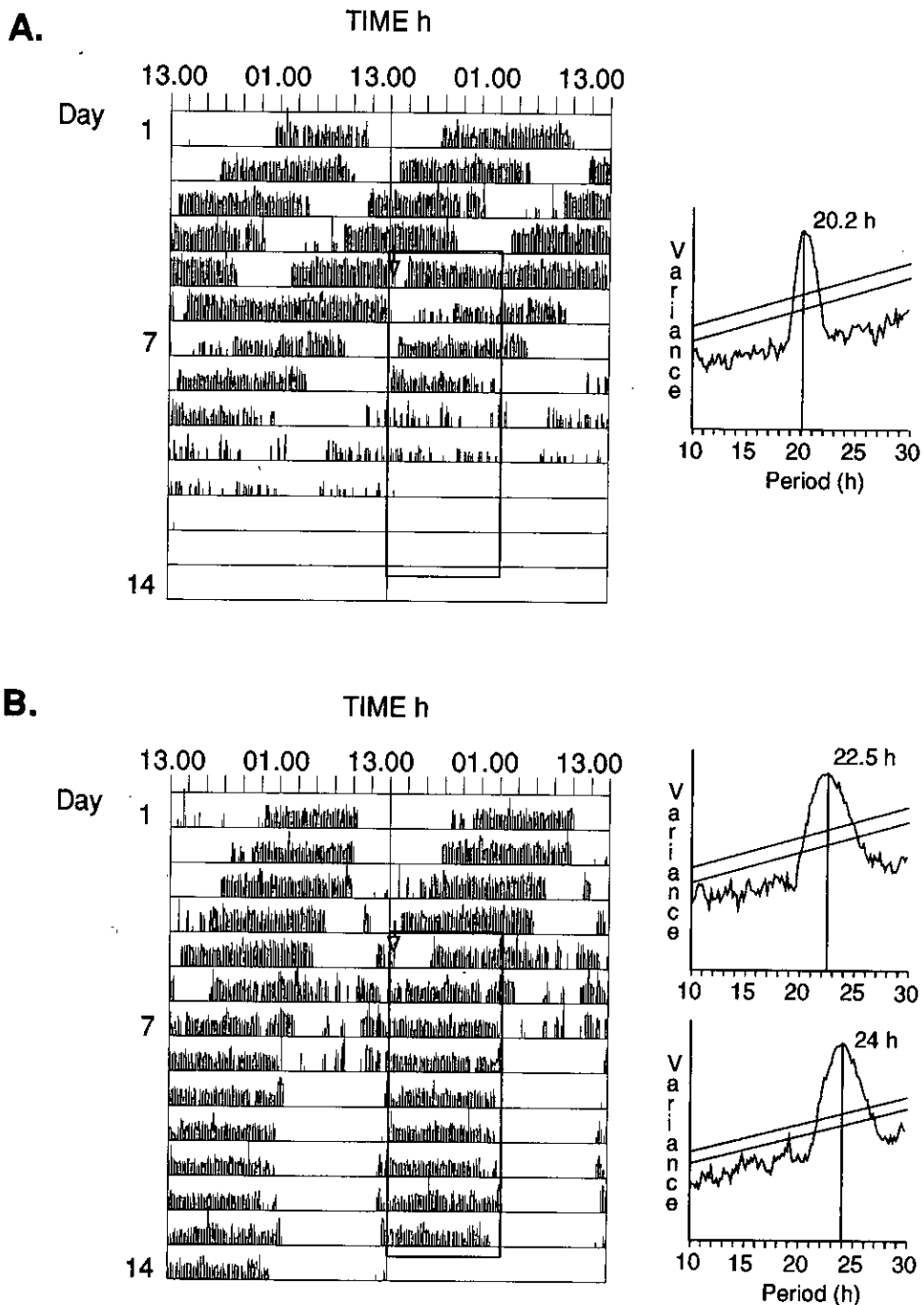
Figure 3.3.2.3. Examples showing free-running behaviour in LD 12:12 observed in flies injected with S-antigen antibody. Both flies were subjected to the same experimental treatment, i.e. injection of antibody (arrows) shortly after the exposure to LD 12:12 (>0.04 Wm⁻²), after the initial free-run in DD. Such free-running behaviour was only observed in a portion of flies (35.7%, N=28), among which some free-ran until they died (A), whereas others free-ran for a few days before entrainment was achieved (B). The latter suggested that injection of antibody does not have a permanent 'blinding' effect and that flies are likely to restore their photoreception ability after several days.
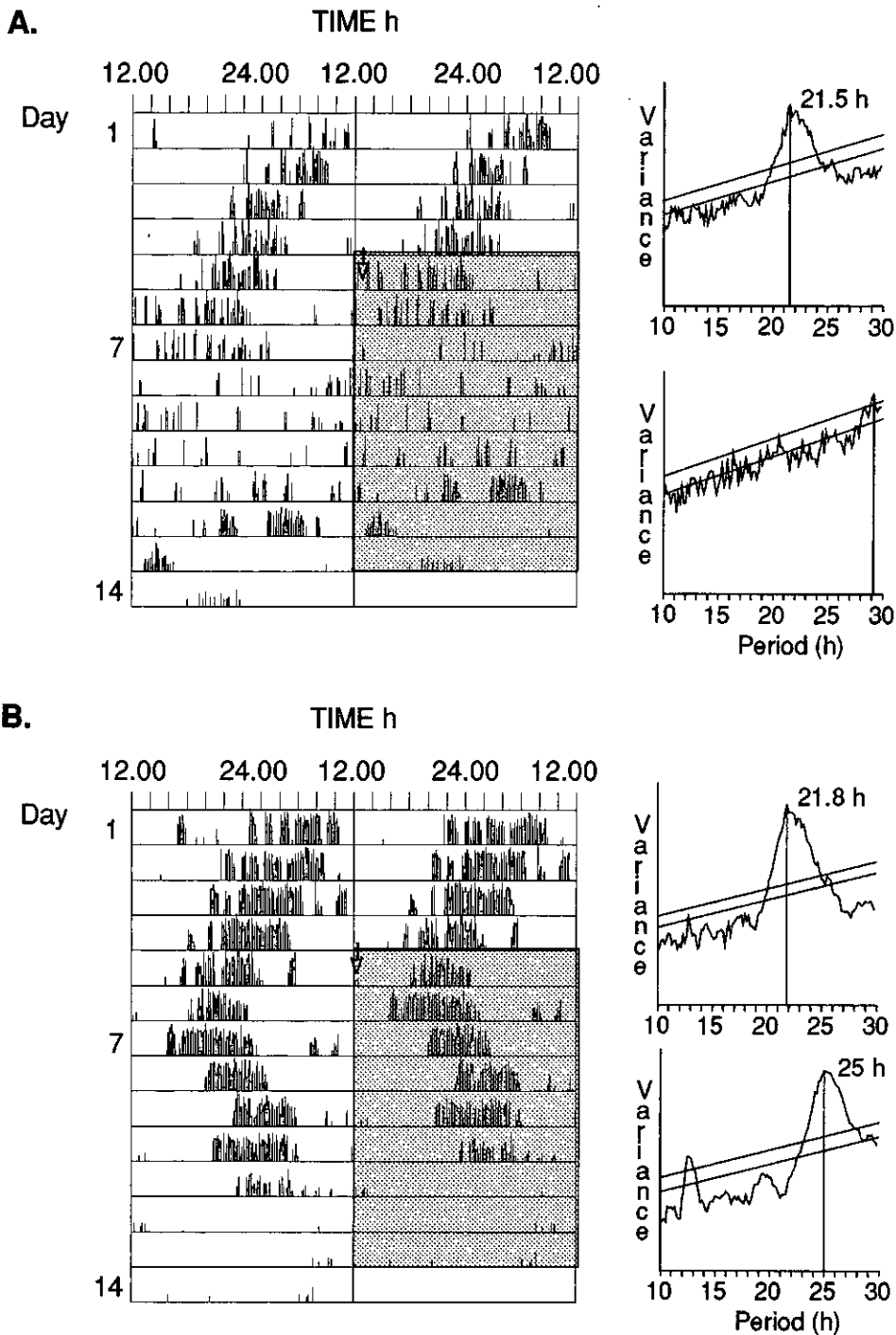
Figure 3.3.2.4. Upon transfer from DD to continuous bright light (bright LL), the majority (83.3%, N=6) of vehicle-injected flies became arrhythmic (A), whereas most (77.8%, N=9) antibody-injected flies free-ran with a lengthened period (B), a behaviour expected in dim LL. After the initial free-run in DD, flies were injected (arrows) shortly after the exposure to bright LL (>0.04 Wm^{-2}) (shadowed areas). The lengthening in period observed in flies injected with antibody suggested a reduction in the fly's sensitivity to irradiance, thereby led to the assumption that the photoreception pathway was only partially blocked by the injection of S-antigen antibody.

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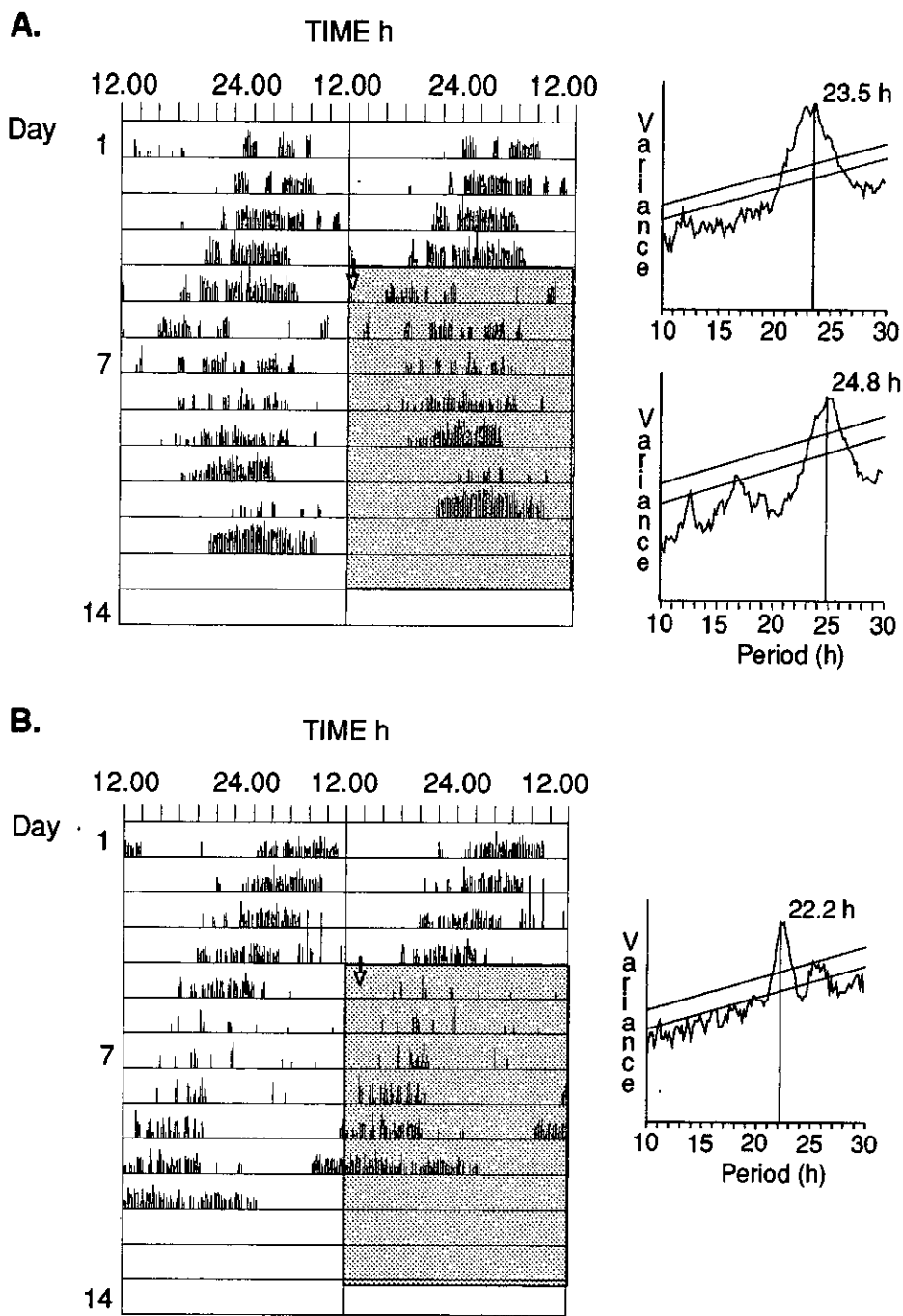
Figure 3.3.2.5. Actograms showing activity rhythms of two flies injected with vehicle (A) or S-antigen antibody (B) shortly after transferring from DD to dim continuous light (dim LL) (shadowed areas). Arrows indicate the time of injections. All flies of vehicle control free-ran in dim LL (<0.02 Wm^{-2}), whereas all flies injected with antibody continued to free-run with a unchanged period. This result provided supportive evidence for the assumption derived from results of an earlier experiment (Fig. 3.3.2.4).

antibody injection on entrainment to LD 12:12 were significantly different from that of the vehicle control ($\chi^2 = 21.991$, d.f. = 4, p < 0.001). Flies categorised as 'doubtful' were those showing such high or low levels of activity that periodogram analysis failed to make meaningful distinction between the free-running or entraining state. The free-running behaviour observed in flies injected with antibody either continued through the light/dark cycles until the flies died (Fig. 3.3.2.3.A) or, after a few days, was substituted by an abrupt entrainment to the light/dark cycle (Fig. 3.3.2.3.B). The abrupt switch between free-run and entrainment occurring 4 days after the injection of antibody suggested that the blockage of photoreception pathway may only be effective within a limited period and the fly is likely to regain its ability for photic perception. These results suggested that the injection of S-antigen antibody may have interfered with the photoreception for entrainment to a light/dark cycle.

The majority of flies (83.3%, N=6) injected with vehicle shortly after transferring from DD to bright LL (irradiance > 0.04 Wm^{-2}) became arrhythmic (Fig. 3.3.2.4.A), which was the expected consequence at irradiance above 0.03 Wm^{-2} (Hong and Saunders, 1994). This was not significantly different from the untreated control (Table 3.3.2.1.B). Flies injected with antibody all remained rhythmic upon transfer to bright LL, the majority (77.8%, N=9) free-ran with a lengthened period (Fig. 3.3.2.4.B), whereas the rest continued with a period the same as or very close to the one prior to the injection in DD (Table 3.3.2.1.B). Statistical analysis showed that the difference between the vehicle control and the antibody-injected group was significant ($\chi^2 = 28.427$, d.f. = 6, p < 0.001). This implied a reduction in the fly's sensitivity to irradiance caused by the injection of S-antigen antibody.

A.

	N	Entraining[*] % (n)	Free-running % (n)	Doubtful % (n)
Untreated control	8	100 (8)	0	0
Vehicle control	6	100 (6)	0	0
S-antigen antibody	28	35.7 (10)	35.7 (10)	28.6 (8)

*: Expected response

B.

	N	Arrhythmic[*] % (n)	$\tau_{LL} \cong \tau_{DD}$ % (n)	$\tau_{LL} > \tau_{DD}$ % (n)	Doubtful % (n)
Untreated control	14	92.9 (13)	0	7.1 (1)	0
Vehicle control	6	83.3 (5)	0	0	16.7 (1)
S-antigen antibody	9	0	22.2 (2)	77.8 (7)	0

*: Expected response

Table 3.3.2.1. Effects of S-antigen antibody injection on locomotor activity rhythms in *C. vicina*, exposed to LD 12:12 (**A**) or continuous light (LL) (**B**). After the initial free-run in DD (5 days), flies were injected with S-antigen antibody or with vehicle (control group) shortly after exposure to LD 12:12 (A), or LL (B) at an irradiance greater than 0.04 Wm⁻².

A similar experiment was carried out in dim LL (irradiance < 0.02 Wm^{-2}) with the result that the difference between the vehicle control and the antibody-injected group was not significant. In vehicle control group, 57% (N=7) of flies showed the expected response, a lengthening of τ (Fig. 3.3.2.5.A), whereas 50% (N=4) of antibody-injected flies free-ran with a unchanged period (Fig. 3.3.2.5.B). This, together with the results obtained with bright LL suggested that the injection of S-antigen antibody only resulted in a partial and temporary 'blinding' effect.

To sum up the effects of S-antigen antibody injection on locomotor activity rhythms, some consequent results are summarised as followed:

(1) in DD, the free-running activity rhythm remained unaltered after the injection;

(2) in bright LL (irradiance > 0.04 Wm^{-2}), the majority of flies free-ran with a lengthened period which was expected to occur only at an irradiance below 0.03 Wm^{-2};

(3) in LD 12:12 (irradiance > 0.04 Wm^{-2}), a noticeable proportion of flies free-ran through the light/dark cycle after the injection.

Although these observations provide no evidence for the location of the photoreceptors involved in the perception of photic stimuli, they suggested that the arrestin-positive neurons demonstrated in the brain of C. *vicina* by Cymborowski and Korf (1995) are involved in the photoreception mechanism. A more integrated discussion including evidence obtained in *Drosophila* will be given in the next chapter.

Chapter 4

General Discussion

4.1. The Circadian System in the Blow Fly *Calliphora vicina*

One of the main aims of the present study was to acquire information on the nature of the circadian system in the adult blow fly, *Calliphora vicina*, in the hope that this will aid the understanding of the underlying mechanisms as well as provide the foundation to construct a model to describe the system. To serve such a purpose, traditional 'black box' approaches were employed. Observations in constant conditions such as constant darkness (DD) or continuous light (LL) revealed the intrinsic characteristics, whereas responses to various external stimuli such as light pulses, temperature steps or light/dark cycles provided clues of how the underlying mechanisms function. This thesis extends the preliminary observations on circadian locomotor rhythms in *C. vicina* made by Kenny (1989) and Kenny and Saunders (1991) and shows that the circadian system of this species presents the array of properties commonly shared by most biological clock systems.

(1) The persistence of the activity rhythm in constant conditions provides strong evidence for the endogeneity of the oscillatory system, which is further supported by the deviation of the free-running period from that of the prime environmental Zeitgeber - a solar day (24 h).

(2) The result that temperature changes had very little effect on the free-running period (Q_{10} is almost always very close to 1) suggests that the system is highly temperature-compensated within the 'normal' temperature range.

(3) Sensitivity of the oscillatory system to a light pulse was illustrated as the phase response curve (PRC) which consists of both delay and advance phase-shifts, therefore implies the entrainability of the activity rhythm to a light/dark cycle; this is also consistent with the entrainment equation $\tau - T = \Delta\phi$.

(4) The commonly observed phenomenon that circadian rhythms also free-run in constant light (LL) is applicable to the locomotor activity rhythm of *C. vicina*, only that the change of the free-running direction violates 'Aschoff's rule'; however, it was consistent with Pittendrigh's prediction that an activity rhythm with a PRC containing a delay portion which greatly exceeds the advance portion should free-run in continuous light with a lengthened period.

In addition, several lines of evidence led to the hypothesis that the circadian locomotor activity rhythm in *C. vicina* may be the result of the coupling between multioscillators. Supportive evidence was obtained when the locomotor activity rhythm (1) changed its free-running direction spontaneously, or (2) split into several components spontaneously or after light stimuli (LD 1:23). The multi-components seen in these cases implied the existence of multioscillators. A heuristic model (Fig. 2.5.1) was, therefore, developed to provide interpretation for such splits. According to this model, when the coupling between individual oscillators is disturbed, spontaneously or by external stimuli, it loosens to a various extent but not necessarily

a complete de-coupling, therefore resulting in a change in period, or a split of the activity rhythm.

An equally important task, searching for the anatomical locations of the clock components, was tackled applying both surgical approaches and injection of S-antigen antibody, a technique only recently applied. Observations on post-operative behaviour provided facts which, although not leading to conclusive statements, may serve as guidelines for future studies.

(1) Role of the compound eyes as the photoreceptors for entrainment to a light/dark cycle was excluded by the unaltered entraining behaviour observed in the absence of both compound eyes. However, the possibility that compound eyes are involved in the direct response to exogenous light stimuli was supported by the elimination of such response in the absence of compound eyes.

(2) Role that the optic lobes play in the photoreception pathway/circadian pacemaking was excluded by the fact that both the free-running rhythm and the entraining behaviour to a light/dark cycle remained virtually unaltered after the complete removal of both optic lobes (bilobectomy).

(3) Role of the S-antigen immunoactive brain neurons as the putative circadian pacemaker(s) was supported by the arrhythmicity observed after S-antigen antibody injection. However, the involvement of such neurons in circadian pacemaking can only be confirmed when further evidence, such as recording of the circadian rhythm in isolated neurons, is produced.

These results, together with the result of other studies (Cymborowski and Korf, 1995; Helfrich-Förster, 1995) have drawn attention in the search for the circadian

pacemaker(s) in higher flies, such as *D. melanogaster* and *C. vicina*, to certain brain neurons, with the main target being PER-containing brain cells in the former, and S-antigen immunoactive brain cells in the latter. Results obtained so far have indicated a bright future for such an approach.

4.2. Multioscillatory Hypothesis

Although it has been generally accepted that circadian rhythmicity of almost all organisms is under the control of a self-sustained oscillatory system, the actual mechanism underlying such a system still arouses much controversy which conceptual models could not settle. The idea that the circadian rhythm is under the control of a population of coupled oscillators which act independently or interact with each other came from several phenomena noted by early researchers, including spontaneous splitting of activity rhythms, spontaneous changes in the free-running period in constant conditions, or non-steady-states (transients) observed before entrainment was achieved. In addition, the fact that circadian rhythms have been observed in multicellular organisms, isolated tissues, and, most provokingly, single cells also suggests the existence of numerous oscillators in a single organism. Several lines of evidence obtained in the present study strongly suggested that the circadian locomotor activity rhythm in *C. vicina* is also under the control of a multioscillatory system. Above all, the most convincing evidence is the splitting of the activity rhythm into several components, which occurred spontaneously or was provoked by light stimuli (LD 1:23). In addition, the spontaneous changes in the free-running period

suggested the existence of a coupling mechanism for the mutual communication between oscillators.

Studies probing the underlying mechanism at a molecular level provided insights into the biochemical regulation of the pacemaker, thereby producing evidence for the existence of multioscillator systems in the circadian makeup of organisms, i.e. many transcriptional pathways are oscillatory. Results of such studies revealed the involvement of certain feedback loops, such as protein and mRNA synthesis, in the regulation of circadian rhythmicity. Page (1994) summarised results of these studies in a modified heuristic model (Fig. 4.1.1) which suggested that the feedback regulation of certain protein transcription is likely a central feature of the clock. Although, so far, no definitive set of criteria can be used to uniquely identify a molecule as a state variable of the oscillator (Page, 1994), two criteria are strongly suggestive. First, the amount of the putative clock molecule should oscillate with a circadian period and abolition of this oscillation should eliminate overt expression of circadian rhythms. Second, transient changes in the amount of the component should, at least at some phases, shift the phase of the oscillation, thus the overt rhythms. In insects, the period gene (*per*) identified in the brain of the fruit fly *Drosophila melanogaster* is one of the best studied examples as well as the foundation of many 'feedback loop' models (Amir and Stewart, 1996; Edery *et al.*, 1994; Hardin *et al.*, 1990; Lee *et al.*, 1996; Myers *et al.*, 1996; Sehgal *et al.*, 1995). With respect to the first criterion, Hardin *et al* (1990) reported that *per* messenger RNA and its protein product (PER) exhibited circadian oscillations. Although the effect of abolition of the oscillation in *per* is less certain, successful demonstration has been reported in *frq*
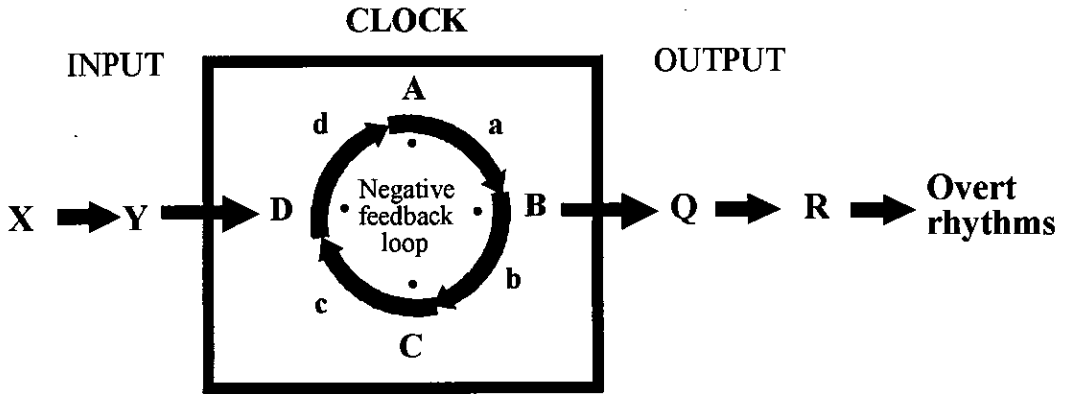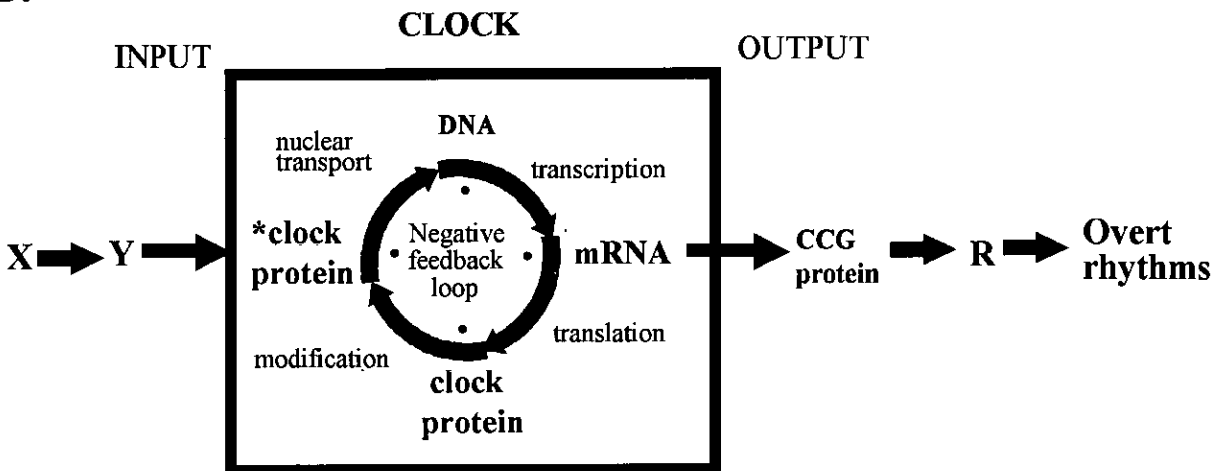
Figure 4.1.1. A modified heuristic model of a circadian clock (Page, 1994). A, B, C and D are state variables; a, b, c and d are parameters of an oscillating loop; X and Y are elements of the input pathway; Q and R are on the output pathway (A). When translated into reality, for example a feedback loop, the state variables and parameters in the model can be interpreted as the main elements consisting the transcription and translation of a clock protein (B).

(Aronson *et al.*, 1994). Edery *et al* (1994) suggested that changes in the amount of *per* expression alone could shift the phase of the oscillation, which fulfil the second criterion.

Although the neuronal network comprising the circadian clock and the pathway by which the pacemaker neurons transfer the circadian information to subsequent cells remains unknown, a new prospect was proposed when a 'double-label' immunohistological technique revealed that the PER-containing brain cells were also immunoreactive against pigment-dispersing hormone (PDH) (Helfrich-Förster, 1995). In this report, the antiserum against PDH revealed the entire arborization of the PER-containing pacemakers and their projections within the brain of *D. melanogaster*. Such results supported both of the propositions that hormonal pathways are involved in mediating the circadian information to the locomotor organs in *Drosophila* (Handler and Konopka, 1979), and that mutual interactions exist between neurons and PER-containing cells. Localisation of the candidate circadian pacemakers and identification of mutual reactions as putative coupling mechanisms, outlines a multioscillatory model for the *Drosophila* circadian system.

As a consequence of the present study, the clear pattern of the locomotor activity rhythm in *C. vicina*, was shown to provide an ideal system for the study of circadian rhythmicity. Future investigations devoted to the search for extra-optic photoreceptors and the circadian pacemaker should, as Cymborowski *et al* (1996) concluded in their very recent report, be focused on the four groups of S-antigen immunoactive brain neurons.

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Acknowledgments

Appendix I. - Circadian locomotor activity rhythms and their entrainment to light-dark cycles continue in flies *(Calliphora vicina)* surgically deprived of their optic lobes.

Appendix II. - Effects of constant light on the rhythm of adult locomotor activity in the blowfly, *Calliphora vicina*.

Appendix III. - S-antigen antibody partially blocks entrainment and the effects of constant light on the circadian rhythm of locomotor activity in the adult blow fly, *Calliphora vicina*.

Circadian Locomotor Activity Rhythms and Their Entrainment to Light–Dark Cycles Continue in Flies (*Calliphora vicina*) Surgically Deprived of their Optic Lobes

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In adult blow flies (*Calliphora vicina*), unilateral or bilateral cuts between the optic lobes and the brain, or complete bilateral lobectomy, failed to interrupt the circadian rhythm of locomotor activity or its entrainment to a daily light:dark cycle. It is concluded that the relevant circadian pacemakers are not within the optic lobes and that the compound eyes are not the photoreceptors for entrainment. As in other holometabolous insects, the pacemakers (the "clock") and the photoreceptors may lie in the brain.

Calliphora vicina   Locomotor rhythmicity   Clock location   Photoreceptors

INTRODUCTION

A number of studies on the organisation of insect circadian systems have focussed on the anatomical locations of the circadian pacemakers, and on the photoreceptors which are necessary for light entrainment (Page, 1981, 1985; Chiba and Tomioka, 1987; Cymborowski, 1988). Surgical approaches to this problem, particularly in orthopteroid insects such as cockroaches (Nishiitsutsuji-Uwo and Pittendrigh, 1968a, b; Roberts, 1974; Page, 1978) and crickets (Loher, 1972; Sokolove and Loher, 1975; Wiedenmann, 1983; Tomioka and Chiba, 1984) have shown that cuts between the compound eyes and the optic lobes result in free-running locomotor rhythmicity (in a light–dark cycle), whereas cuts between the optic lobes and the brain, or complete lobectomy, lead to arrhythmicity. In all of these studies, therefore, the pacemakers could be traced to the optic lobes and the relevant photoreceptors to the compound eyes. Similar results were obtained for the beetles *Carabus problematicus* (Balkenohl and Weber, 1981) and *Pachymorpha* (= *Anthia*) *sexguttata* (Fleissner, 1982). The clock of the tree weta *Hemideina thoracica* (Waddell *et al.*,

1990), was also located in the optic lobes, and the eyes were probably the principal photoreceptors. Light entrainment was however observed in blinded animals, suggesting additional extra-optic entrainment pathways.

More detailed experiments with the cockroach *Leucophaea maderae* (Roberts, 1974; Sokolove, 1975; Page, 1978) suggested that the pacemaker is located in the lobula region of the optic lobe, whereas in the cricket *Gryllus bimaculatus* (Tomioka and Chiba, 1984) it is in the lamina-medulla region and, in the weta, the medulla was crucial (Waddell *et al.*, 1990). Arrhythmicity resulting from surgical isolation of the pacemaker tissues from the brain suggests that expression of overt rhythmicity requires neural connection, although a humoral influence via the axons cannot be ruled out. The importance of neural connections was demonstrated by elegant optic lobe transplantation experiments with *L. maderae* (Page, 1981) in which rhythmicity only reappeared after regeneration of the connectives between the brain and optic lobes.

Evidence from moths and flies is, however, different. Extirpation of the optic lobes of silkmoths (*Antheraea pernyi*, *Hyalophora cecropia* and *Samia cynthia*) had no effect on the persistence of the flight activity rhythm, but removal of the cerebral lobes led to arrhythmicity (Truman, 1974). Similarly, in the mosquito *Culex pipiens* (Kasai and Chiba, 1987) and the house fly *Musca domestica* (Helfrich *et al.*, 1985) the brain and not the optic lobe appears to be the site of the clock and

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entrainment is effected via extra-optic, probably mid-brain, photoreceptors. Work using mutants of *Drosophila melanogaster* with lesions to the eyes and optic lobes has led to the same general conclusion (Helfrich, 1987; Helfrich and Engelmann, 1983).

With respect to clock location, therefore, insects appear to fall into two groups, those in which the pacemaker is in the optic lobe (orthopteroid insects and beetles) and those in which the pacemaker may be brain centred (moths and flies). Since, however, this view is based on rather few studies, the present work describing effects of microsurgery (optic nerve section and lobectomy) on the persistence and entrainment of the locomotor activity rhythm in the blow fly, *Calliphora vicina*, was carried out.

MATERIALS AND METHODS

Insects

The strain of *Calliphora vicina* (= *C. erythrocephala*) used in this study was collected in Musselburgh, Scotland, in 1984. Maintenance of the stock culture was as previously described (Saunders, 1987). Experimental flies were all females kept at 20°C and in continuous darkness unless otherwise stated.

Recording locomotor activity

Adult locomotor activity was recorded in 9 cm Petri dishes, with the recording device comprising an infra-red light beam (of 5 mm dia) passing vertically through one side of the dish on to a phototransistor. Sugar solution was provided at the opposite side of the dish, away from the light beam. Pairs of dishes were placed on a wooden platform which also provided support for the infra-red emitters (Radio Spares, type 306-077) and detectors (RS type 306–083). The whole assembly was then placed in a light-tight wooden box held in a walk-in constant temperature room at $20 \pm 0.5°C$. Each box was provided with a 4 W fluorescent lamp, water jacketted to suppress a temperature rise when the light was on. Light–dark cycles were controlled by 24 h timers.

Activity events were registered as the number of times the infra-red light beam was broken by a moving fly within successive 10-min intervals. These numbers were recorded using a BBC B plus microcomputer and assembled into the familiar "double-plotted" actogram format (Kenny and Saunders, 1991). Subsequent periodogram analysis and calculations such as the duration of daily activity (α) and total activity per cycle were carried out using an Acorn Archimedes 400 series computer (Cymborowski et al., 1993).

Surgical procedures

Procedures to remove the optic lobes or to cut the optic tracts were similar to those used by Helfrich et al.

(1985) for the house fly, *M. domestica*. Each fly was mounted in soft plasticine without anaesthesia and the head bent forward to facilitate access to the rear of the head capsule. The head was then opened under a dissecting microscope, making incisions with a fine piece of razor blade along cuticular "landmarks" on the rear of the head (Helfrich et al., 1985). A flap of integument was then lifted to expose the brain and optic lobes. Unilateral or bilateral optic tract section was performed by making cuts between the optic lobe(s) and the brain. Lobectomy was achieved by making similar cuts, then separating the optic lobes from the eye cups with fine scissors and removing the optic lobes with forceps. Ocelli were cauterized with a hot needle and then covered with black wax. In sham-operated flies all of these procedures were followed except severing the optic tracts or lobectomy. After surgery the wounds were treated with penicillin-streptomycin and sealed with a low melting-point wax. Operations were conducted under white light, the duration of exposure being 5–8 min.

Experimental design

Newly emerged flies were placed in the activity monitors at 20°C and in continuous darkness (DD) to record their initial free-running circadian rhythms of activity. Flies to receive experimental and sham operations were then removed from their activity recorders after 7–9 days to undergo surgery, then replaced (in DD) for a further 6–8 days to assess any affects of the operations. All flies were then exposed to a daily cycle of 12 h of light and 12 h of darkness (LD 12:12) for another 6 or 7 days before a final free-run in DD. Control (unoperated) flies received identical treatment except surgery. Free-running and entrained rhythm periods were calculated by periodogram analysis.

Postmortem histological examination

At the end of each experiment, histological examination was made of representative flies to assess the precision of the cuts between optic lobes and brain, or whether complete lobectomy had been achieved. Heads were fixed in Bouin's fluid and mounted in paraffin wax. Sections were cut at 10 μm and stained with paraldehyde fuchsin (Dogra and Tandan, 1964). Flies

TABLE 1. The effects of optic tract section and lobectomy on the free-running rhythm of locomotor activity in the blow fly, *Calliphora vicina*

	N	Rhythmic	Arrhythmic	Doubtful
Control, normal	17	16(94.1%)	0	1(5.9%)
Control, sham	26	25(96.2%)	0	1(3.8%)
Optic tract section unilateral	6	5(83.3%)	0	1(16.7%)
Optic tract section, bilateral	23	19(82.6%)	1(4.3%)	3(13.0%)
Lobectomy, bilateral	37	30(81.1%)	2(5.4%)	5(13.5%)

subjected to operations which were judged to be unsuccessful (e.g. incomplete optic lobe removal) were excluded from the results.

RESULTS

Cumulative data for locomotor rhythmicity in experimental and control blow flies are shown in Table 1. Among 17 normal (unoperated) control flies, all but one (94.1%) showed clear free-running rhythms. Under constant darkness at 20°C the mean period of these flies ($\tau_{DD} = 22.43 \pm 0.83$ h) showed little change during the 7–14 days of their initial free-run. A representative actogram for a control fly is shown in Fig. 1. Exposure to LD 12:12 (days 15–21) brought about immediate entrainment to the light cycle ($\tau \rightarrow T = 24$ h), and in subsequent darkness (days 22–28) free-running behaviour resumed.

Similar results were obtained with 26 sham-operated flies. In this group, only one had a "doubtful" post-operative rhythm; the rest (96.2%) showed relatively undisturbed rhythmicity following the operation, normal entrainment to LD 12:12, and an unaltered final free-run in darkness (Fig. 2). Among a group of 16 sham-operated flies, the post-operated mean free-running period in DD ($\tau = 23.42 \pm 0.79$ h) was not significantly different from that before operation ($\tau = 22.84 \pm 0.61$ h) (Wilcoxon, $T = 32.5$, $N = 16$, not significant). The mean duration of daily activity (α) also remained unchanged following the operation ($T = 33.5$) but the mean amount of daily activity was, however, significantly reduced ($T = 16$, $P < 0.01$).

Unilateral or bilateral optic tract section had little effect on the rhythm of locomotor activity (Table 1), clear rhythms being observed in about 83% of both groups. Combined data for these two groups of optic tract sectioned flies were no different in this respect from the sham-operated controls ($\chi^2 = 0.2653$, d.f. = 1).

Of the 37 flies whose optic lobes were completely removed, 30 (81.1%) showed clear post-operative rhythmicity, 2 (5.4%) showed activity patterns judged to be arrhythmic by periodogram analysis, and 5 (13.5%) showed very weak or doubtful rhythms. The proportion of flies remaining rhythmic in DD after bilateral lobectomy was not significantly different from that in the sham-operated controls ($\chi^2 = 0.3968$, d.f. = 1). Further analysis of these bilobectomized flies showed non-significant post-operative changes in τ (Wilcoxon, $T = 16.5$) and α ($T = 17.0$) but, as with the sham-operated controls, a significant post-operative reduction in activity ($T = 2.0$, $P < 0.01$).

Representative actograms for sham-operated and bilaterally lobectomized flies are shown in Figs 2–4. In Fig. 2 (sham) the operation, or the brief light pulse experienced by the fly during the operation, caused a phase advance (day 9). The fly subsequently became entrained to LD 12:12 (days 15–21) and showed a final free-run in DD. The two examples of bilobectomized

flies were operated upon in the middle of the subjective night (Fig. 3) or in the middle of the subjective day (Fig. 4), respectively. In neither example was rhythmicity significantly disturbed, although some "splitting" of the activity band may have occurred in the latter. Both of the examples illustrated showed entrainment to the light cycle and a final free-run in DD.

Figure 5 shows that some of the control (37.5%, $N = 8$) and sham-operated flies (36.4%, $N = 11$) expressed increased activity immediately following light off. This was never observed in lobectomized flies ($N = 37$) or in flies with their optic lobes disconnected from the brain ($N = 29$).

Post-mortem histological examinations were made of those optic tract sectioned and bilobectomized flies that remained alive at the end of the 28 days of the experiment. In 10 flies showing clear rhythms after bilateral severance of the optic tracts, such examination showed that transection was complete and in the correct position on each side [Fig. 6(A)]. During 1–3 post-operative weeks there was no sign of connections re-establishing between the optic lobes and the brain. Some degenerative processes, however, were observed at the point of nerve section. In eight of the 30 lobectomized flies that survived until the end of the experiment and showed clear post-operative rhythms, histological examination confirmed that the optic lobes has been completely removed [Fig. 6(B)]. In the few cases where portions of the optic lobes remained [Fig. 6(C)] no differences in rhythm persistence and light entrainment were observed.

Histological examination also revealed that successfully lobectomized flies showed median neurosecretory cells heavily stained by the paraldehyde-fuchsin method [see Figs 6(B) and (C)] while control and sham-operated flies never showed such heavily stained cells.

DISCUSSION

The present results show that circadian locomotor rhythmicity in the blow fly *C. vicina*, and its entrainment to the light cycle, continue in flies in which the optic lobes were surgically disconnected from the brain or completely removed. Although such operations reduced the amount of activity, the free-running period of the rhythm in darkness and the duration of daily activity were largely unaltered. Optic lobes and the brain are clearly demarcated in higher flies (Strausfeld, 1976) and post-mortem histological examinations indicated successful isolation of optic lobes from brain tissues in most cases; it seems unlikely, therefore, that parts of the optic lobes were left attached to the brain in these experimental flies [see Fig. 6(B)]. Post-operative rhythms in flies retaining a small part of an optic lobe [Fig. 6(C)] were no different from those of flies with complete removal.

These observations demonstrate that the optic lobes in *C. vicina* are not essential for the expression of rhythmicity and that the compound eyes cannot be the principal
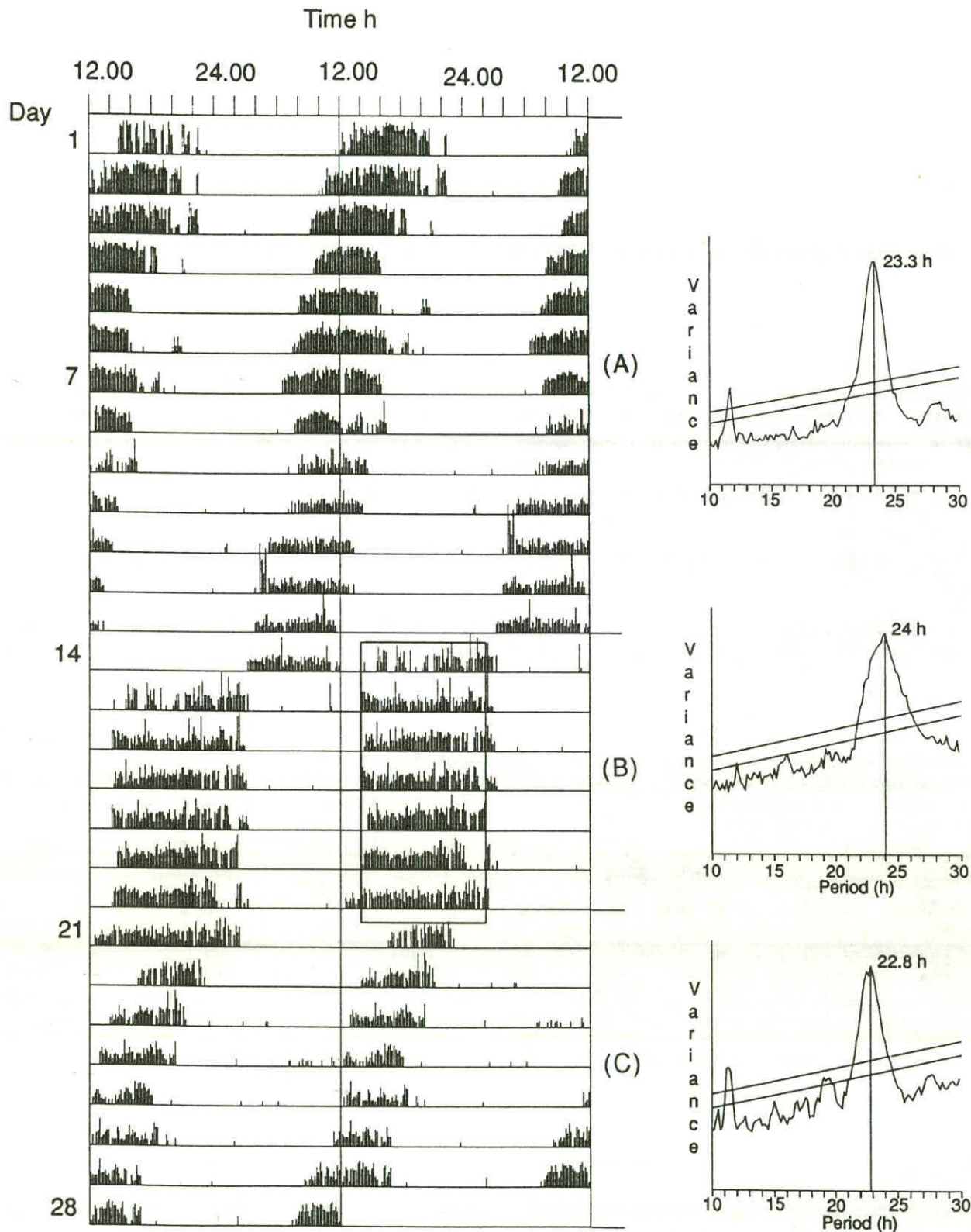
FIGURE 1. Activity record (actogram) and associated periodogram analyses for an unoperated control female of *C. vicina* at 20 ± 0.5°C, showing a free-running rhythm of locomotory behaviour in continuous darkness (DD) (days 1–14), entrainment to a light cycle (LD 12:12) (days 15–21) and a final free-run in DD (days 22–28). The data are presented in a "double plotted" format to facilitate visual inspection. The closed square in the right-hand panel of this plot indicates when the light was on. Periodograms for DD free-runs (A and C) show τ values of 23.3 and 22.8 h, respectively; that for the entrained section (B) shows a period of 24 h, the same as that of the light cycle.
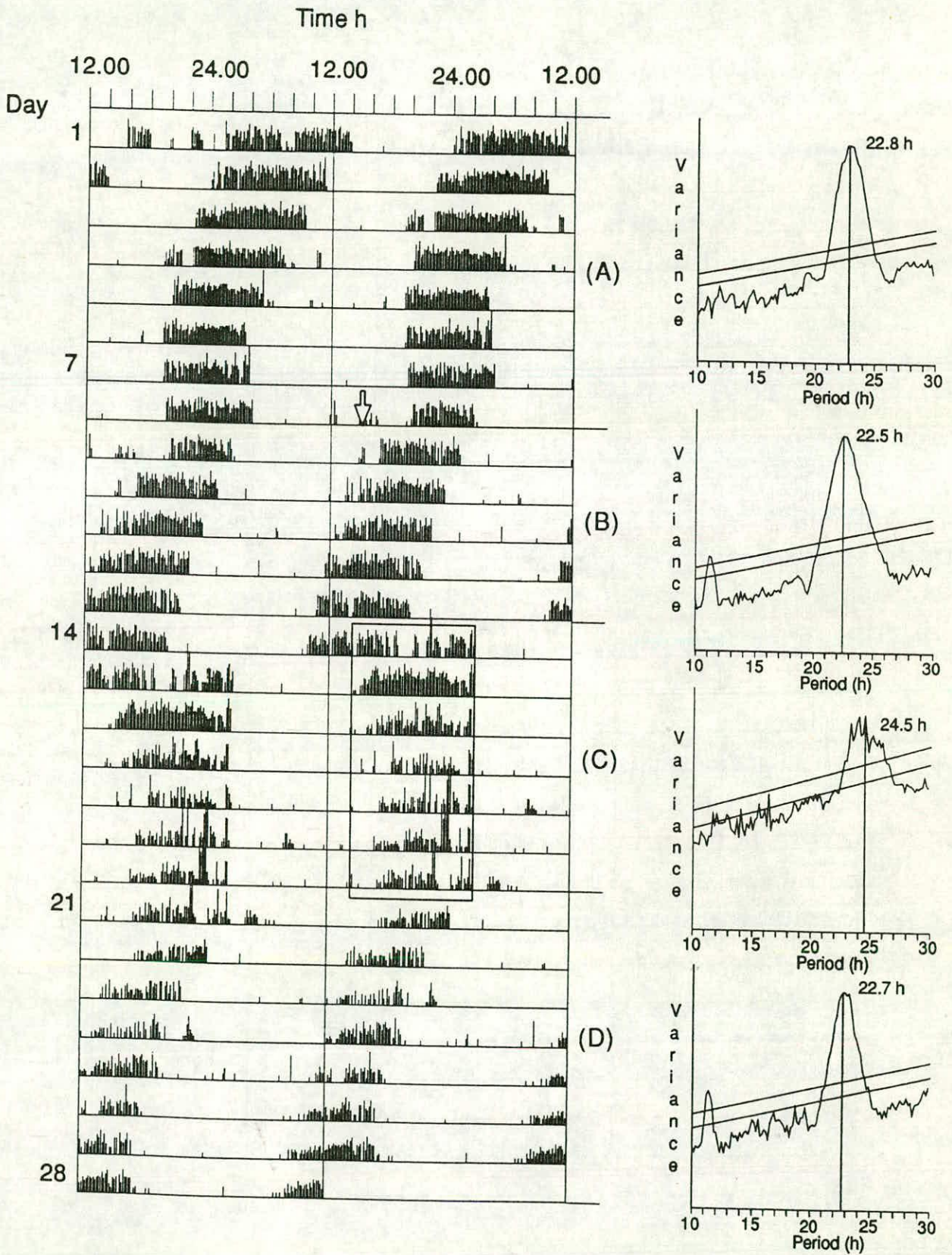
FIGURE 2. Activity record and periodogram analyses for a sham-operated fly. Arrow shows the time of the operation. Periodograms (A) and (B) compare pre- and post-operative free-running rhythms, (C) the entrained rhythm and (D) its final free-run. Other details as in Fig. 1. Pre-operative, post-operative and post-entrainment τ values are similar.

FIGURE 3. Activity record and associated periodogram analyses for a fly whose optic lobes were completely removed during the subjective night (arrow on day 7). Bilobectomy failed to eliminate the free-running rhythm, or entrainment to the light–dark cycle. Other details as in Fig. 1.
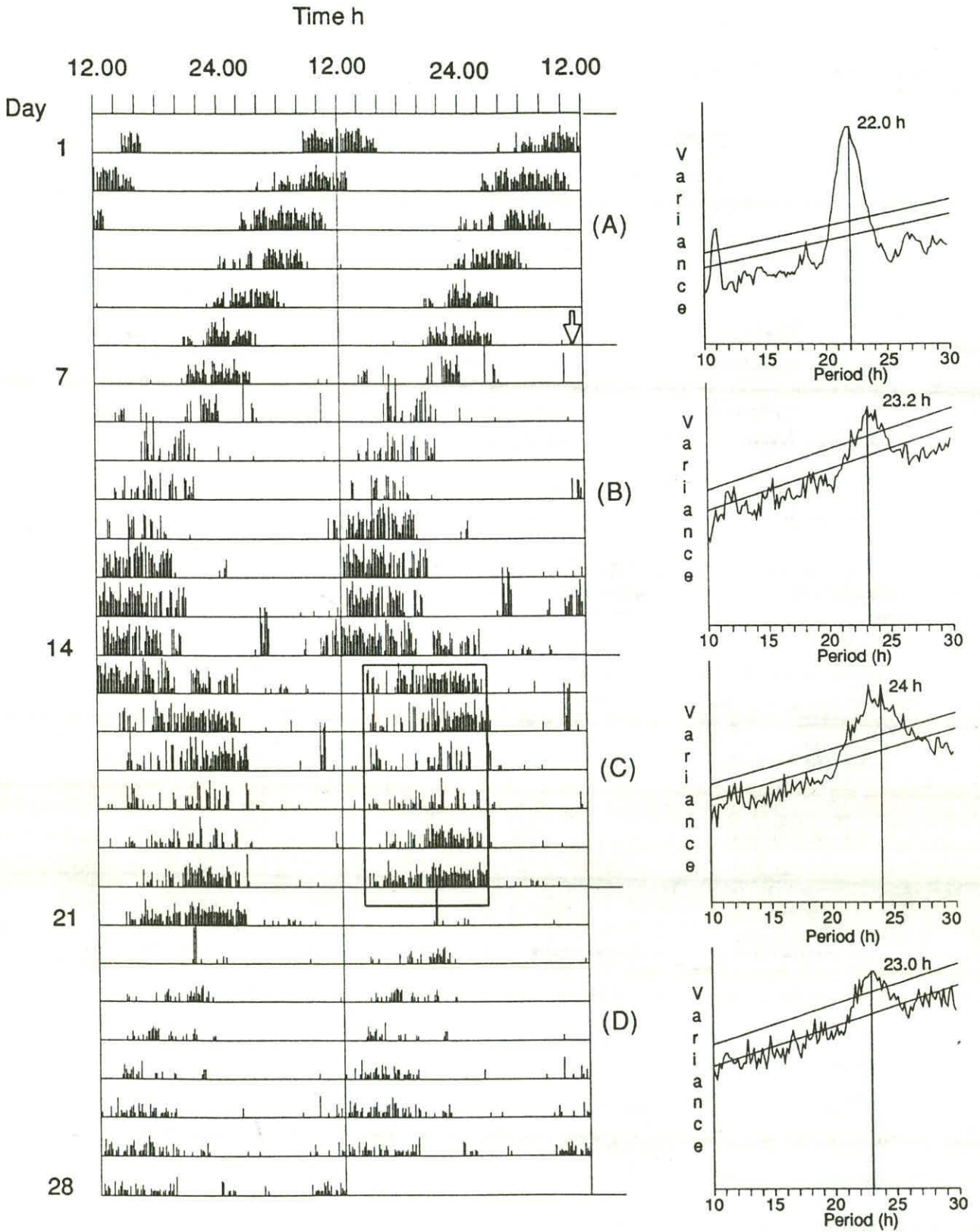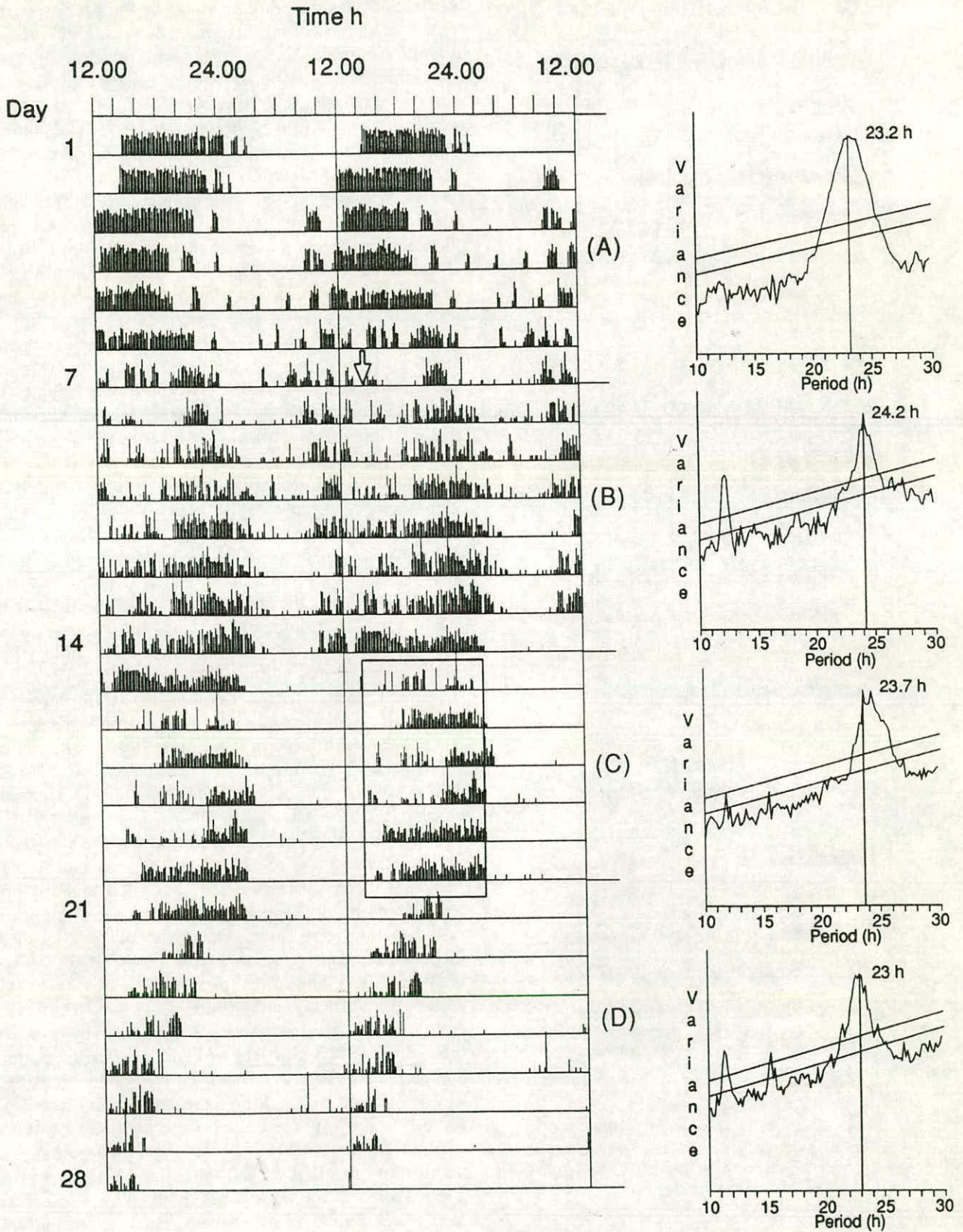
FIGURE 4. Activity record and associated periodogram analyses for a fly whose optic lobes were completely removed during the subjective day (arrow on day 8). Bilobectomy failed to eliminate the free-running circadian rhythm or entrainment to the light–dark cycle. The experimental treatment, however, led to a temporary "splitting" of the activity record. Other details as in Fig. 1.

(A)

Time h

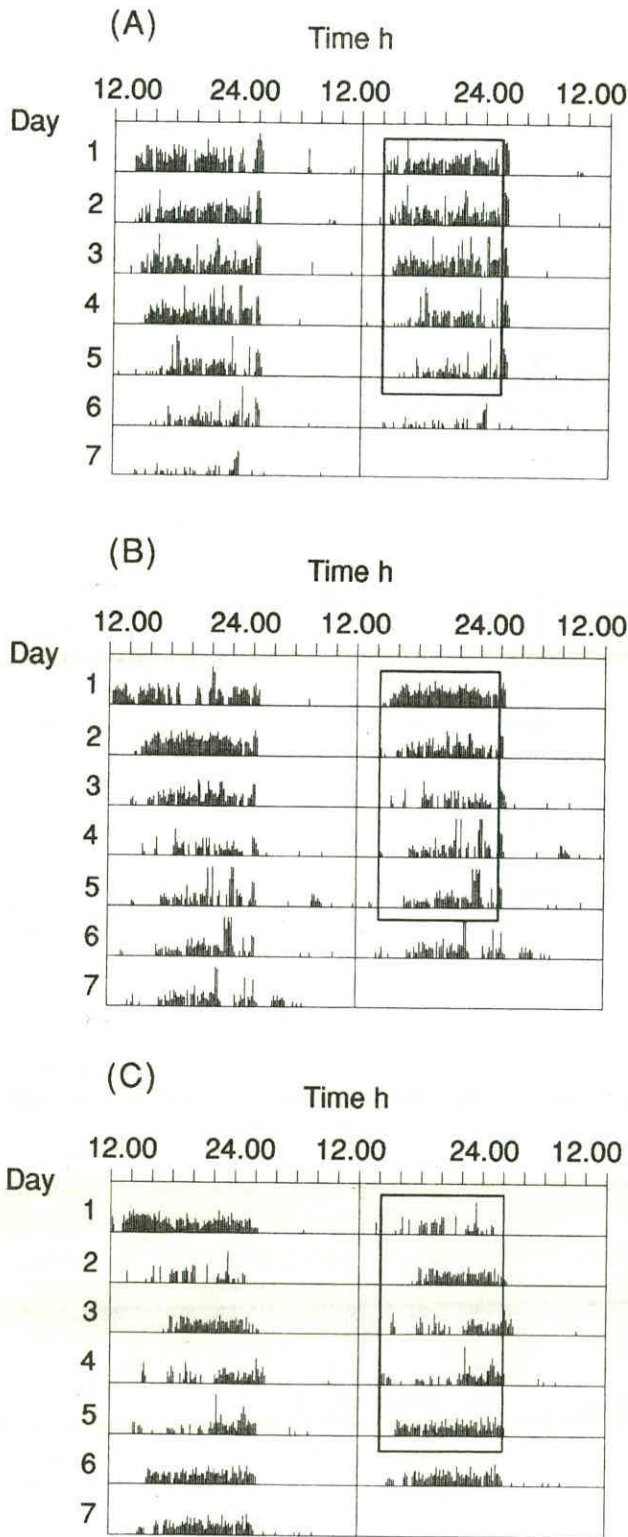**(B)**

Time h

(C)

Time h

FIGURE 5. Exogenous effects of light on the locomotor activity rhythm in LD 12:12. (A) Control and (B) sham-operated flies show an increase in activity close to light-off. Bilobectomized flies (C), however, never show such a response.

or only photoreceptors for rhythm entrainment. Since the ocelli were heat cauterized and occluded in the experimental flies, it is also unlikely that these structures are the photoreceptors involved. For these reasons, the results suggest that the circadian·pacemaker(s) for locomotor rhythmicity may lie within the brain tissue, and that the brain may also be the site for extra-optic photoreception (Cymborowski and Korf, 1994). Confirmation of these presumed roles for the brain in *C. vicina*, however, must await further surgical experiments to isolate the brain from the thoracic centres, or transplantation studies (e.g. Handler and Konopka, 1979; Page, 1982) if these prove possible with the present material.

The increased activity immediately following light off in control and sham operated flies (Fig. 5) and its absence in lobectomized and optic tract severed flies, suggests that this activity is a direct or "masking" effect of light via the eyes and optic lobes, similar to that described for the eclosion rhythm in the silkmoth *Hyalophora cecropia* (Truman, 1972).

The conclusion that the circadian clock and its associated photoreceptor are in the brain of *C. vicina* is in agreement with observations on other Diptera (Handler and Konopka, 1979; Helfrich *et al.*, 1985), but in contrast with observations on orthopteroid insects (Nishiitsutsuji-Uwo and Pittendrigh, 1968a, b; Page, 1984; Sokolove and Loher, 1975; Tomioka and Chiba, 1984; Waddell *et al.*, 1990) and beetles (Balkenohl and Weber, 1981; Fleissner, 1982) in which the pacemakers are in the optic lobes and photoreceptors in the eyes. This apparent difference, however, may not hold true since evidence exists for a brain centred pacemaker in the cricket *Acheta domesticus* (Cymborowski, 1981) and specialized extraretinal photoreceptors have been identified in the optic lobes of beetles (Fleissner *et al.*, 1993).

The nearest comparable study is that by Helfrich *et al.* (1985) on the house fly *M. domestica*. These authors also found that circadian rhythmicity and its photic entrainment continued in flies surgically deprived of their optic lobes, and concluded that rhythm control and photoreception were functions of the brain rather than the optic lobes. However, only 50% of their lobectomized flies retained simple rhythms: of the remainder, 20% showed complex post-operative activity patterns, and 30% were considered to be arrhythmic. Apart from the appearance of complex rhythmicity, lobectomized flies also showed an increase in period and an increase in activity. Lobectomized flies entrained to a light cycle, but with an increased activity time and a more negative phase angle to the light. The authors concluded that the brain of *M. domestica* contains a number of circadian oscillators making up the central pacemaker, but the optic lobes might play a role with respect to mutual coupling between these oscillators. The present results with *C. vicina* are much simpler. Although the ablation experiments described here cannot exclude the possibility of endogenous oscillators in the optic lobes, the data suggest that such a role for the optic lobes is not required.
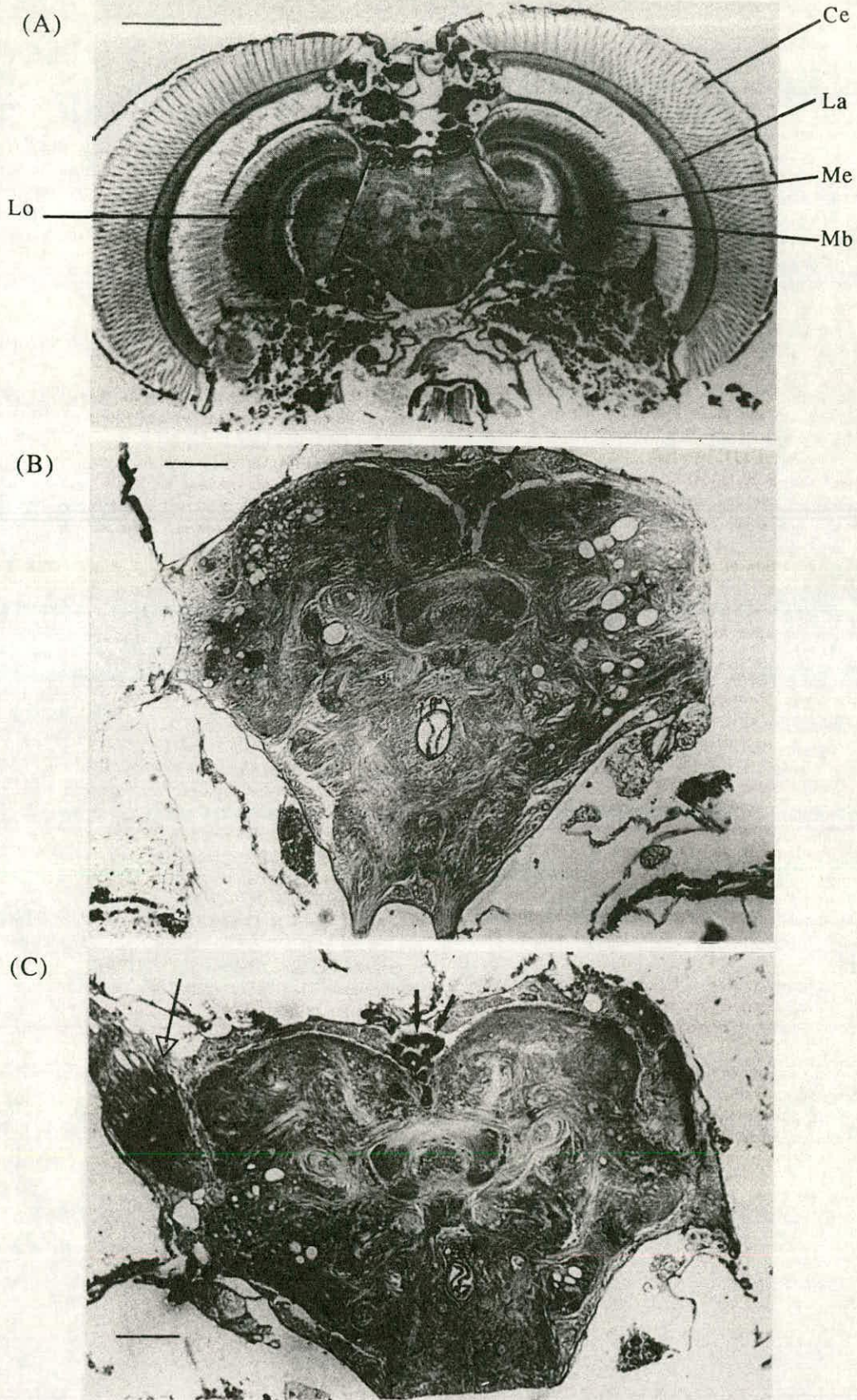
FIGURE 6. Frontal sections of the head and brain of *C. vicina*. (A) Section of the head of an intact fly showing positions of the cuts (black lines) between optic lobes and mid-brain. (B) Section of mid-brain from a lobectomized fly showing complete removal of both optic lobes. (C) Section of mid-brain of a fly with incomplete removal of the optic lobe (open arrow) from the left side. Sections were cut at 10 μm and stained by the paraldehyde-fuchsin method. Small closed arrows indicate heavy staining of neurosecretory material in the *pars intercerebralis* of lobectomized flies (B and C). Lobectomized flies frequently showed vacuolated (degenerated) areas close to the cuts (☆). Ce, Compound eye; La, lamina; Lo, lobula; Me, medulla; Mb, midbrain. Scale bars: A, 400 μm; B and C, 100 μm.

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Effects of constant light on the rhythm of adult locomotor activity in the blowfly, *Calliphora vicina*

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Abstract. Females of the blowfly, *Calliphora vicina*, showed an increase in the free-running period (τ) of their locomotor activity rhythm when transferred from continuous darkness (DD) to continuous 'dim' light (LL) at an irradiance below about $0.03 \, \mathrm{Wm^{-2}}$. Transfer to LL of this intensity also caused a reduction in the duration of the active phase (α) of the cycle. Transfer to 'bright' light ($>0.03 \, \mathrm{Wm^{-2}}$), however, lead to arrhythmicity. Data suggest that constant light of this intensity does not 'stop' the circadian pacemaker but imposes behavioural arrhythmicity at a more superficial level.

Key words. *Calliphora vicina*, locomotor rhythm, arrhythmicity, continuous light, light intensity.

Introduction

One of the fundamental properties of a circadian rhythm is that it persists ('free-runs') in constant conditions, almost always in continuous darkness (DD) and usually also in continuous light (LL), provided that it is below a threshold intensity (Saunders, 1982). The free-running period (τ) of the rhythm is, by definition, close to but rarely equal to 24 h. In night-active mammals (and a few birds), Aschoff (1979, 1981) showed that τ in continuous darkness lengthened upon transfer to continuous light (i.e. $\tau_{DD} < \tau_{LL}$), and further lengthened with increased light intensity. In day-active species (mainly birds), on the other hand, τ shortened upon transfer to LL (i.e. $\tau_{DD} > \tau_{LL}$). As intensity increased, night-active species frequently showed reduced activity and a shortened activity time per cycle (α), whereas day-active species showed the opposite. These observations have been dubbed 'Aschoff's rule' or the 'Circadian rule'.

Although τ_{DD} in nocturnally active insects such as cockroaches (Roberts, 1960; Lohmann, 1967; Saunders, 1982), crickets (Nowosielski & Patton, 1963; Loher, 1972; Sokolove, 1975) and the New Zealand weta, *Hemideina thoracica* (Christensen & Lewis, 1982), lengthened upon transfer to continuous light, and in the beetle *Tenebrio molitor* τ_{LL} lengthened from 24.3 h in 0.01 lux to 26.07 h in

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100 lux (Lohmann, 1967), many other insects appear to violate Aschoff's rule (Saunders, 1982). For example, in the beetle *Carabus problematicus*, usually regarded as night-active, τ_{DD} 23.07 h became τ_{LL} 26.0 h in light at 1.5 lux, but then systematically *shortened* as the light intensity rose from 1.5 to 250 lux (τ_{LL} 23.6 h) (Weber, 1967). Furthermore, in the apparently night-active antlion *Myrmeleon obscurus* τ_{LL} was shorter than τ_{DD} (Youthed & Moran, 1969).

Studies on the effects of continuous light on activity rhythms in Diptera are few. In the mosquitoes *Aedes aegypti* and *Culex pipiens fatigans* τ lengthened upon transfer to LL (Taylor & Jones, 1969; Jones, 1976). Working with the adult locomotor activity rhythm in *Drosophila melanogaster* (wild type), Konopka *et al.* (1989) showed that τ_{LL} in most flies also increased with light intensity, but activity became arrhythmic above about 3 lux. With blowflies, studies on locomotor activity rhythms have been performed with two species, *Calliphora vicina* (Grosse, 1985) and *Protophormia terraenovae* (Aschoff & von Saint Paul, 1982). In the former, average τ in DD was 22.5 h, and activity became arrhythmic in LL (500 lux); *P. terraenovae*, however, were totally inactive in DD and, although active and rhythmic in LL, their circadian period showed no obvious relationship to light intensity. Similar studies on 'population rhythms' in Diptera (e.g. pupal eclosion, oviposition, egg hatch and pupation) are widespread and generally show that these, too, become arrhythmic in LL above a certain intensity (see Saunders, 1982, and Discussion). However, given the paucity of data for

locomotor rhythmicity in 'higher' flies and their apparent diversity, the present experiments were conducted using adults of *Calliphora vicina*.

Materials and Methods

Flies. The strain of *Calliphora vicina* R.-D. used in this study was collected in Musselburgh, Scotland (55°N) in 1984. Maintenance of the stock culture was as previously described (Saunders, 1987).

Recording locomotor activity. A 5 cm diameter Petri dish was glued to the centre of a larger, 9 cm, Petri dish to form a circular track. The inner dish contained cotton wool, soaked in sugar solution, with some of the cotton drawn through lateral holes to provide a source of sugar and water for the fly. A single female was placed within the track. Two such Petri dishes were mounted on a wooden platform which also provided support for the infra-red emitters (Radio Spares, type 306-077) and detectors (RS, type 306-083). The infra-red light beam was arranged vertically across the side of the circular track containing the fly. The whole assembly was then enclosed in a light-tight wooden box held in a constant temperature room at $20 \pm 0.5°C$. Each box was provided with a 4 W fluorescent lamp, water jacketted to suppress a temperature rise when the light was on. At the level of the experimental fly, irradiance from this lamp was about $0.7\,Wm^{-2}$ ($\sim 480\,lux$) unless reduced light intensities were used (see below).

Reduction in light intensity. The reduced light intensities used in this study were achieved by wrapping paper of various thicknesses around the fluorescent tubes, with both ends of the tubes tightly wrapped in aluminium foil to prevent leakage of light. Reduced light intensities were measured with a Tektronix J 16 digital photometer in Wm^{-2}. The range of light intensities achieved by this method was from about $0.017\,Wm^{-2}$ ($\sim 11.6\,lux$) to about $0.07\,Wm^{-2}$ ($\sim 47.9\,lux$).

Experimental design. Female flies within 24 h of emergence were placed in the recording devices in continuous darkness (DD) for 7 days to record their initial free-running activity rhythms. They were then exposed to constant light (LL) at various intensities for a further 11 days before a final free-run in DD. In different experiments the time of final release into DD was varied.

Activity events were registered as the number of times the moving fly broke the infra-red light beam within successive 10 min intervals. These numbers were recorded using a BBC B-plus microcomputer and assembled into the conventional 'double-plotted' actogram format (Kenny & Saunders, 1991). Free-running periods (τ), durations of the 'active' phase per cycle (α), or arrhythmicity, were calculated, as appropriate, either by periodogram analysis (based on Enright, 1965, and Williams & Naylor, 1978) or by using specially-written software in which the cursor was used to select the beginning and the end of each day's activity, and a least-squares regression line computed through the mid-points of the activity 'band' (Saunders *et al.*, 1994).

Results

Fig. 1 shows representative actograms for three females of *C. vicina* exposed, after 7 days in DD, to continuous light of different intensities; on day 19 each was then returned to darkness. In Fig. 1A the fly was exposed to continuous light at an irradiance of $0.018\,Wm^{-2}$ ($\sim 12.3\,lux$) which caused the free-running period in DD (23.7 h) to lengthen to 24.3 h. On return to darkness the free-running period of this fly shortened again to 23.3 h. In Fig. 1C the fly was exposed to continuous light at a higher irradiance ($0.033\,Wm^{-2}$; $\sim 22.6\,lux$) which caused overt behavioural arrhythmicity while the light was on, as shown by the relevant periodogram (Fig. 1C', middle panel). The third example (Fig. 1B) is a fly exposed to LL of an intermediate irradiance ($0.024\,Wm^{-2}$; $\sim 16.4\,lux$). Upon initial exposure to this level of illumination behaviour was overtly arrhythmic, but after 4 days rhythmic locomotor activity appeared with $\tau > 24\,h$. This finally shortened to $\tau < 24\,h$ after illumination was terminated.

Changes in τ after transfer from DD to dim LL (less than about $0.035\,Wm^{-2}$; $\sim 24\,lux$) and back to DD, are shown for a group of thirteen flies in Fig. 2. All thirteen flies remained rhythmic in LL of this intensity and showed an increase in τ. Average initial τ_{DD} was $22.95 \pm 0.56\,h$ which increased to an average τ_{LL} $24.61 \pm 0.89\,h$ ($t = 5.0930$, df = 12, $P \ll 0.001$). After return to darkness, τ_{DD} became $22.97 \pm 0.61\,h$, not significantly different from its initial value ($t = 0.1209$).

The duration of the 'active' phase of the cycle (α) was found to shorten in nine (69.2%) of these thirteen flies, but to lengthen in four. This experiment was repeated with an identical result (i.e. nine of thirteen showing reduced α); the two groups were therefore combined. For those flies showing a shortening of activity, α_{DD} $9.33 \pm 1.88\,h$ became α_{LL} $7.26 \pm 2.08\,h$ ($t = 6.09$, df = 17, $P \ll 0.001$); for those showing a lengthening of activity, α_{DD} 9.16 ± 1.66 h became α_{LL} $10.18 \pm 1.23\,h$ ($t = 3.51$, df = 7, $P < 0.01$). The degree of lengthening or shortening of α in individual flies varied from 3% to 50% of the α_{DD} value, but bore no relationship to intensity. The data, however, clearly indicate that transfer from DD to LL (low intensity) caused an increase in τ and, in most cases, a reduction in α.

Fig. 3 presents data for twenty-nine flies transferred from DD to LL at irradiances between 0.018 and 0.055 Wm^{-2} (~ 12.3 to 37.7 lux). In those flies that remained rhythmic in LL, τ increased with light intensity, up to about 14% increase under $0.03\,Wm^{-2}$ ($\sim 20.6\,lux$). Above this value, most flies became overtly arrhythmic.

A number of flies were transferred from LL to DD at different times. For flies transferred from 'dim' light, in which locomotor rhythmicity persisted with a lengthened period, this period shortened in DD with no change of phase (see Fig. 1A), regardless of the time of light-off in relation to the activity band. Consequently, the interval between the LL/DD transition and the first activity band in DD was extremely variable (5.2–21 h, $n = 22$). For flies which were arrhythmic in 'bright' LL, the LL/DD
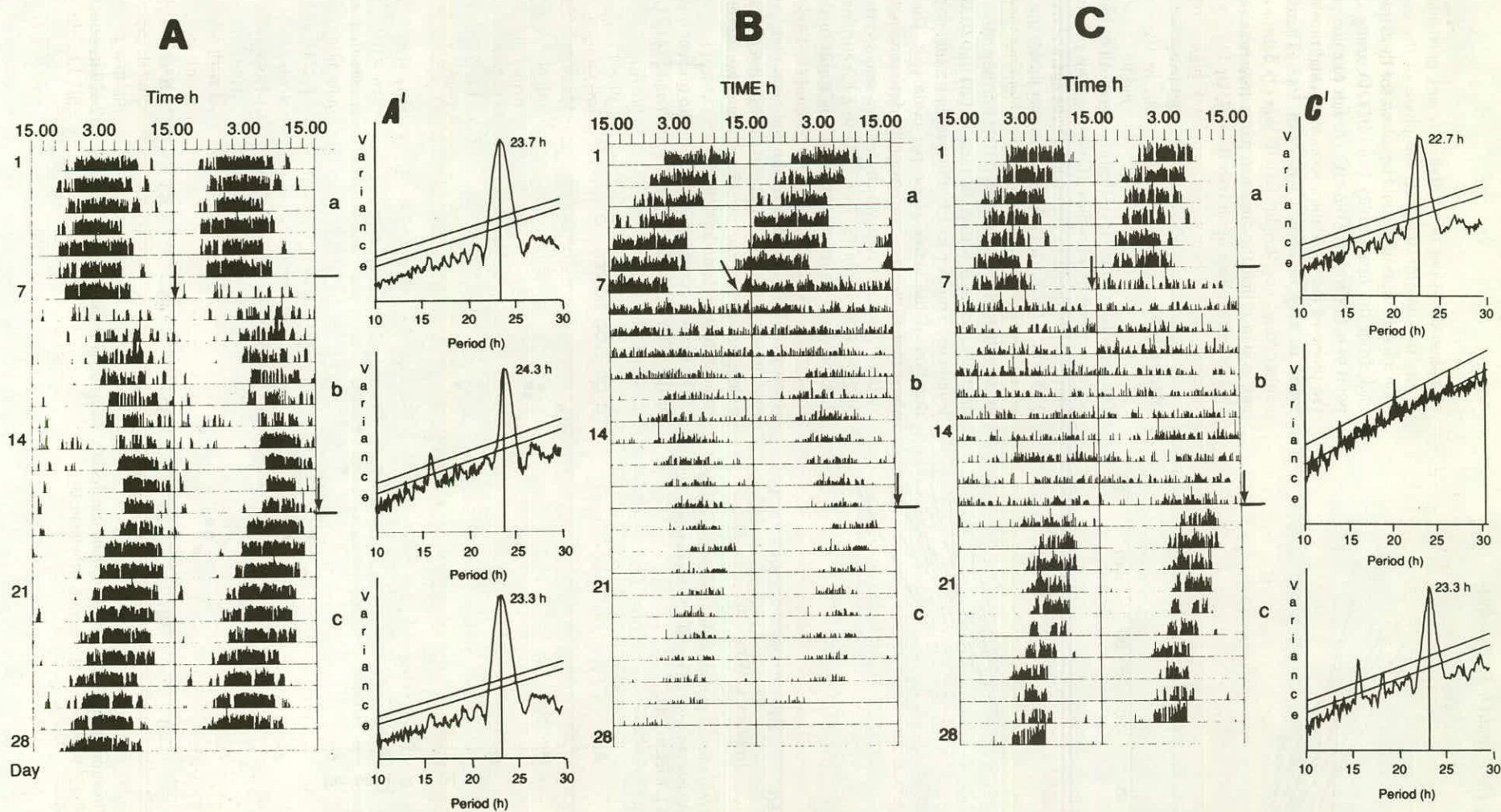
Fig. 1. Actograms for three females of *Calliphora vicina* at 20°C, held for the first 7 days in DD (a), then for 11 days in LL at various light intensities (b), and finally in DD (c). (A) fly in LL at $0.018\,\mathrm{Wm^{-2}}$, (B) fly in LL at $0.024\,\mathrm{Wm^{-2}}$; (C) fly at $0.033\,\mathrm{Wm^{-2}}$. A′ and C′ show periodogram analyses for the appropriate sections of actograms A and C. Arrows show the times of light on and light off.
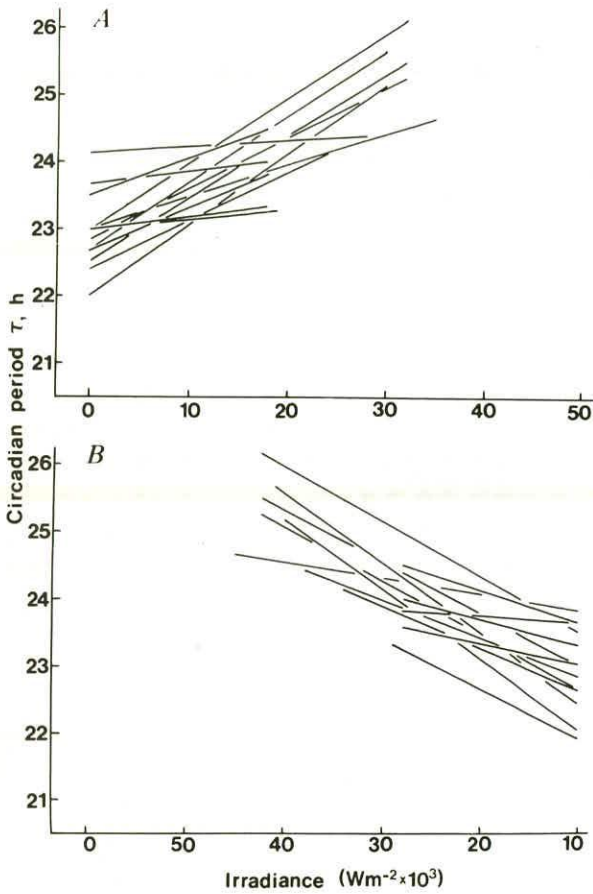
Fig. 2. Changes in free-running periods(τ) for thirteen females of *C.vicina* at 20°C: (A) transfer from DD to LL of various intensity; (B) transfer from LL back to DD.

transition could not be attributed to any particular phase of the oscillation. However, the phase of the oscillation soon *after* the transition could be calculated by extrapolation back from the first activity band in DD, using the mid-point of α as Circadian time (Ct) 6. For thirteen such flies the phase of the oscillation soon after this transition was found to vary between Ct 6.4 and 16.3. Therefore, in neither 'dim' nor 'bright' ($>0.03\,Wm^{-2}$) light was there any evidence that the circadian pacemaker was reset to a characteristic phase upon transfer to DD.

Discussion

The results of this investigation show that locomotor activity of adult blowflies (*Calliphora vicina*) becomes overtly arrhythmic in continuous light (LL) above about $0.03\,Wm^{-2}$ (~20.6 lux). Below this threshold, the rhythm of activity persists but with an endogenous period (τ) greater than that in darkness (DD). Furthermore, as light intensity increases within the range 0.018 to $0.03\,Wm^{-2}$, τ lengthens. Transfer to LL also causes a reduction in the duration of the active phase per cycle (α). These data show that *C.vicina* — an obviously day-active species — violates 'Aschoff's rule' which (for day-active vertebrates) suggests that a transfer from DD to LL shortens τ, and that increased light intensity shortens τ still further, but lengthens α (Aschoff, 1979, 1981; Menaker, 1968, for the sparrow). This is consistent with the view that 'Aschoff's rule' is not applicable to insects, nearly every species of which, day- or night-active, shows a lengthening of τ upon transfer to LL (Saunders, 1982).

The nearest comparable study to the present one is that on the related blow fly, *Protophormia terraenovae* (Aschoff
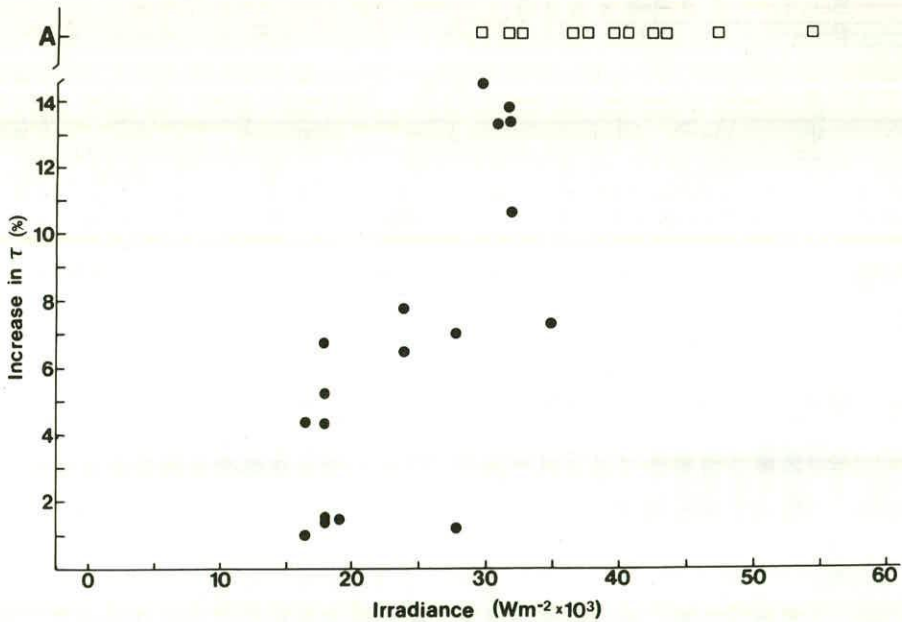

Fig. 3. Increase in free-running period (τ) of twenty-nine females of *C.vicina* upon transfer from DD to LL of various intensities. Above about $0.03-0.035\,Wm^{-2}$ the flies activity became arrhythmic ($\square$).

& von Saint Paul, 1982). The two flies, however, show radically different responses to DD, LL and light intensity. For example, *P.terraenovae* showed total inactivity in darkness (and in LL below 1.0 lux) and no obvious relationship between τ_{LL} and light intensity. The two studies were conducted using different recording methods: *C.vicina* in a circular track with the fly breaking an infra-red light beam, and *P.terraenovae* in small running wheels. It is unlikely, however, that the observed differences may be attributed to differences in technique; they are more likely to represent differences in species behaviour.

Work with 'population rhythms' such as pupal eclosion (Pittendrigh & Bruce, 1957; Chandrashekeran & Loher, 1969; Saunders, 1979), oviposition (Gillett *et al.*, 1959), egg hatch (Minis & Pittendrigh, 1968) and pupation (Nayar, 1968) also shows intensity related period changes in LL giving way to arrhythmicity above a threshold. With the pupal eclosion rhythm in *Drosophila pseudoobscura*, Pittendrigh & Bruce (1957) were among the first to describe such effects. Later, Winfree (1974) showed that τ_{DD} in *D.pseudoobscura* (normally close to 24 h) lengthened to 24.7 h in continuous (blue) light below 0.001 erg cm^{-2} s^{-1}. In light above 0.1 erg cm^{-2} s^{-1} emergence from the cultures was continuous and arrhythmic. Between these two intensity values the emergence peaks broadened with little change in τ, although towards the upper part of the range the number of persistent cycles before arrhythmia fell.

Pittendrigh (1974, 1981) reported that cultures of *D.pseudoobscura* transferred from 'bright' (i.e. above threshold intensity) LL to DD resumed rhythmic eclosion activity immediately, with the first peak of eclosion occurring a fixed time (15 h) after the LL/DD transition. Extrapolation back to LL/DD suggested that the circadian pacemaker was initiated at that point at a characteristic phase (called Circadian time, Ct 12). The initial interpretation of this phenomenon was that the pacemaker's motion ceased in 'bright' light until transfer to DD. However, transfer of cultures from 'dim' (i.e. below threshold) LL to DD also led to a resetting of the pacemaker to Ct 12, no matter *when* (at what phase of the persisting LL rhythm) the step into darkness occurred. This raised the possibility that bright light suppressed overt eclosion rhythmicity but not the underlying circadian pacemaker. Thus the pacemaker's motion might have persisted in bright LL, although perhaps with a reduced amplitude. Similar interpretations were offered for the effects of protracted light on the flight activity rhythm of *Culex pipiens quinquefasciatus* (Peterson & Jones, 1979; Peterson, 1980a, b) and for eclosion rhythmicity in *Sarcophaga argyrostoma* (Peterson & Saunders, 1980).

In the present investigation few flies survived beyond their time in 'bright' LL (irradiance >0.035 Wm^{-2}) or, if they did, their levels of activity in the final DD free-run were too weak to establish period and phase. Nevertheless, in thirteen surviving flies the time interval between the step from LL and the first activity band in DD was very variable (13.5−22 h), and extrapolation back from the mid-point of α (Ct 6) to light off showed that the phase of the oscillation at the start of DD ranged from Ct 6.4 to Ct

16.3. Step-downs from 'dim' LL to DD (twenty-two flies) lead to a lengthening of τ with no change of phase; once again, therefore, the interval between light off and the first activity band in DD was very variable (5.2−22 h). There is no evidence, therefore, that steps into darkness reset the circadian pacemaker to a characteristic phase. Furthermore, although very bright light may reduce the amplitude of the pacemaker to zero (i.e. 'stop' the clock), present evidence suggests that light just above the threshold for arrhythmicity leaves the pacemaker in motion. Evidence to support this view may be obtained from flies in the intermediate intensity range (Fig. 1B) in which activity was initially arrhythmic but then showed a lengthened τ. In these examples, extrapolation of the LL activity band back to the initial period in DD indicates that the pacemaker was still running during the time of behavioural arrhythmicity. If this interpretation is valid, one should conclude that 'bright' light just above threshold causes overt arrhythmicity at a level downstream of the circadian pacemaker which continues its motion through the light. More data would be needed to settle this point.

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S-Antigen Antibody Partially Blocks Entrainment and the Effects of Constant Light on the Circadian Rhythm of Locomotor Activity in the Adult Blow Fly, *Calliphora vicina*

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Abstract Injection of S-antigen (arrestin) antibody into the brain of the blow fly, *Calliphora vicina*, appeared to reduce sensitivity to the photic effects of a light:dark cycle (LD; entrainment) or continuous "bright" light (LL; arrhythmicity). In LD, a proportion of the injected flies evaded entrainment or showed delayed entrainment. In bright LL, flies continued with a free-running rhythm, which remained unchanged (as in continuous darkness) or lengthened (as in "dim" LL). These results focus attention on four groups of arrestin-positive neurons in the fly's brain as potential components of the photoreceptive system.

Key words circadian rhythms, *Calliphora vicina*, extraretinal photoreceptors, photic entrainment, S-antigen

INTRODUCTION

The circadian rhythm of locomotor activity in the adult blow fly, *Calliphora vicina*, free-runs in continuous darkness (DD) with a mean period (τ) between 22 and 23 h, but becomes entrained to a light:dark cycle (LD) (Kenny and Saunders, 1991). In constant bright light (LL), activity is continuous and arrhythmic, but in dimmer LL, τ lengthens systematically with light intensity. The threshold light intensity between rhythm persistence and arrhythmicity is about 0.03 Wm^{-2} (Hong and Saunders, 1994). These three effects of light—entrainment, lengthened τ, and arrhythmicity—are thought to be mediated by photoreceptors on the input pathway to the circadian clock.

Microsurgery involving cuts between the optic lobes and the brain, or complete bilateral lobectomy, leaves the free-running rhythm and its photic entrainment virtually unaltered (Cymborowski et al., 1994), demonstrating that the compound eyes are not the only relevant photoreceptors and that the clock is not in the optic lobes (as in cockroaches, see Page, 1984). The brain of *C. vicina* thus emerges as a likely site for both clock and the "extra-retinal" photoreceptors.

Candidate extra-retinal photoreceptor neurons have been identified in *C. vicina* by an immunocytological technique using an antibody raised against the S-antigen (arrestin) (Cymborowski and Korf, 1995), an important element in the phototransduction cascade in both vertebrate and invertebrate photoreceptors (Korf and Wicht, 1992; Van Veen et al., 1986; Mirshahi et al., 1985; Bentrop et al., 1993). Apart from major neuropil areas within the optic lobes, and a prominent group of neurons in the ventral part of the

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optic lobe, all of which may be eliminated as putative photoreceptors because the optic lobes are unessential for entrainment (Cymborowski et al., 1994), positive S-antigen immunoreactivity was found in four distinct bilaterally arranged groups of neurons in the proto-, deuto-, and tritocerebrum of the fly's brain.

This article reports some results of injecting S-antigen antibody into the brain of *C. vicina*, particularly on the various effects of light on the locomotor activity rhythm, with an a priori expectation that flies may be partially "blinded" if the antibody interferes with phototransduction.

MATERIALS AND METHODS

Flies

Three strains of *Calliphora vicina* (= *C. erythrocephala*) were used: the Edinburgh strain, which was collected in Musselburgh, Scotland, in 1984; the Warsaw strain collected in 1992; and a Finnish strain (Nallikari) collected near Oulu in 1993. Maintenance of the stock cultures was as described in an earlier article (Saunders, 1987). The flies used for each experiment (all females) came from the same larval culture, emerged at the same time, and were thus of the same age.

Recording Locomotor Activity

Adult locomotor activity was recorded, as described by Kenny and Saunders (1991), in 9 cm petri dishes. The recording device comprised an infrared light beam (of 5 mm diameter) passing vertically across one side of the dish on to a phototransistor. Sugar solution was provided through cotton wool wicks. Pairs of dishes were placed on a wooden platform that provided support for the infrared emitters (Radio Spares, type 306-077) and detectors (RS type 306-083). The whole assembly was then placed in a light-tight box in a walk-in constant temperature room at $20 \pm 0.5\ °C$. Each box was provided with a 4 W fluorescent lamp, water-jacketted to suppress a temperature rise when the light was on.

Flies were exposed to either DD, LL, or to a light:dark cycle (LD 12:12). The intensity of the light in LL was either just above $0.04\ Wm^{-2}$, referred to here as "bright" LL, or about $0.018\ Wm^{-2}$, referred to as "dim" LL. These light intensities were obtained by wrapping paper of various thicknesses around the fluorescent

tubes and were chosen because the value for bright light was just above, and the value for dim light just below, the previously established threshold between lengthened τ and arrhythmicity (Hong and Saunders, 1994). Intensity of the light in LD 12:12 was also above this threshold. All light intensities were measured using a Tektronix J 16 photometer.

Activity events were registered as the number of times the infrared light beam was broken by a moving fly. Numbers in consecutive 10-min bins were then recorded automatically using a BBC B plus microcomputer, and assembled into the familiar "doubled plotted" format (Kenny and Saunders, 1991). Subsequent periodogram analyses (based on Enright, 1965, and Williams and Naylor, 1978) were carried out using an Archimedes 400 series computer.

Injection of Antibody

Experiments were performed on adult female flies within 2 to 3 days of emergence. In one experimental design, free-running locomotor activity was monitored for at least 5 days in DD prior to the experimental manipulations. After exposure to DD, flies were subjected to one of the following treatments: (1) S-antigen antibody injection within minutes of transfer to LD 12:12, (2) vehicle alone and LD 12:12, (3) antibody injection in DD, (4) vehicle alone and DD, (5) antibody injection within minutes of transfer to constant bright light (> $0.04\ Wm^{-2}$), (6) vehicle alone and the same constant bright light, (7) antibody injection within minutes of transfer to constant dim light (about 0.018 Wm^{-2}), and (8) vehicle alone and the same constant dim light. In a second experimental design, flies were exposed to LD 12:12 without the initial DD free-run. Data from the two experimental groups of flies were analyzed separately. No essential differences between the three strains of flies were observed.

A polyclonal antibody against bovine S-antigen (arrestin) raised in rabbits (NEI 04111083; dilution 1: 4000) (the kind gift of Professor Horst-W Korf of Johann Wolfgang Goethe-University, Frankfurt/ Main, Germany) was used. $0.5\ \mu l$ of antibody was injected through the right compound eye into the optic lobe using a very fine glass capillary. Control flies were handled by the same procedure, but received only vehicle ($0.5\ \mu l$ PBS plus 1% BSA). Each fly was subjected to treatment once. Injections were carried out shortly after lights on in LD 12:12, or shortly after transfer of the fly to constant bright or
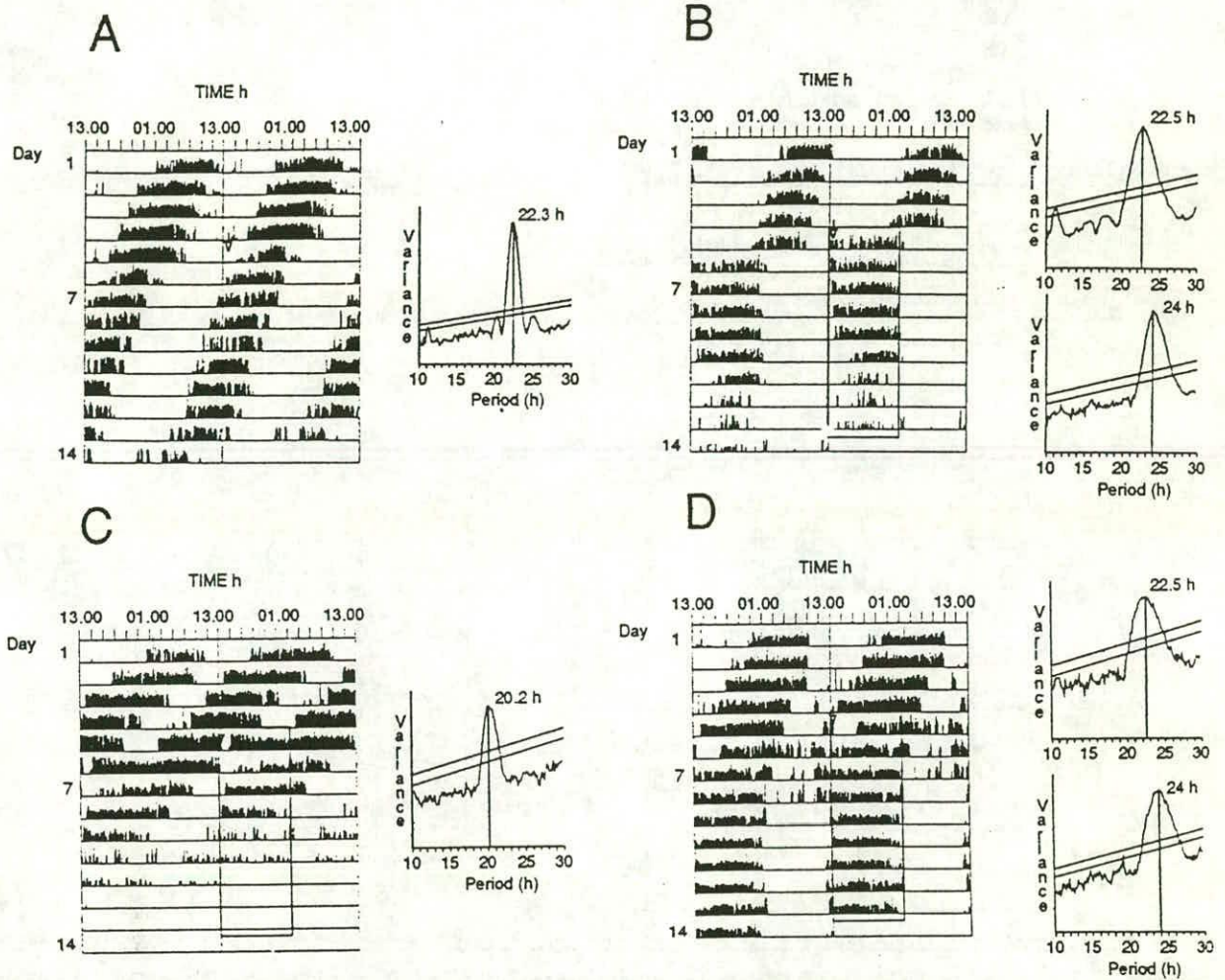
Figure 1. Double-plotted locomotor activity rhythms of the blow fly *Calliphora vicina* (all females at 20 °C). (A) Fly free-running in DD. Injection with S-antigen antibody (arrow) on Day 6 caused no lasting effect on period or phase. (B) Fly exposed to LD 12:12 from Day 6 and injected with vehicle shortly after first light-on; it entrains to LD 12:12 immediately, as in uninjected controls (not shown). (C and D) Flies exposed to LD 12:12 from Day 6 and injected with S-antigen antibody shortly after light-on. In C, fly free-runs through the light cycle; in D, fly free-runs through light cycle for about 4 days and then abruptly entrains. Periodograms for the appropriate sections of the activity records are shown alongside the actograms. Open boxes denote times when the lights were on.

dim light, the injection taking about half a minute. Immediately after injection with the antibody (but not with its vehicle alone), flies showed behavioral signs of unilateral blinding (running in circles). Within a few minutes of injection, flies appeared totally blind to visual stimulation, indicating that the injected material had infiltrated across to the contralateral side.

RESULTS

Representative actograms of *C. vicina* receiving 0.5 μl of the S-antigen antibody or its vehicle are shown in Figs. 1 to 3; numerical data for all treated and control flies are shown in Tables 1 and 2.

Injection of antibody into flies in DD had little or no effect on their free-running behavior, the example shown in Fig. 1A continuing with the same circadian period (22.3 h) after injection. Some flies injected with antibody or its vehicle, however, suffered a shortened life span (e.g., Figs. 1C, 3B, 3C, 3D) presumably caused by the influx of foreign protein; premature mortality, however, rarely prevented analysis.

In Experiment 1, flies transferred from DD to LD 12:12 (bright light: irradiance > 0.04 Wm^{-2}) and then receiving vehicle (Fig. 1B) entrained to the light cycle in a manner identical to the untreated controls (not shown). Of those receiving antibody, however, a proportion (35.7%) free-ran through the light cycle either until they died (Fig. 1C) or free-ran for a few days
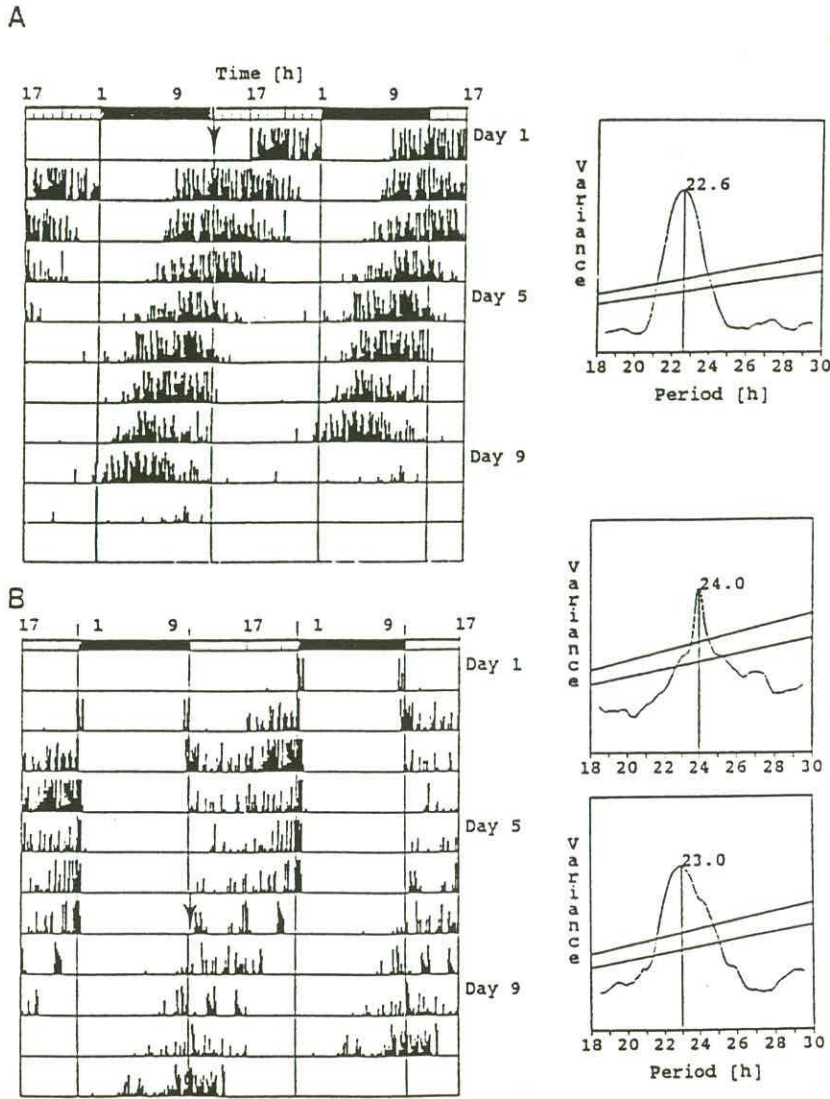
Figure 2. Females of *C. vicina* kept in LD 12:12 throughout and injected with S-antigen antibody (arrows) on (A) Day 1 or (B) Day 6. After injection the flies free-run through the light cycle without entrainment. Periodograms show average period of the rhythm before (2B) and after (2 A, B) injection.

until entrainment was abruptly restored (Fig. 1D). Such effects were not seen in vehicle-only controls, which entrained immediately; this difference between antibody-injected and control flies is statistically significant (combined $\chi^2 = 21.991$, $df = 4$, $p < 0.01$). In flies exposed to LD 12:12 throughout and receiving antibody injections soon after lights-on (Experiment 2), a higher proportion (67.3%) free-ran through the light cycle (Fig. 2) and only 14.5% clearly entrained (Table 1) ($\chi^2 = 27.389$, $df = 1$, $p < 0.001$). Flies categorized as "doubtful" in both experiments were those that developed high levels of arrhythmicity, or whose activity levels became too low to allow a meaningful distinction to be made between the entrained and free-running states.

Out of the 6 flies, 5 transferred to bright LL (irradiance > 0.04 Wm^{-2}) before receiving 0.5 µl of vehicle became arrhythmic (Fig. 3A; Table 2) as in the untreated controls (not shown). Of those receiving antibody, however, all remained rhythmic (Table 2); in 2/9 (22.2%) the circadian period remained unaltered, as if in DD, whereas in the remaining 7 flies (77.8%), τ lengthened as if in dim LL (Hong and Saunders, 1994) (Fig. 3B). This difference is significant ($\chi^2 = 28.427$, $df = 6$, $p < 0.001$). Eleven further flies were transferred to dim LL (irradiance < 0.02 Wm^{-2}) before vehicle or antibody injection. All remained rhythmic: For those receiving vehicle, 4/7 showed a lengthening of τ as expected (Hong and Saunders, 1994) (Fig. 3C) and in
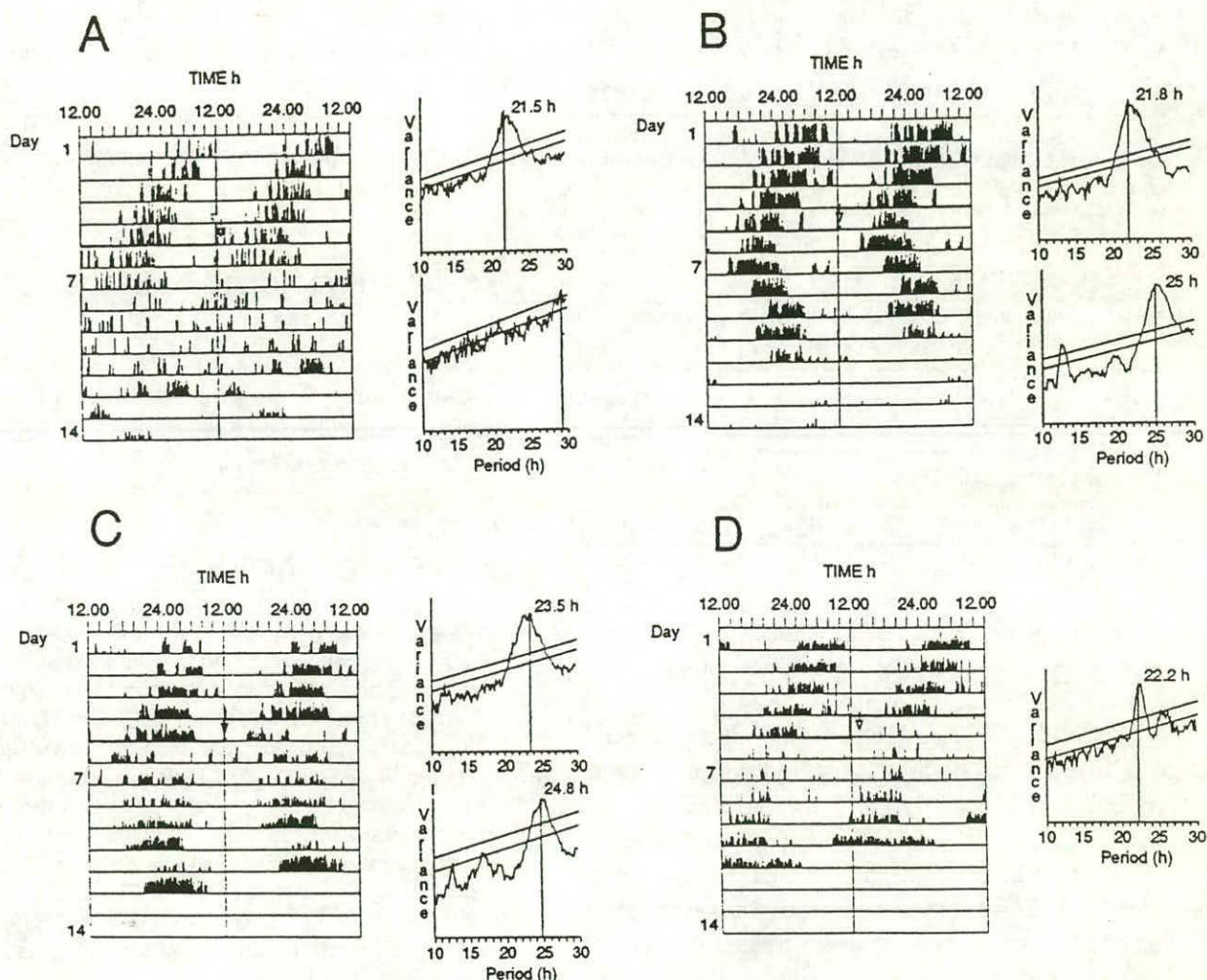
Figure 3. (A) Female of *Calliphora vicina* transferred to continuous bright light (> 0.04 Wm^{-2}) on Day 6 and injected with vehicle shortly after light-on. Activity in LL became arrhythmic, as in uninjected controls (not shown). (B) Fly transferred to continuous bright light on Day 6 and injected with S-antigen antibody, showing period lengthening more characteristic of a control fly in dim LL. (C) Fly transferred to dim LL (~ 0.018 Wm^{-2}) on Day 6 and injected with vehicle shortly after light-on. Period lengthens as in uninjected controls (not shown). (D) Fly transferred to dim LL on Day 6 and injected with S-antigen antibody shortly after light-on. Period of the rhythm remains unaltered at 22.2 h, characteristic of a fly in DD. Periodograms for the appropriate sections of the activity records are shown alongside the actograms. Arrows show the times of injection.

3/7 τ remained unaltered, and for those receiving antibody, 2/4 τ lengthened and 2/4 τ remained unchanged (Fig. 3D). The proportions of flies whose free-running periods remained unaltered in dim LL between the antibody treated and vehicle control groups were not significantly different.

DISCUSSION

Injection of S-antigen antibody or its vehicle into the brain of *C. vicina* had no lasting effect on the fly's

activity rhythm in DD. In some flies activity was either delayed or accelerated on the day of injection, probably due to the shock of the injection, but subsequent free-running activity continued undisturbed.

When combined with LD exposure, however, injection of antibody caused a proportion of the flies to evade the entraining effects of the light cycle (Table 1). This effect was more frequent in those flies transferred to LD on the first day of the experiment (Experiment 2) than in those allowed an initial free-run in DD before exposure to the light:dark cycle (Experiment 1). This difference might be attributable to light effects on the

Table 1. Behavior of *Calliphora vicina* in LD 12:12 (L > 0.04 Wm^{-2}) after injection of S-antigen antibody.

	N	Entraining* n (%)	Free-Running n (%)	Doubtful n (%)
Untreated control	8	8 (100)	0	0
Vehicle control	6	6 (100)	0	0
Experiment 1	28	10 (35.7)	10 (35.7)	8 (28.6)
Experiment 2	55	8 (14.5)	37 (67.3)	10 (18.2)
Experiment 1 + Experiment 2	83	18 (21.7)	47 (56.6)	18 (21.7)

NOTE: Experiment 1: Flies maintained in DD for 5 days before exposure to LD 12:12 and antibody injection. Numbers are totals for two replicates. Experiment 2: Flies maintained in LD 12:12 from start of the experiment. Numbers are totals for two replicates.
* Expected response

Table 2. Behavior of *Calliphora vicina* in continuous light (LL > 0.04 Wm^{-2}) after injection of S-antigen antibody.

	N	Arrhythmic* n (%)	$\tau = \tau_{DD}$ n (%)	τ Lengthens n (%)	Doubtful n (%)
Untreated control	14	13 (92.9)	0	1 (7.1)	0
Vehicle control	6	5 (83.3)	0	0	1
Antibody	9	0	2 (22.2)	7 (77.8)	0

* Expected response

photoreceptive pigments (Schwemer, 1984), but much more experimentation is required for a fuller understanding of this phenomenon.

Antibody also interfered with the effects of continuous illumination, such as arrhythmic locomotor activity in bright LL (> 0.04 Wm^{-2}) or the lengthening of τ in dim LL (~ 0.018 Wm^{-2}). Plangger et al. (1994) have shown that in the *C. vicina* compound eye, S-antigen binds to light-activated rhodopsin (= metarhodopsin), facilitating phosphorylation and inactivation of the metarhodopsin. The present results, therefore, suggest that injection of S-antigen antibody into the central nervous system interferes with the process of light perception, effectively lowering the sensitivity of the photoreceptor.

These results provide no evidence relating to the location of the photoreceptors involved in the mediation of the LD and LL effects. However, as mentioned earlier, they are unlikely to be in the compound eyes or the optic lobes in that these structures are dispensable (Cymborowski et al., 1994). This fact and the present results focus attention on the four groups of arrestin-positive neurons observed in the brain (Cymborowski and Korf, 1995) as candidates for the extra-optic, and probably brain-centered, photoreceptors in *C. vicina*.

Extraoptic photoreception has been described in insects, birds, reptiles, amphibia, and fish (Menaker and Underwood, 1976; Truman, 1976; Binkley, 1993) in which light may entrain circadian rhythms or mediate photoperiodic responses in animals deprived of their eyes. The location of the extraoptic photoreceptors in birds has remained elusive, but the ventral forebrain is a likely site (Kuenzel, 1993). If the arrestin-positive cells observed in the brain of *C. vicina* (Cymborowski and Korf, 1995) are part of the relevant photoreceptors, their identification as such represents one of the few observations of this type in the insects.

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