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CIRCADIAN ORGANIZATION IN THE REGULATION OF
LOCOMOTOR ACTIVITY AND REPRODUCTION IN RATTUS EXULANS

PHILIPPA H. GANDER.

Zoology Department,
University of Auckland.

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ABSTRACT

The role of the circadian time-keeping system in regulation of locomotor activity and certain aspects of reproduction has been investigated in wild Polynesian rats, *Rattus exulans*.

Locomotor activity is under circadian control and data are consistent with a general model of the pacemaker mechanism as a weakly interacting population of circadian oscillators. Experimental studies and field observations indicate that the action of light in entrainment of this rhythm is primarily non-parametric.

Female *R. exulans* continue to ovulate during prolonged periods in constant conditions and undergo a pattern of change in vaginal cytology through the estrous cycle which closely resembles that of laboratory *R. norvegicus*. These findings are consistent with the hypothesis that the estrous cycle in *R. exulans* is regulated by a similar circadian mechanism to that controlling the timing of ovulation, and hence the duration of estrous cycle, in laboratory rats.

Female *R. exulans* do not exhibit regular fluctuations in either the period of the activity rhythm or intensity of the active phase in association with the estrous cycle. Ovariectomy also has no significant effect on the period of the activity rhythm and no discernible effect on the distribution or intensity of activity. It is therefore concluded that there is no feedback action of the ovaries or estradiol on the circadian pacemaker regulating locomotor activity in *R. exulans*, which thus differs from laboratory rodents. This proposition is further supported by the observation that there are no significant changes in either period or variability of the activity rhythm in association with the degenerative changes that occur in the female reproductive system in old age. The adaptive significance of these findings is considered.

Field studies on breeding patterns of *R. exulans* throughout its distribution provide several lines of indirect evidence in support of the hypothesis that the onset of breeding in this species in temperate latitudes is regulated by seasonal changes in photoperiod. Accelerated attainment of puberty occurs in juvenile females collected during the non-breeding part of the year and housed in LD 16:8. Juvenile females collected at the same times but housed in LD 8:16 for an identical duration remain immature. Groups of mature females collected during the breeding
season do not show a differential response to these light regimes. These results are discussed in relation to field data on breeding patterns in the population from which experimental animals were collected. It is concluded that the onset of breeding in this population is controlled primarily by a photoperiodic mechanism regulating the attainment of reproductive maturity in females.

Information on the physiological organization of circadian systems in mammals is reviewed, with particular emphasis on the relationships between locomotor activity rhythms, the estrous cycle, and the effects of photoperiod on reproductive function in rodents.
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SYMBOLS AND ABBREVIATIONS

\( \tau \)  
The period of an overt circadian rhythm in conditions of constant light intensity and temperature. In this study \( \tau \) is the mean period calculated over 10 consecutive cycles.

CT  
Circadian time. A relative time scale in which one unit = 24/\( \tau \).

\( \Delta \phi \)  
The steady-state phase shift produced in an overt rhythm by a perturbation.  
+ \( \Delta \phi \) indicates a phase advance.  
- \( \Delta \phi \) indicates a phase delay.

\( T \)  
The period of a zeitgeber cycle.

\( \Upsilon \)  
The phase angle in steady-state entrainment between an overt circadian rhythm (or the pacemaker driving it) and the zeitgeber cycle. In this study \( \Upsilon \) is measured between activity onset and the onset of the dark phase of the LD cycle.

LL  
Constant light.

DD  
Constant darkness.

LD  
x:y x hours of light alternating with y hours of darkness.

See also:

1, 31 - 64.
FRONTISPICE

Adult female *Rattus exulans*.

(Photograph courtesy of Professor C.R. Austin.)
1.1 INTRODUCTION

The daily temporal niche of a species is presumably determined by selection pressures arising from daily fluctuations in certain critical factors in the environment. These may include food availability, predation, mate location and other inter- and intraspecific behavioural interactions, and the optimum range within the daily cycle of the physical environment for efficient physiological functioning (Kenagy, 1976). Circadian time-keeping systems are one of the mechanisms moulded by selection to optimize efficiency of temporal niche utilization in many species of eukaryotes. It seems reasonable to infer that they have functional properties which distinguish them from alternative mechanisms such as direct responses to environmental fluctuations.

Circadian rhythms formally resemble autonomous oscillators. Their oscillatory properties have been extensively investigated and a number of empirical generalizations have emerged from which functional explanations have been extrapolated (Pittendrigh and Daan 1976 b,c). Circadian pacemakers effectively impose a pattern of temporal change on many aspects of the physiology and behaviour of an organism, which persists in the absence of cyclic environmental input. Appropriate phasing of this temporal pattern with respect to daily environmental fluctuations is ensured by entrainment of the pacemaker(s) by particular environmental cycles (zeitgebers). This phase control, which is a property of entrainment of all self-sustained oscillators, is considered to be one of the distinguishing characteristics of circadian time-keeping systems. It enables them to function as a mechanism for the recognition of local time (Pittendrigh and Daan, 1976 b). Because the internal organization of the organism follows a programmed temporal sequence, necessary preparation for effective reaction to regular environmental changes can begin in advance of the critical external events (Aschoff, 1964). The other commonly recognized function of circadian time-keeping systems is measurement of the passage of time, for example in time-compensated suncompass orientation (Pittendrigh and Daan, 1976 b). This function requires stable angular velocity of the oscillating mechanism and is proposed as an explanation for the temperature compensation of period of circadian rhythms (e.g. Gander, 1979) and for the postulated general homeostasis of their frequency (Pittendrigh and Caldarola, 1973).
pacemakers have also been developed on the basis of laboratory-based experimental analyses (Pavlidis, 1978 a,b; Gander and Lewis, 1979).

The actual role played by circadian systems in regulating the physiology and behaviour of organisms in the field is an area which has received comparatively little attention. Functional considerations have often tended to be little more than a speculative appendix to experimental results. For fuller understanding of the physiological organization of behaviour, both lines of investigation should have an integrative share in the development of hypothetical guidelines for research (Daan and Slopsema, 1978). Particular cognizance was taken of this dual approach in the design of this project.

Studies in which several rhythmic functions have been monitored simultaneously, particularly in laboratory rodents, primates and man, have revealed that a diversity of behavioural and physiological functions are regulated by circadian time-keeping systems in mammals. The complex integration of these functions and the mechanism(s) whereby they are synchronized by environmental fluctuations, are areas of major interest in current research. Three basic models of circadian organization in mammals have been proposed (Moore-Ede et al., 1976).

In the first (Model I), it is postulated that the circadian system consists of a network of cellular subsystems which are driven by a single self-sustained oscillator (or synchronous population of oscillators). This master oscillator receives input from the sensory apparatus and thus mediates the effects of zeitgebers on the system. In the second model (the hierarchical model), the circadian system is envisaged as a network of cellular self-sustained oscillators. These unit oscillators are synchronized by a master oscillator or pacemaker, which is again proposed to be the sole recipient of information from the sensory apparatus and thus the exclusive mediator of environmental entraining stimuli. A network of self-sustained cellular oscillators is also envisaged in Model III (the non-hierarchical model) but no one oscillator acts as an overall pacemaker. Several oscillators may receive inputs from different sensory systems and thus be susceptible to zeitgebers. Internal synchrony is postulated to be maintained by feedback interactions among the subsystems.

In most steady-state situations, the various rhythmic functions within an individual exhibit identical periods and maintain a constant phase relationship with respect to one another i.e. they are internally synchronized (Moore-Ede et al. loc. cit.). Internal synchrony was
originally interpreted as evidence in support of Model I (Mills, 1966, 1973). A single master oscillator model cannot, however, accommodate the breakdown of synchrony which is observed under certain experimental conditions.

Spontaneous internal desynchronization occurs when different rhythmic functions within an individual simultaneously exhibit different free-running periods and thus have continually changing phase relationships with respect to one another. This phenomenon has been reported between body temperature and activity rhythms in man (Wever, 1975; Aschoff and Wever, 1976) and between feeding and colonic temperature rhythms, and rhythms of urinary excretion in the squirrel monkey Samiri sciureus (Moore-Ede et al., 1977; Sulzmann et al., 1977, 1978). On the other hand spontaneous internal desynchronization does not appear to be a characteristic of the circadian system of the laboratory rat (Rattus norvegicus). Plasma corticosterone and food intake rhythms persist in phase for seven weeks in constant darkness (DD) and until the corticosterone rhythm damps out in constant light (LL 700 - 750 lux) (Takahashi et al., 1977). No desynchronization is evident between the rhythms of locomotor activity, body temperature and corticosterone in rats in LL (200 lux) (Honma and Hiroshige, 1978 a). By the end of three months, all three rhythms decompose into phase-locked ultraradian rhythms with 4 - 6 h periodicities (Honma and Hiroshige, 1978 b). Ultraradian components are also reported in rhythms of locomotor activity, heartbeat rate and body temperature during the dark phase of LD 12:12 (Plonait et al., 1979).

Several rhythms in an individual may be forced to desynchronize temporarily following phase shifts in environmental light regimes, as certain physiological variables take longer to resynchronize than others. Forced internal desynchronization of this type has been observed in man (A.L. Elliot et al., 1972), squirrel monkeys (Moore-Ede et al., 1977) and the hamster Mesocricetus auratus (Zucker and Stephan, 1973; Finkelstein et al., 1978). Alternatively, rhythmic functions within an individual can be forced from their normal phase relationships if they have different ranges of entrainment. Exposure to a non-24 h light cycle, which entrains some rhythms but not others, causes forced internal desynchronization in humans (Wever, 1975; Aschoff and Wever, 1976) and squirrel monkeys (Moore-Ede et al., 1979). The activity and drinking rhythms in hamsters have different phase relationships in dim LD (L < 4 lux)
and bright LD (L > 60 lux) entrainment (Wolterink et al., 1977). This suggests different sensitivity of their respective controlling mechanisms to light, which might also result in different ranges of entrainment.

Internal desynchronization indicates that circadian rhythms in mammals are controlled by a multioscillatory system. This proposition is also supported by the observation that some isolated tissues in vitro exhibit free-running rhythms e.g. the adrenals (Andrews, 1968), heart cells and cell networks (Thorp and Folk, 1965) and the liver (Moore-Ede et al., 1976 cite Rintoul, 1975).

Whether the circadian system is predominantly hierarchical or non-hierarchical is comparatively difficult to establish in practice. In the hierarchical model, a single oscillator or population of oscillators in a distinct anatomical location is postulated to act as a pacemaker to the system and to be essential for entrainment. Damage to the suprachiasmatic nuclei (SCN) disrupts a diversity of behavioural and physiological rhythms in laboratory rodents (Table 5.2). This suggests that the SCN serve a critical function in circadian organization in these animals. Several recent reports indicate residual circadian or ultraradian rhythmicity in a number of functions following SCN ablation (Rusak, 1977 a; Watson-Whitmyre and Stetson, 1977; Boulos and Terman, 1979; Powell et al., 1979; Stephan et al., 1979; Wiegand et al., 1979). These findings suggest that the SCN do not drive all circadian rhythmicity in these animals (i.e. they are not a Model I-type pacemaker). They would seem rather to perform a hierarchical synchronizing function among other autonomous oscillating systems. The SCN also receive direct bilateral projections from the retinae, which are implicated in the entrainment of circadian rhythms in rodents (Moore and Eichler, 1972; Stephan and Zucker, 1972 a, b; Moore and Klein, 1974; Mosko and Moore, 1978; Stephan and Nunez, 1978).

In the non-hierarchical model, internal synchrony is not endowed by a single pacemaker, but instead relies on mutual synchronization of a number of self-sustained oscillating systems in various anatomical locations. While the variety of endocrine and behavioural rhythms influenced by the SCN suggest they are an important circadian regulating centre, the possibility cannot be excluded that there may be other centres regulating rhythmic functions which were not monitored in SCN ablation experiments. It is also postulated in the non-hierarchical model that environmental timing cues may influence the circadian time-keeping system
through more than one route and that different types of zeitgebers may influence different oscillators. The existence of several zeitgebers which have differential influences on various rhythms would therefore tend to support this model. Light and/or sound cycles and social interactions have been found to be effective zeitgebers of human circadian rhythms (Aschoff, Patranska et al., 1971; Wever, 1975; Aschoff and Wever, 1976). Whether or not they entrain separate oscillators, or affect different rhythms differentially does not appear to have been established. In squirrel monkeys, feeding schedules are a stronger zeitgeber than LD cycles for the circadian drinking and urinary rhythms, while LD cycles are a stronger entraining agent for colonic temperature. In 24 h LD cycles and a 25 h feeding regime, these rhythms can be forced to desynchronize (Moore-Ede et al., 1978). It is difficult to envisage how the two zeitgebers could act through a common master oscillator to produce these effects. In laboratory rats, restricted feeding schedules result in anticipatory running behaviour prior to expected feeding times (Edmonds and Adler, 1977 a, b). Fixed interval feeding does not, however, appear to constitute a true zeitgeber of the activity rhythm, but may affect the activity generating apparatus directly (Gibbs, 1979). On the other hand, fixed interval feeding is reported to alter sleep patterns (Mouret et al., 1973) which are under circadian control (Borbely and Neuhaus, 1978 a, b). It also synchronizes the circadian rhythms of food intake and plasma corticosterone (Takahashi et al., 1977). The effects of conflicting feeding and lighting regimes on the activity and corticosterone rhythms might provide a test of the non-hierarchical model. The ability of such conflicting temporal information to cause desynchronization would, however, depend on the strengths of the entraining cycles relative to the strength of internal coupling between the two rhythmic systems (Moore-Ede et al., 1976). Failure to cause dissociation would not necessarily disprove the non-hierarchical model.

In the circadian organization of mammals there are probably various levels of hierarchical and mutual interaction between autonomous oscillating systems. In addition, some rhythmic functions appear to be passive, driven responses to others e.g. the circadian rhythm of urinary potassium excretion in rats appears to be regulated by the rhythm of corticosterone secretion (Hilfenhaus and Herting, 1979). When a number of rhythmic functions undergo correlated fluctuations, it is often very
difficult to establish the causal relationships (if any) among them. For example cyclic changes in activity have often been proposed to be the cause of the accompanying variation in heartbeat rate. Simultaneous monitoring of heartbeat, activity and deep body temperature in rats indicates, however, that a significant amount of the daily variation in body temperature and heart rate is not accounted for by corresponding changes in activity (Meinrath and D'Amato, 1979). Deep body temperature is also at least as good an indicator of heart rate as is activity.

Each overt rhythmic function is not necessarily regulated by a single oscillator. Several characteristics of free-running locomotor activity rhythms suggest that they are regulated by populations of weakly interacting oscillators (Pavlidis, 1978 a, b).

The complexity of circadian organization in mammals, and the difficulties involved in resolving the causal nexus of such a system, provided a stimulus to this thesis.
1.2 AIMS

This study was designed to examine the role of circadian time-keeping in the regulation of locomotor activity and reproduction in wild *Rattus exulans* (Polynesian rats). The topic was selected because of its potential as a system in which functional considerations might be useful in elucidation of the nature of interactions between two circadian rhythms in a mammal.

The estrous cycles and the locomotor activity rhythms of laboratory rats (*Rattus norvegicus*) and hamsters (*Mesocricetus auratus*) have been demonstrated to be under circadian control, and it is proposed that a common circadian mechanism may regulate both functions (see Sections 3.1, 4.1, 5.1). It has also been suggested that linkage between these two rhythms may be important in synchronizing mating in these species (Richter, 1970; Stetson and Watson-Whitmyre, 1976).

The first aim of the research reported in this thesis was to provide comparative laboratory studies on the regulation of activity patterns and the estrous cycle in a wild rodent. Experimental animals were collected from a local island population (on Tiritiri Matangi - see Chapter Two) which has been the subject of ongoing ecological, demographic and behavioural studies in the Zoology Department, University of Auckland. Considerable data from field observations were thus available as a framework within which to assess information derived from laboratory-based experiments. The second aim of this research was to relate the circadian mechanisms revealed in laboratory studies to what is known of the patterns of activity and reproduction of this species in the field i.e. to consider them in their functional context. Four specific hypotheses were examined:

1) that locomotor activity of *Rattus exulans* is under circadian control;
2) that the estrous cycle is under circadian control;
3) that there are interactions between these two rhythms, possibly mediated by estradiol;
4) that the breeding season of *R. exulans* on Tiritiri Island is regulated by a circadian-based photoperiodic mechanism controlling the onset of estrous cycling in the females.
2.1 THE EXPERIMENTAL ANIMAL

*Rattus exulans* (Peale), (the Polynesian rat, Maori name kiore) is common on islands of the central and western Pacific and on the mainland of South East Asia (Egoscue, 1970). It is thought to have spread throughout the Pacific in association with Polynesian colonization, and is assumed to have arrived in New Zealand, the southernmost limit of its distribution, by this means. It causes damage of considerable economic importance in some areas, primarily to coconuts, sugar cane and rice and is a known vector of the typhus-carrying mite and of the plague flea (Williams, 1973).

*R. exulans* had apparently become abundant and widespread throughout both the North and South Islands of New Zealand prior to European settlement. By 1840, however, it was reported to be rare over much of the North Island, although remaining prevalent in the South Island until around 1900 (Williams, 1973). By 1922 it was considered extinct, but has subsequently been identified from Stewart Island, a number of offshore islands, and in the Fiordland region of the South Island (Watson, 1956). There is some evidence that kiore may have a significant impact on the flora and fauna of New Zealand's offshore islands (Moller, 1977), some of which are now important reserves for native species.

Animals used in this study were live-trapped on Tiritiri Matangi Island (174° 54' E longitude, 36° 36' S latitude) in the Hauraki Gulf. This population of *R. exulans* has been the subject of two recent ecological studies (Moller, 1977; Bunn, 1979) and patterns of movement related to resource utilization of animals in the field are currently being investigated by M. Nicholas of the Zoology Department University of Auckland. Moller (1977) provides references on the geology and climate, and details of the current vegetation and faunal history of Tiritiri. Maori habitation of the island is thought to have continued up to about 1821-22 and probably explains the presence of *R. exulans*. Tiritiri was farmed from at least as early as the erection of the first lighthouse in 1865, until the early nineteen seventies. At present all but the 20 hectares forming the Lighthouse Reserve (at the south end of the island) is administered by the Hauraki Gulf Maritime Park Board as a recreational reserve. In June 1971 grazing leases to the Park Reserve were terminated to allow the island
to revert to native bush and the majority of stock were removed about February, 1972. The only stock now remaining are confined to the Lighthouse Reserve. *R. exulans* is evidently the only mammal now present on the rest of Tiritiri, despite the presence in the past of grazing stock, goats, rabbits, feral cats and possibly other rodent species.

With the exception of animals living around the Lighthouse Reserve, and possibly around the University of Auckland field hut, *R. exulans* on Tiritiri are thus living in a "wild" state in as much as they are not subject to major human interference and are away from human habitation.
2.2 ANIMAL COLLECTION AND MAINTENANCE

Rats were live-trapped in dome-shaped wire cage traps, each with a spring-loaded door triggered by a bait hook (Tiaka brand, see Moller, 1977 plate 4). Males and/or females were retained as required.

On arrival in the laboratory, animals were weighed, sexed and allocated a numbered cage as described in Appendix I, part A. Before being transferred to the experimental cages, all rats were totally immersed for several seconds in a 0.5% solution of Mal Diaz insecticide. This proved a very effective treatment against the numerous ectoparasites carried by wild R. exulans, and no subsequent reinfestations were observed.

Individually caged females were housed in one of three ventilated cabinets with independently controlled lighting (Appendix I, part B), in a temperature controlled room at 21 ± 1°C (Experimental Room I). Since this room was rodent-proof, interference from feral rats and mice in the building may be precluded as a factor in these studies. Suckling males born to females in the experimental cabinets were the only males ever housed in Experimental Room I. Twenty-one days after first being observed (i.e. at a maximum of 35 days old) young male rats were weaned and removed from Experimental Room I and housed individually.

Individually caged male R. exulans were housed in a single ventilated light-controlled cabinet in a darkroom at 20 ± 1°C (Experimental Room II) which was four floors below Experimental Room I. Possible pheromonal or acoustic influences of male R. exulans on the experimental females were thus avoided. Activity records show no evidence of socially induced synchrony among individually caged males or females in their respective cabinets.

Fans providing ventilation in the cabinets, and the room air-conditioning units created a continuous low level background noise which masked much of the daily variation in ambient noise levels. The non 24 h free-running activity rhythms recorded indicate that sounds associated with the daily routine in the building were not a significant synchronizing agent in these studies.

Each rat had access ad libitum to food (New Zealand Stockfoods Diet 86, laboratory rat and mouse pellets) and tap water. The floor
of each cage was covered with a layer of sawdust roughly 1 cm deep and at irregular 12 to 16 day intervals, animals were transferred to clean cages and the food and water supplies replenished. This procedure was performed in deep red (> 650 nm) light if animals were in constant darkness and did not cause any disruption to the experimental lighting regimes. It had no phase-shifting effect on the activity rhythms of *R. exulans*.

Lighting in each of the experimental cabinets was provided by two 20 watt 0.61 m Philips cool white fluorescent tubes, resulting in an average intensity of 520 lux when the lights were on. In the cabinets in Experimental Room I, there was also a continuous low intensity emission of white (tungsten filament) light from the ventilation holes in the mounting of the light beam activity-detecting apparatus (Section 3.2). This resulted in a constant intensity ranging from 0.02 to 0.07 lux at the level of the cages.

All times given in this study refer to New Zealand Standard Time.
3.1 INTRODUCTION

Locomotor activity rhythms have probably been investigated more thoroughly than any other circadian regulated function in animals (Aschoff, Gurecke et al., 1971; Aschoff, 1979). In the case of rodents, information is rapidly accumulating on both the physiology and the oscillatory properties of the pacemaker mechanism involved.

The suprachiasmatic nuclei (SCN) are implicated as the anatomical location of the circadian pacemaker regulating locomotor activity in laboratory rats and hamsters (Stephan and Zucker, 1972 a; Stetson and Watson-Whitmyre, 1976; Raisman and Brown-Grant, 1977; Rusak, 1977 a; Stephan and Nunez, 1977; Watson-Whitmyre and Stetson, 1977, Mosko and Moore, 1978). Direct bilateral projections from the retinae to the SCN apparently mediate the entraining effects of environmental light, which is the major zeitgeber for activity rhythms in these animals (Stephan and Zucker, 1972 b; Rusak, 1977 b; Mosko and Moore, 1978; Rusak and Zucker, 1979). The pathways whereby the circadian pacemaker exerts its effects on the locomotor apparatus remain, however, essentially unknown (Gibbs, 1979).

The duration and precision of activity records obtained from various rodents have permitted detailed analyses of both free-running rhythms and their responses to zeitgebers. The multioscillatory nature of the pacemaker regulating rodent activity rhythms is indicated by several types of period lability observed in prolonged free-runs (Pavlidis, 1978 b). Rhythm splitting has been described in running wheel activity rhythms of the ground squirrel *Spermophilus undulatus* (Pittendrigh, 1960), the squirrels *Funambulus palmarum* (Pohl, 1972 a) and *Glaucomys volans* (Pittendrigh and Daan (1976 c)) cite Daan unpublished), and the hamster *Mesocricetus auratus* (Pittendrigh, 1974; Pittendrigh and Daan, 1976 c). After-effects on the period of activity rhythms following various light treatments have also been reported in the squirrels *Glaucomys volans* (De Coursey, 1960) and *Tamiasciurus hudsonicus* (Kramm, 1975 b), the hamster *Mesocricetus auratus* (Pittendrigh, 1960, 1974; Pittendrigh and Daan, 1976 a), the ground squirrel *Ammospermophilus leucurus* (Kramm, 1976), the mouse *Mus musculus* and the deer mouse *Peromyscus leucopus* (Pittendrigh and Daan, 1976 a). Spontaneous loss and re-establishment of rhythmicity is observed in activity rhythms of *M. auratus* (Pittendrigh
1974). A comprehensive model has been proposed in which the pacemaker controlling rodent activity rhythms is envisaged as two coupled oscillators, or two populations of oscillators (Pittendrigh, 1974; Pittendrigh and Daan, 1976 c; Daan and Berde, 1978).

The nature of the action of light (whether predominantly parametric or non-parametric), and the mechanism(s) of entrainment have been investigated in a number of nocturnal and diurnal species (Swade and Pittendrigh, 1967; Swade, 1969; De Coursey, 1972, 1973; Kramm, 1973, 1974, 1975 a,b, 1976; Pohl, 1976; Pittendrigh and Daan, 1976 b). Kenagy (1976) has conducted the first systematic study in a mammal of the times of activity onset and end in field and laboratory conditions, in the nocturnal kangaroo rats Dipodomys merriami and D. microps. Seasonal patterns of change in the timing of activity have also been examined in several diurnal and nocturnal species of rodents housed in activity recording apparatus in natural light cycles (Daan and Aschoff, 1975; Kenagy, 1978). These studies have provided a new perspective on the complexity of interactions between the circadian pacemaker mechanism controlling activity and natural zeitgebers.

To test the hypothesis that locomotor activity of Rattus exulans is under circadian control, activity patterns of male and female rats were monitored in constant conditions. The effects of single light pulses and light cycles were subsequently investigated to enable a comparison of the oscillatory properties of this rhythm with those of activity rhythms of other rodents. The mechanism of entrainment was examined with regard to its function in regulating the timing of activity in the field.
3.2 METHODS OF DETECTING AND RECORDING ACTIVITY.

In the majority of laboratory studies of activity rhythms in rodents, running wheel activity has been monitored. Close scrutiny of the literature, however, reveals a number of inherent disadvantages in this technique.

When the activity of golden hamsters (Mesocricetus auratus) recorded in spring-suspension cages is compared with their activity simultaneously recorded in attached running wheels, a number of significant differences are observed (Aschoff et al., 1973). Onset of activity in the cage precedes the onset of running wheel activity in LD 12:12 and in dim LL and this phase angle difference is positively correlated with light intensity in LL. Some activity is recorded from the spring-suspension cage during the light phase of the LD 12:12 cycle but there is no running wheel activity during this phase and in some instances (e.g. ibid Fig.2) a circadian periodicity may continue in cage activity when there is little or no running wheel activity. These observations suggest that running wheel activity may not be the most reliable indicator of the underlying circadian pacemaker. Running wheels fail to detect the numerous other types of activity e.g. feeding, drinking, excretion, grooming, which may be performed throughout the day and which may or may not be under circadian clock control. (Chapter One).

Mongolian gerbils Meriones unguiculatus recorded in running wheels show a unimodal nocturnal pattern of activity, whereas the same species shows a bimodal crepuscular pattern in tilt cages. In addition there are marked differences between the sexes in the distribution and total amount of running wheel activity throughout 24 h, and in the day-to-day consistency of this activity (Roper, 1976).

The amount and distribution throughout the day of running wheel activity in the vole Microtus agrestis is significantly affected by cage size and layout (Lehmann, 1976). Since running wheel activity can be elicited in this species by disturbances or continuous exposure to stressful conditions (very small cage size), Lehmann proposes that it may in fact be a quite unspecific response to a certain level of excitation, which is expressed in totally different types of behaviour in nature.
Kenagy (1976) compares the patterns of running wheel activity in natural light and temperature cycles with field activity of male kangaroo rats *Dipodomys merriami* throughout the year. Running wheel activity in these animals is under circadian clock control (Kenagy, 1978) and always begins at light intensities 2 to 3 orders of magnitude lower than corresponding values for surface activity in the field. Light intensity would appear to be a major factor controlling activity onset and end in *D. merriami* which live solitarily in subterranean burrows and whose food supply does not vary in its availability during the course of the day.

With suitable cage configurations, it is possible to obtain very long running wheel activity records from rodents (e.g. Daan and Pittendrigh 1976 a, b; Pittendrigh and Daan, 1976 a,b, c) and such records may provide much information leading to empirical generalizations on the behaviour of the underlying circadian clock mechanism. The relevance of running wheel activity to natural behaviour patterns is, however, questionable and interpretations of the "functional significance" of such rhythms must be viewed with reservation.

Taking these considerations into account, in this study activity of *R. exulans* was detected by interruption of either a deep red or an infra-red beam bisecting the cage at a height of 3 cm and not visible to the animal (Figs II.1 and II.2). While the problems of social isolation and artificial environment were not overcome in this apparatus, some of the disadvantages of running wheels were avoided. In particular, the food channel in the cage lid was always aligned so that it lay above the activity detecting beam. Feeding activity was thus detected as well as general movement around the cage. The nozzle of the water bottle was directed away from the "nest box" so that the animal had to pass through the beam when leaving the nest box to drink. Although activity in the nest box itself, or restricted movements either side of the beam were not detected, most gross motor activity, whatever its motivation, was probably registered.

The deep red light apparatus consisted of a focused beam from a tungsten filament bulb passed through a Kodak Wrattan 70 filter (transmitting wavelengths greater than 650 nm) and detected by a light-dependent resistor. The IR system comprised a focused beam from an Optron Inc. OPL33 IR emitting diode (with a narrow band spectral emission centred on 940 nm) detected by a spectral matched N-P-N silicon
phototransistor (OP805, see Appendix II). In both systems, changes in state of the detector opened or closed a relay which in turn triggered an event recorder pen (Esterline Angus A620X 20 channel recorder, chart speed 30 cm per day; Goetz Miniscript Z 10 channel recorder, chart speed 24 cm per day; or Edgecumbe Peebles (Glasgow) 12 channel recorder, chart speed 12 ins per day).
3.3 RESULTS

3.3.1 THE FREE RUNNING RHYTHM

When monitoring an overt circadian rhythm as an indicator of the behaviour of its underlying timing mechanism, it is crucial to distinguish which observed responses of the overt rhythm reflect unambiguously the behaviour of the pacemaker. Some observed behaviour may be attributable to effects of experimental manipulations on the physiological systems linking pacemaker and rhythm, or to direct effects on the overt rhythmic process. The free-running period ($\tau$) of an overt circadian rhythm is the characteristic assumed to reflect most directly the behaviour of the underlying pacemaker mechanism. Investigations of the sources of spontaneous and experimentally induced variability in $\tau$ provide information on pacemaker properties which enables discrimination between possible models of circadian time-keeping systems.

3.3.1.1 INTERINDIVIDUAL VARIATION IN PERIOD

A typical free-running locomotor activity rhythm of a female $R$. exulans is illustrated in Fig. 3.1. The active phase characteristic-ally began fairly abruptly with comparatively intense bursts of activity. The intensity and duration of activity bouts tended to decline towards the end of the active phase, which was less clearly defined than activity onset. Activity onset has therefore been adopted as the phase reference point for the measurement of free-running periods and of phase changes following perturbations. In the most common alternative pattern, albeit observed infrequently, the intensity of activity tended to remain fairly constant throughout the active phase, but activity onsets still gave a less variable estimate of period than activity ends. In one animal (Rat 52, Fig. 3.2) in extreme old age, the reverse of the typical pattern was observed, i.e. the most intense and prolonged bout of activity occurred at the end of the active phase and the period as measured from end to end of activity was less variable than that measured from onset to onset. Since the end of the active phase was often indistinct and generally very variable, active phase lengths were not measured in these studies.
FIGURE 3.1

Typical Activity Record of a Female *Rattus exulans*

Section A: LL dim (0.02 to 0.07 lux)
Section B: LD 16:8 (Lights on from 0500 h to 2100 h)
Section C: LL dim (0.02 to 0.07 lux)

In the free-running rhythm, the most intense and consistent bout of activity occurs at the onset of the main active phase. A second component at 180° antiphase to the main active phase becomes evident after prolonged exposure to constant conditions. This record also illustrates the characteristic pattern of activity in LD 16:8. Activity begins to build up prior to the onset of darkness and finishes before the next light pulse. Times of activity onset are less variable than times of cessation of activity.
Part of an activity record in constant conditions (0.02 to 0.07 lux, 21 \pm 1^\circ C). Shaded areas indicate exposures to bright light (520 lux). This animal exhibits the reverse of the typical free-running pattern, with the most intense and consistent bout of activity occurring at the end of the active phase.
All animals exhibited brief bouts of activity throughout the "inactive" phase, the total amount varying considerably between individuals and tending to increase with age. In 12 of 32 females and 1 of 7 males, a distinct second activity component was sometimes discernible in the middle of the inactive phase (e.g. Fig. 3.1). This second component maintained the same overall period as the main active phase, but showed much greater day-to-day period variability. It was generally observed only after prolonged exposure to constant conditions. In free-runs following entrainment, the total amount of activity in the inactive phase tended to be reduced and a regular second activity component was not evident for many cycles. Because of the requisite of prolonged exposure to constant conditions for expression of the component, the potential frequency of its occurrence is probably underestimated in the ratios quoted above, since not all animals received such treatment in the experimental programme.

The distribution of free-running periods of the locomotor activity rhythms of all R. exulans recorded in this study is presented in histogram form in Fig. 3.3. Each \( \bar{T} \) value is the mean of 10 consecutive periods measured from activity onsets. Period values measured when a rhythm was obviously undergoing transients or after-effects are not included. The 118 estimates of \( \bar{T} \) from the activity records of 31 female R. exulans give a species mean period (\( \bar{T} \)) for females of 24.5 ± 0.32 (standard deviation) h. The shaded area represents the distribution of 16 \( \bar{T} \) estimates from the activity records of 6 males, the resulting \( \bar{T} \) for males being 23.7 ± 0.11 h. The mean period for males is significantly shorter (Pt < 0.001) than that for females.

3.3.1.2 INTRAINDIVIDUAL VARIATION IN PERIOD

I Endogenous Sources of Period Variability

a) Day-to-Day Period Instability.

The standard deviations (S.D.) of each of the 10 day \( \bar{T} \) estimates
FIGURE 3.3

Distribution of Observed Free-Running Periods

Shaded area indicates $\bar{T}$ values of males
- $\bar{T}$ for females $= 24.5 \pm 0.32$ h
- $\bar{T}$ for males $= 23.7 \pm 0.11$ h
  - $t_i = 10.464$
  - $P_t = < 0.001$

FIGURE 3.4

The Relationship Between $\bar{T}$ and S.D. $\bar{T}$

0 denote values for males
X denote values for females
- $\bar{T} \geq 24$ h $r_s = 0.179$
  - $0.1 > P > 0.05$
  - $n = 111$
- $\bar{T} \leq 24$ h $r_s = 0.213$
  - $0.9 > P > 0.5$
  - $n = 26$

(See Table III.1)
are plotted as a function of their respective periods in Fig. 3.4. It seems probable that day-to-day variability in the chain of physiological events initiated by the pacemaker and ultimately regulating the timing of locomotor activity, contributes to the overall variability of the period of the overt rhythm. Thus the standard deviation of the period of the overt rhythm is likely to be an overestimate of the day-to-day instability of period of the pacemaker (Pittendrigh and Daan, 1976a). Pittendrigh and Daan propose that high day-to-day precision is a functional requisite for circadian rhythms with periods close to 24 h but is less critical for rhythms with periods further from that of the natural zeitgeber. If this hypothesis holds, then there should be a significant correlation between \( \bar{T} \) and S.D. \( \bar{T} \) which is positive for rhythms with \( \bar{T} \geq 24 \text{ h} \) and negative for rhythms with periods \( \leq 24 \text{ h} \). The 111 period estimates \( \geq 24 \text{ h} \) do not show a significant positive correlation \((r_s = 0.179, 0.1 > P_{ts} > 0.05)\) between \( \bar{T} \) and S.D. \( \bar{T} \), nor do the 26 period estimates \( \leq 24 \text{ h} \) show a significant negative correlation \((r_s = 0.213, 0.9 > P_{ts} > 0.5)\). Five females, for which 12 or more period estimates were obtained, and whose \( \bar{T} \) values were always \( > 24 \text{ h} \), also fail to show the proposed relationship (Table 3.1).

**TABLE 3.1**

Intra-Individual Relationships Between \( \bar{T} \) and S.D. \( \bar{T} \)

<table>
<thead>
<tr>
<th>Rat Number</th>
<th>n</th>
<th>( r_s )</th>
<th>( t_s )</th>
<th>( P_{ts} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td>12</td>
<td>-0.226</td>
<td>-0.732</td>
<td>0.5 &gt; P &gt; 0.4</td>
</tr>
<tr>
<td>23</td>
<td>16</td>
<td>-0.059</td>
<td>-0.220</td>
<td>0.9 &gt; P &gt; 0.5</td>
</tr>
<tr>
<td>25</td>
<td>16</td>
<td>0.334</td>
<td>1.325</td>
<td>0.4 &gt; P &gt; 0.2</td>
</tr>
<tr>
<td>31</td>
<td>14</td>
<td>0.431</td>
<td>1.654</td>
<td>0.2 &gt; P &gt; 0.1</td>
</tr>
<tr>
<td>52</td>
<td>15</td>
<td>0.254</td>
<td>0.945</td>
<td>0.4 &gt; P &gt; 0.2</td>
</tr>
</tbody>
</table>

\( n \) is the number of estimates of \( \bar{T} \) and S.D. \( \bar{T} \)

\( r_s \) is the Spearman’s rank correlation coefficient.

\[ t_s = r_s \sqrt{\frac{n - 2}{1 - r_s^2}} \] (n - 2 degrees of freedom)
b) The Effects of Aging on the Period and Stability of Free-Running Rhythms.

The effects of aging on the period and day-to-day stability of the free-running activity rhythms of seven female *R. exulans* monitored for in excess of 200 days are summarized in Table 3.2. The average life expectancy of *R. exulans* on Tiritiri is about one year. Only animals born during the 3-4 month breeding season beginning in November, would be expected to survive the population collapse the following autumn and winter and breed the next year (Moller, 1977). The weights at capture of Rats 23, 25, 31 and 52 suggest that they were all born in the 1977-78 breeding season. Rats 18 and 30 could possibly have been born the season before, although their subsequent longevity makes this unlikely. Rat 79 was probably born in the 1978-79 breeding season. At the end of its record in Table 3.2 the latter animal would have been approaching the end of its expected life span. The remaining animals exceeded their expected life span, the oldest surviving in excess of 21 months in captivity. Except for the intervals of entrainment indicated in Table 3.2 a), the animals were in constant conditions interrupted by single light pulses at 20-30 day intervals. For each rat $\bar{C}$ was measured at approximately 50 day intervals, over 10 consecutive cycles when no transients or after-effects of light pulses or prior entrainment were evident.

No animal showed significant changes with age in either free-running period or in its day-to-day variability (Tables 3.2 b and c). As mentioned previously, there was a tendency to increased activity during the "inactive" phase with increasing age i.e. the amplitude of the free-running rhythm tended to decrease. The age of experimental animals is therefore not a significant source of inter-and intra-individual variability in free-running locomotor activity rhythms of female *R. exulans*, in contrast to several other rodent species (Pitman-Drigh and Daan, 1974).

II Induced Period Variability

a) Effects of Constant Bright Light.

The activity records of two female rats before, during and after exposure to 100 h of bright (520 lux) light are shown in Figs. 3.2 and
<table>
<thead>
<tr>
<th>RAT NUMBER</th>
<th>WEIGHT AT CAPTURE</th>
<th>DAYS</th>
<th>DAYS</th>
<th>DAYS</th>
<th>DAYS</th>
<th>DAYS</th>
<th>DAYS</th>
<th>DAYS</th>
<th>DAYS</th>
<th>DAYS</th>
<th>DAYS</th>
<th>DAYS</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td>80g+</td>
<td>45-55</td>
<td>93-103</td>
<td>104-146</td>
<td>190-200</td>
<td>236-246</td>
<td>303-313</td>
<td>341-351</td>
<td>400-410</td>
<td>Dead</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1/6/78</td>
<td>24.5 0.52</td>
<td>24.8 0.56</td>
<td>LD 16:8</td>
<td>24.5 0.39</td>
<td>24.6 0.43</td>
<td>24.3 0.69</td>
<td>24.6 0.48</td>
<td>24.3 1.07</td>
<td>440</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>69g</td>
<td>15-25</td>
<td>49-275</td>
<td>295-305</td>
<td>341-351</td>
<td>395-405</td>
<td>438-448</td>
<td>495-505</td>
<td>549-559</td>
<td>600-610</td>
<td>648-658</td>
<td></td>
</tr>
<tr>
<td>1/6/78</td>
<td>24.2 0.69</td>
<td>LD 8:16</td>
<td></td>
<td>24.2 0.51</td>
<td>24.4 0.45</td>
<td>24.5 0.53</td>
<td>24.6 0.48</td>
<td>24.6 0.42</td>
<td>24.5 0.34</td>
<td>24.4 0.52</td>
<td>24.3 0.46</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>62g</td>
<td>17-27</td>
<td>49-275</td>
<td>309-319</td>
<td>341-351</td>
<td>396-406</td>
<td>438-448</td>
<td>495-505</td>
<td>549-559</td>
<td>600-610</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1/6/78</td>
<td>24.7 0.36</td>
<td>LD 8:16</td>
<td></td>
<td>24.4 0.52</td>
<td>24.7 0.48</td>
<td>24.6 0.46</td>
<td>24.6 0.36</td>
<td>24.6 0.31</td>
<td>24.7 0.20</td>
<td>24.6 0.42</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>80g+</td>
<td>1-104</td>
<td>145-155</td>
<td>194-204</td>
<td>236-246</td>
<td>Dead</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1/6/78</td>
<td>LD 16:8</td>
<td>24.1 0.55</td>
<td>24.4 0.81</td>
<td>24.4 0.57</td>
<td></td>
<td>260</td>
<td></td>
<td></td>
<td></td>
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<td>31</td>
<td>56g</td>
<td>1-104</td>
<td>145-155</td>
<td>197-207</td>
<td>251-261</td>
<td>297-307</td>
<td>341-351</td>
<td>396-406</td>
<td>440-450</td>
<td>495-505</td>
<td>532-542</td>
<td></td>
</tr>
<tr>
<td>1/6/78</td>
<td>LD 16:8</td>
<td>24.3 0.24</td>
<td>24.5 0.46</td>
<td>24.8 0.20</td>
<td>24.8 0.17</td>
<td>24.8 0.20</td>
<td>24.9 0.38</td>
<td>24.7 0.18</td>
<td>24.9 0.44</td>
<td>24.7 0.52</td>
<td></td>
<td></td>
</tr>
<tr>
<td>52</td>
<td>74g</td>
<td>31-41</td>
<td>43-161</td>
<td>195-205</td>
<td>245-255</td>
<td>295-305</td>
<td>335-345</td>
<td>395-405</td>
<td>445-455</td>
<td>497-507</td>
<td>551-561</td>
<td></td>
</tr>
<tr>
<td>12/9/78</td>
<td>24.6 0.70</td>
<td>LD 16:8</td>
<td>24.7 0.26</td>
<td>24.7 0.32</td>
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Prior to day 1 all animals were in LD natural.
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<td>P&gt;0.1</td>
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* For RAT 30, the comparison is $t_{3,4}$, $t_{3,5}$, etc.
  and $F_{3,4}$, $F_{3,5}$, etc.

# For RAT 31, the comparison is $t_{4,5}$, $t_{4,6}$, etc.
  and $F_{3,4}$, $F_{3,5}$, etc.

(because between days 145 and 155 the rhythm is still undergoing after-effects of the previous LD 16:8 entrainment.)
3.5. Two of three animals became arrhythmic under these conditions, while in the third (illustrated in Fig. 3.5) the free-running period apparently lengthened during the LL exposure. On return to DD, two animals showed several abnormally long cycles and then $\overline{T}$ gradually decreased to a period shorter than the pre-treatment value. In Fig. 3.5, $\overline{T}$ was still shorter than the pre-treatment value at the end of the record 31 to 41 days after lights off ($0.1 > PT > 0.05$).

A further animal exposed to bright light for over 22 days continued to free-run with a period of $25.5 \pm 1.34 \text{ h}$, which was significantly longer ($0.02 > PT > 0.01$) than that of the prior free-run in DD ($\overline{T} = 24.3 \pm 0.43 \text{ h}$). During LL the active phase tended to be more diffuse than that in constant darkness.

b) After-effects of Single Light Pulses.

Recognition of the phenomenon of after-effects, first noted by Pittendrigh (1960), has introduced the concept of the history-dependence of the free-running period of circadian rhythms. The lighting regime to which an organism has previously been exposed may produce long-term effects on its subsequent free-running rhythm.

The after-effects produced by single 8 h and 16 h light (520 lux) pulses on the subsequent period of $R. \text{ exulans}$ activity rhythms are summarized in Tables 3.3 and 3.4. (The phase response curves derived from the same data are presented in Section 3.3.2). Following the convention of Pittendrigh and Daan (1976 a), only phase shifts greater than $10^\circ$ of arc i.e. 40 min are included. The mean period of the 10 cycles immediately before each pulse is compared with the mean of the subsequent 10 cycles. Twenty to 30 days of constant conditions were allowed between consecutive 8 h pulses, while an interval of at least 27 days was allowed between 16 h pulses. By the end of this time $\overline{T}$ had generally returned to its value prior to the previous pulse.

Following 8 h light pulses that produce phase advances, $\overline{T}$ shortens in 6 out of 8 cases. The average period after such a perturbation is significantly shorter ($0.05 > PT > 0.02$) than the average period before. There is no significant change in the average period before and after 8 h light pulses which produce phase delays. Although in all 3 cases $\overline{T}$ apparently shortens following 16 h pulses producing phase advances, there is not a significant change in the average period before and after such pulses. Following 16 h pulses that produce phase delays,
FIGURE 3.5

Effects of Constant Light (520 lux)
on a Free-Running Rhythm

Part of the activity record of a rat in constant conditions (0.02 to 0.07 lux, 21 ± 1° C). Shaded area indicates a 100 h exposure to bright light (520 lux) which results in subsequent shortening of the free-running period.

\[ \tau \text{ before the light exposure} = 24.6 \text{ h} \]
\[ \tau \text{ over the last 10 cycles illustrated} = 24.4 \text{ h} \]
\[ t_i = 2.020 \quad 0.1 > p > 0.05 \]
TABLE 3.3

After-Effects of 8 h Light Pulses

<table>
<thead>
<tr>
<th>RAT NUMBER</th>
<th>CT PULSE</th>
<th>$\Delta \phi$</th>
<th>$\bar{\tau}$ before</th>
<th>$\bar{\tau}$ after</th>
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<td>1.5</td>
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<td>24.3</td>
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<td>1.3</td>
<td>24.2</td>
<td>24.3</td>
<td>0.1</td>
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<tr>
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<td>3.4</td>
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$t_i (\bar{\tau}_{after} : \bar{\tau}_{before}) = 2.468$

$0.05 > P_t > 0.02$

$\Delta \phi$ vs $\Delta \bar{\tau}$

$r_s = -0.780$

$P < 0.05$

<table>
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<tr>
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<th>$\Delta \phi$</th>
<th>$\bar{\tau}$ before</th>
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$t_i (\bar{\tau}_{after} : \bar{\tau}_{before}) = 0.154$

$0.9 > P_t > 0.5$

$\Delta \phi$ vs $\Delta \bar{\tau}$

$r_s = 0.679$

$P > 0.05$

Probabilities from Table of Critical Values of $r_s$.
The Spearman Rank Correlation Coefficient, Siegel (1956).
TABLE 3.4

After-Effects of 16 h Light Pulses

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$t_i (\bar{\tau}_{after} : \bar{\tau}_{before}) = 0.522$

$0.9 > \text{Pt} > 0.5$

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TABLE 3.4 cont.

<table>
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<tr>
<th>RAT NUMBER</th>
<th>CT PULSE</th>
<th>$\Delta \phi$</th>
<th>$\bar{\tau}$ before</th>
<th>$\bar{\tau}$ after</th>
<th>$\Delta \bar{\tau}$</th>
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<td>52</td>
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<td>24.7</td>
<td>25.2</td>
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<tr>
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<td>-8.8</td>
<td>24.5</td>
<td>25.0</td>
<td>0.5</td>
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<td>-5.6</td>
<td>24.6</td>
<td>25.1</td>
<td>0.5</td>
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<tr>
<td>79</td>
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<td>-1.4</td>
<td>24.7</td>
<td>25.0</td>
<td>0.3</td>
</tr>
</tbody>
</table>

\[ t_1 \left( \bar{\tau}_{\text{after}} : \bar{\tau}_{\text{before}} \right) = -6.464 \]
Pt < 0.001

$\Delta \phi$ vs $\Delta \bar{\tau}$

\[ r_s = 0.435 \]
\[ t_s = 2.602 \]
\[ 0.02 > P > 0.01 \]
lengthens in 29 out of 31 cases, and the average period after such perturbations is significantly longer \((Pt < 0.001)\) than that before.

The relationship between the circadian phase with which the light pulse coincides and the subsequent after-effect on \(\bar{T}\) that it produces is depicted in Fig. 3.6 for 8 h pulses and Fig. 3.7 for 16 h pulses. From the 8 h period response curve (Harker, 1964) it is evident that maximum period lengthening occurs in the region of maximum phase delays in the phase response curve (i.e. around CT 8) and maximum period shortening coincides with the region of maximum phase advances (i.e. shortly after CT 8). The magnitude of the period after-effect produced by an 8 h pulse is significantly correlated (Spearman's rank correlation) with the magnitude of the phase change it produces for phase advances \((P<0.05)\) but not for delays \((P>0.05)\).

In the 16 h period response curve, maximum period lengthening also occurs in the region of maximum phase delays (i.e. around CT 24) and maximum shortening occurs in the region of maximum phase advances (i.e. around CT 12). The magnitude of the period after-effect produced by a 16 h light pulse is significantly correlated with the magnitude of the phase change it produces for phase delays \((0.02 > Pt_s > 0.01)\). These regularities confirm that the period changes observed following single light pulses are not spontaneous and unpredictable, but are responses of the underlying pacemaker to the perturbation.

c) After-Effects of Entrainment.

There are four aspects of an entrainment regime which have been demonstrated to influence the subsequent free-running period of a circadian rhythm: a) the period \(T\) of the entraining zeitger; b) its L:D ratio; c) the duration (number of cycles) of exposure to it; d) whether entrainment of the rhythm is achieved through phase advances or phase delays (Pittendrigh and Daan, 1976 a; Aschoff, 1979; Christensen and Lewis, b, in preparation). Data from \textit{R. exulans} activity records confirm that the first three of these aspects are effective in producing after-effects on this rhythm.

The effect of the period \(T\) of the entraining cycle on the subsequent \(\bar{T}\) of rat activity rhythms is summarized in Fig. 3.8 for LD 8:16, 8:18 and 8:20 cycles. The minimum length of exposure to the light regime was 10 cycles (see Table III.2) Data for LD 8:18 and 8:20 are derived
FIGURE 3.6

Period Response Curve To 8 h Light Pulses

x denote after-effects of pulses that produced phase delays.

o denote after-effects of pulses that produced phase advances.

FIGURE 3.7

Period Response Curve To 16 h Light Pulses

x denote after-effects of pulses that produced phase delays.

o denote after-effects of pulses that produced phase advances.
FIGURE 3.8

After-Effects of Entrainment to LD 8:16, 8:18
and 8:20

$\bar{T} - T$: the free-running period before entrainment minus the period of the entraining light cycle.

$T_{\text{after}} - T_{\text{before}}$: the change in free-running period before and after entrainment.

Circles denote period changes that occurred following exposure to light cycles that failed to entrain the activity rhythm. (See Table III.2.)

FIGURE 3.9

After-Effects of Entrainment to LD 16:8

$\bar{T} - T$: the free-running period before entrainment minus the period of the entraining light cycle.

$T_{\text{after}} - T_{\text{before}}$: the change in free-running period before and after entrainment.

(See Table III.3.)
from the activity records of males, while data for LD 8:16 (and 16:8, see below) are from records of both males and females. Points denoted by circles are after-effects (on the activity rhythms of males) when entrainment failed at LD 8:20 and 8:22. From Fig. 3.8 it is evident that when $\bar{T}-T > 0.0$ h for entrainment to cycles of 8 h light pulses, $\bar{T}$ in the first 10 days after entrainment is shorter than for the 10 days immediately prior to entrainment. When $\bar{T}-T \leq -0.2$ h, the subsequent free-running period is unchanged or longer than that before entrainment.

The effect of the LD ratio on after-effects can be ascertained by comparing the after effects produced by entrainment to LD 8:16 ($n = 8$, $\bar{T}$ before range 23.5 h to 24.7 h) with those resulting from entrainment to LD 16:8 (Fig. 3.9, $n = 15$, $\bar{T}$ before range 23.6 h to 24.8 h). Entrainment to LD 16:8 produces a subsequent shortening of period in all cases and mean $\bar{T}$ after is significantly shorter than mean $\bar{T}$ before ($0.01 > P_t > 0.001$). Following entrainment to LD 8:16 however, $\bar{T}$ remains unchanged in two animals ( $\bar{T}$ before of 23.7 and 23.8 respectively) and lengthens in two animals ( $\bar{T}$ before of 23.5 and 23.7 respectively). In the one animal common to both experiments (Rat 54 C, male), $\bar{T}$ lengthens following exposure to 10 cycles of LD 8:16 but shortens following exposure to LD 16:8. The after-effects produced by entrainment show significant negative (Spearman's rank) correlation with $\bar{T}$ before for both LD 16:8 ($r_s = -0.713, P < 0.01$) and LD 8:16 ($r_s = -0.905, P < 0.01$). There is no a priori reason to suppose that entrainment to LD 16:8 does not produce period lengthening in animals where $\bar{T}$ is shorter than 23.6 h (i.e. $\bar{T}-T < -0.4$), however from the species distribution of $\bar{T}$ values (Fig. 3.3) it is evident that such $\bar{T}$ values are uncommon. Alternatively, $\bar{T}-T$ values $< -0.4$ could be achieved by entrainment to 16 h light pulses in cycles longer than 24 h, and it is possible that such entrainment would produce subsequent period lengthening. Over comparable $\bar{T}$ ranges (in 24 h cycles) however, entrainment to LD 8:16 produces after-effects that are different in magnitude and (when $\bar{T}$ before $\leq 23.8$ h) different in sign, to those produced by entrainment to LD 16:8.

In the experiments specifically designed to examine after-effects of 8 h pulses in 24 h, 26 h, 28 h and 30 h cycles (using male R. exulans) a minimum interval of 30 days was allowed between successive entraining schedules which were of 10 cycles duration. By the end of this time, $\bar{T}$ had always returned to its pre-entrainment value. Data on after-effects of LD 8:16 also include records of females who were exposed for much and 16:8.
longer periods to these light regimes in the course of other experiments and these after-effects were consistently of much longer duration (e.g. Section C of Fig. 3.1, where after 34 days $\tau$ has still not returned to its pre-entrainment value). Although a systematic survey of the effects of the duration of exposure to an entraining cycle on the subsequent duration of after-effects has not been undertaken, these data suggest a possible positive correlation between these two variables. Further investigation of this phenomenon would require a very long-term experimental programme, since inter-individual variability in after-effects would make it desirable to use the same individuals throughout, and prolonged intervals between entrainment regimes would be necessary for $\tau$ to return to its pre-entrainment value, particularly following long exposures to LD cycles. Nevertheless, due to their longevity in experimental conditions, R. exulans would be ammenable to such an experimental programme.

Included in Fig. 3.8 are after-effects of exposures to LD 8:20 and 8:22 when entrainment failed. The period changes observed are small and do not fit the overall pattern for after-effects resulting from entrainment to LD cycles containing 8 h light pulses.

The regularities evident in entrainment after-effects indicate that the period changes observed following entrainment represent long-term responses of the pacemaker to the prior treatment, not spontaneous pacemaker lability.
3.3.2 PHASE RESPONSE CURVES

The second aspect (in addition to the free-running period) of an overt circadian rhythm which can be assumed to reliably reflect the behaviour of its underlying pacemaker mechanism is its phase-dependent sensitivity to standard perturbations. If the free-running period of an overt rhythm is a reliable indicator of the underlying pacemaker, then a phase shift produced by a perturbation, as measured in the overt rhythm after it has regained a steady state, must reflect a phase change in the pacemaker. Information derived from phase response curves enables models for the mechanism of entrainment of a rhythm to be tested. The behaviour of a rhythm in response to pulses administered near the so-called "singularity" (Pavlidis, 1967 a) also aids in discrimination between various structural models for the underlying pacemaker (Pavlidis, 1978 a,b).

The phase response curves for the locomotor activity rhythms of female R. exulans to 4 h, 8 h, and 16 h light pulses are presented in Figs. 3.10, 3.11 and 3.12. Circadian time zero (CT 0) is defined as activity onset, which is used as the phase reference point for measuring phase shifts. The midpoint of a light pulse is taken as its phase reference point. Phase advances generally occur through more cycles of transients than phase delays. These phase response curves conform to the typical circadian pattern with phase delays occurring in response to light pulses falling late in the subjective day and early in the subjective night, and phase advances in response to pulses late in the subjective night and early in the subjective day (Daan and Pittendrigh, 1976 a).

In all three response curves the maximum delays are greater than the maximum advances, and delays occur over about 240° of the circadian cycle. To permit a quantitative comparison with the phase response curves for other rodents (Daan and Pittendrigh, 1976 a, Table 1) the data have been averaged in 2 h bins for each curve and the product of bin width (in circadian hours) and bin average (in degrees of arc) calculated. Summing these values for advances and for delays gives estimates for the areas under the respective parts of the curve.
FIGURE 3.10

Normalized Phase Response Curve to 4 h Light Pulses

(See Table III.4.)
FIGURE 3.11

Normalized Phase Response Curve to 8 hr Light Pulses

-o-o Phase shifts measured in two separate activity components observed in a rhythm following a light pulse at CT 8.2.
- A light pulse occurring at CT 9.0 that produced temporary arrhythmicity (Figure 3.13).
+++- Phase changes in the activity rhythm of Rat 31.
(See Table III.5)
Normalized Phase Response Curve to 16 h Light Pulses

- Rhythm dissociated into several components following the light pulse and a discrete phase shift could not be measured.

(See Table III.6.)
TABLE 3.5

Areas Under the Delay (D) and Advance (A) Sections of R. exulans Phase Response Curves.

<table>
<thead>
<tr>
<th>PULSE</th>
<th>D</th>
<th>A</th>
<th>D - A</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 h</td>
<td>11,534.4</td>
<td>4,363.2</td>
<td>+ 7,171.2</td>
</tr>
<tr>
<td>8 h</td>
<td>13,096.8</td>
<td>8,344.8</td>
<td>+ 4,752.0</td>
</tr>
<tr>
<td>16 h</td>
<td>35,985.6</td>
<td>6,372.0</td>
<td>+ 29,613.6</td>
</tr>
</tbody>
</table>

Daan and Pittendrigh (1976a) Table 1 summarizes data for the nocturnal rodents Mesocricetus auratus, Peromyscus leucopus, P. maniculatus, Mus musculus and the diurnal Ammorspermophilus leucurus, which indicate that species and individuals with the smallest periods show the largest delays (D - A positive), while those having the longest T show largest advance phase shifts (D - A negative). Female R. exulans do not conform to this trend, since T = 24.5 h but all three phase response curves have positive D - A values.

In all three curves the transition from advances to delays occurs at around CT 18. In the 8 h and 16 h curves in the region of transition from delays to advances, effects such as those illustrated in Figs. 3.13 and 3.14 are observed. Responses such as the shattering of rhythm in Fig. 3.13 following an 8 h pulse, or the splitting off of components following 8 h or 16 h pulses (e.g. Fig. 3.14) result in a region of discontinuity in the respective phase response curves which are therefore considered to be of the strong type (Winfree, 1970, 1971).

This interpretation is reinforced by the diagrams in Fig. 3.15 where the same phase shift data are plotted in terms of CT before and after the pulse. This procedure derives from the "corkscrew" topographical model for the phase-resetting surface of the circadian eclosion rhythm of populations of Drosophila pupae (Winfree, 1970, 1971). In this model the phase shift is measured in each of the transient cycles following the pulses. For the R. exulans data the steady-state CT after time is extrapolated back (at the prevailing T) to the cycle immediately after the pulse. Because these data are from the activity rhythms of individuals which show variable patterns of transients (and after-effects) after comparable pulses, plotting CT after for each transient cycle contributing to an overall phase shift does not produce
FIGURE 3.13

Temporary Arrhythmicity Following an 8 h Light Pulse at CT 9.0

Part of an activity record in constant conditions (0.02 to 0.07 lux, 21 ± 1°C). Shaded area indicates an 8 h light pulse (520 lux).
FIGURE 3.14

Dissociation of an Activity Rhythm into Several Components Following a Light Pulse at CT 8.2

Part of an activity record in constant conditions (0.02 to 0.07 lux, 21 ± 1°C). Shaded area indicates an 8 h light pulse.
FIGURE 3.15

CT before Versus CT after

4 h light pulses
\[ r_s = 0.960 \quad t_s = 18.767 \]
\[ P < 0.001 \]

8 h light pulses
\[ r_s = 0.288 \quad t_s = 0.651 \]
\[ 0.9 > P > 0.5 \]

16 h light pulses
\[ r_s = -0.288 \quad t_s = -1.672 \]
\[ 0.2 > P > 0.1 \]
any consistent picture. It is not equivalent to the procedure for the *Drosophila* eclosion rhythm for which all data are means of large samples (populations of pupae) and inter-batch variability is negligible. A phase response curve is defined as weak or Type 1 if the plot of CT before versus CT after has a slope of 1. Such a plot would be expected to show a strong positive correlation between CT before and CT after. Conversely a phase response curve is defined as strong or Type 0 if the plot of CT before versus CT after has a slope of 0, in which case a significant correlation would not be expected. Only the 4 h phase response curve for the *R. exulans* activity rhythm shows significant correlation between CT before and CT after \( (r_s = 0.960, P_{rs} < 0.001) \), i.e. it is Type 1 while the 8 h and 16 h curves are Type 0.

### 3.3.3 ENTRAINMENT

#### 3.3.3.1 INTRODUCTION

The major debate on the mechanisms of entrainment of circadian rhythms by light cycles centres on the relative importance of parametric and non-parametric actions of light on the pacemaker system in producing the necessary period change in each cycle to convert the pacemaker period \( (\tau) \) to that of the entraining cycle \( (T) \). The entrainment experiments undertaken in this study were designed to approach this question through examining the applicability to the *R. exulans* activity rhythm of the non-parametric entrainment model designed for the *Drosophila* eclosion rhythm (Pittendrigh 1965, 1966, 1974, 1976). It is therefore appropriate at this stage to outline briefly the possible effects of light on a circadian pacemaker system and to examine the specific assumptions involved in this non-parametric model.

In nearly all species tested (including *R. exulans*) \( \tau \) has been shown to be a function of light intensity. This effect of light on the angular velocity of circadian rhythms is, by definition, parametric. For parametric entrainment to occur, the effects of light on the angular velocity of the pacemaker must be phase-dependent. i.e. it should be possible to derive a velocity response curve for the overt rhythm (Aschoff, 1979). A dependence of \( \tau \) on light intensity does not necessarily imply the existence of a velocity response curve. Conversely, where \( \tau \) is independent of light intensity, a velocity response curve
is not necessarily precluded, since the phase-dependent effects of light on angular velocity would cancel each other out in LL if the area under the decelerating section of the response curve equaled that under the accelerating section.

The phase angle in entrainment ($\Psi$) of a rhythm is dependent on its free-running period. Therefore, whether or not parametric entrainment occurs, a dependence of $\mathcal{T}$ on light intensity will result in $\Psi$ being dependent on the mean light intensity of the entraining cycle. A dependence of $\Psi$ on mean light intensity has been observed in two chaffinches and two nocturnal rodents, but was found to be minimal or absent in two species of diurnal rodents (Pohl, 1976).

Short light pulses are the most widely tested perturbations known to reset the phase of circadian rhythms. The dependence of this resetting effect on the phase of the rhythm with which the light pulses coincide is described in phase response curves. Non-parametric entrainment occurs as a consequence of this phase-dependent phase-resetting effect of rapid transitions in light intensity. The specific model of non-parametric entrainment, developed for the Drosophila eclosion rhythm, is also based on the assumption that short light pulses instantaneously reset the phase of the underlying pacemaker. Observed transients in the overt rhythm are attributed to the rhythm regaining its steady-state phase relationship with the reset pacemaker.

It follows from this assumption that the phase response curve for single light pulses of a certain intensity and duration also describes the behaviour of the system for a combination of such pulses. If these assumptions are valid, then it should be possible to predict from the phase response curve, the phase at which a light pulse must fall to produce the necessary $\Delta \phi = \mathcal{T} - T$ in each cycle, for entrainment to occur. It should, therefore, also be possible to predict the phase relationship $\Psi$ between an entrained rhythm and the entraining cycle for any known combination of $T$ and $\mathcal{T}$.

3.3.3.2 GENERAL FEATURES OF ENTRAINED RHYTHMS

The activity record in Fig. 3.1 shows the typical pattern of entrainment of an R. exulans activity rhythm to LD 16:8. As is expected for a nocturnal animal, the bulk of activity is confined to the dark phase. The short bursts of activity preceding the intensive activity
bouts associated with lights off are a common feature of such records. The phase angle in entrainment \( \Psi \) is measured from the onset of the intensive activity to lights off since this is the most consistent and readily identifiable phase point in the entrained rhythm. (The selection of this phase reference point is vindicated in the predictions of \( \Psi \) from the non-parametric entrainment model - see below). The intensity of activity tends to decline towards the end of the active phase which generally ceases before lights off.

A typical activity pattern in LD 8:16 is illustrated in Fig. 3.16. Activity onset always lags lights off, thus the intensive activity associated with this transition in LD 16:8 is not evident in LD 8:16. Again, \( \Psi \) is measured from activity onset to lights off. Both activity onset and end tend to be more variable in rhythms entrained to LD 8:16 than in those entrained to LD 16:8. During entrainment there is generally less diffuse activity during the inactive phase than is observed in free-running rhythms and consequently the amplitude of the rhythm tends to be greater under entrainment.

3.3.3.3 THE DEPENDENCE OF \( \Psi \) ON T AND \( \tau \)

In the entrainment of self-sustained oscillators, a qualitative relationship is observed between the phase angle \( \Psi \) and the periods \( T \) of the driving oscillation and \( \tau \) of the driven oscillation, such that the driven rhythm phase leads the driver more or phase lags the driver less, the shorter \( \tau \) and the longer \( T \) i.e. the smaller \( \tau - T \). (Aschoff and Pohl, 1978). The relationships between \( \Psi \) and \( \tau - T \) for \textit{R. exulans} activity rhythms entrained by 8 h of light per cycle (T range 24 h to 28 h) or by LD 16:8 are depicted in Figs. 3.17 and 3.18. Negative values indicate that activity begins after lights off i.e. that the rhythm lags the light cycle. From Fig. 3.17 it is evident that, in 8 h light pulse entrainment, as \( \tau - T \) decreases the rhythm does lag the driver less, although the relationship is not linear. A much narrower range of \( \tau - T \) values is covered in Fig. 3.18 but there would appear to be a similar overall relationship with \( \Psi \) in LD 16:8 entrainment. There is considerable interindividual variability in \( \Psi \) e.g. for \( \bar{\tau} = 23.7 \) h there is a range of \( \Psi \) values from +1.5 h to -0.4 h. This represents a variation of 1.9 circadian h in the CT times of pulses producing the same phase change. (The corresponding region on the 16 h PRC has a comparatively shallow slope - see Section 3.3.3.5.)
Section A: LD 8:16 (lights on from 0900 h to 1700 h).

vs - intervals during which daily vaginal smears were taken.

nvs - intervals during which animals remained undisturbed
(Chapter Four).

Section B: LL dim (0.02 to 0.07 lux).

Typical pattern of entrainment to LD 8:16. Activity is confined to
the dark phase and the times of activity onset are less variable
than the times of cessation of activity.
FIGURE 3.17

The Relationship Between $\psi$ and $\bar{T} - T$ in LD 8:16, 8:18 and 8:20 Entrainment.

$\bar{T} - T$: the free-running period before entrainment minus the period of the entraining light cycle.

$\psi$ measured from activity onset to lights off.

o denote values for males.

x denote values for females.

(See Table III.7.)

FIGURE 3.18

The Relationship Between $\psi$ and $\bar{T} - T$ in LD 16:8 Entrainment.

$\bar{T} - T$: the free-running period before entrainment minus the period of the entraining light cycle.

$\psi$ measured from activity onset to lights off.

o denote values for males.

x denote values for females.

(See Table III.8.)
3.3.3.4 THE RANGE OF ENTRAINMENT

All animals exposed to LD 16:8 or 8:16 attained steady-state entrainment in these regimes (Appendix III, Tables III. 7 and III. 8). Similarly 4 out of 4 rats had achieved an entrained steady-state by the end of 10 cycles of LD 8:18. Exposure to LD 8:20 for 10 or 15 cycles resulted in entrainment in 3 out of 6 cases, while all 3 animals tested failed to become entrained by the end of 10 cycles of LD 8:22. Longer exposures to these cycles may have eventually produced entrainment, although in every case the pulse did scan through the entire circadian cycle, albeit rapidly, in the course of the light treatment. Relative coordination (Enright, 1965) was observed in activity rhythms that failed to entrain (Figs 3.19 and 3.20). The shortest light pulse tested was 10 min per 24 h. The activity rhythms of the 3 female R. exulans exposed to this cycle were all entrained by it.

3.3.3.5 TESTING THE PREDICTIONS OF THE NON-PARAMETRIC ENTRAINMENT MODEL

The overlays to Figs. 3.21 and 3.22 show the phase changes ($\Delta \varphi = \tau - T$) produced by 8 h of light per cycle, or LD 16:8 cycles, plotted as a function of the circadian time of the midpoint of the light pulse in steady-state entrainment. (The midpoint of a pulse was taken as its phase reference point in the phase response curves — Section 3.3.2). Although there are insufficient data to allow quantitative estimation of goodness of fit, there are no conspicuous departures from the predictions of the non-parametric entrainment model. The phase changes produced by light pulses in entraining cycles are not detectably different from those produced by single pulses of the same intensity and duration. This is consistent with the assumption of the model that phase shifting in the pacemaker is effectively instantaneous, but it does not necessarily imply a primarily non-parametric action of light in entrainment.
FIGURE 3.19

Relative Coordination in LD 8:20

Part of an activity record of a male rat in constant conditions (DD, 20 ± 1°C). Open boxes indicate 8 h light pulses.

\[ \tau_{\text{before}} = 23.8 \text{ h} \]
\[ \tau_{\text{after}} = 23.6 \text{ h} \]
FIGURE 3.20

Relative Coordination in LD 8:22

Part of an activity record of a male rat in constant conditions (DD, 20 ± 1°C). Open boxes indicate 8 h light pulses.

\[ \bar{T}_{\text{before}} = 23.6 \text{ h} \]
\[ \bar{T}_{\text{after}} = 23.8 \text{ h} \]
FIGURE 3.21

Phase Changes Produced by 8 h Light Pulses
In Entrainment Compared With the 8 h Phase
Response Curve

Overlay: phase changes produced in LD 8:16, 8:18 and 8:20
entrainment.
o denote values for males.
x denote values for females.
Figure: phase response curve to 8 h light pulses
(See Figure 3.11).

FIGURE 3.22

Phase Changes in LD 16:8 Entrainment
Compared with the 16 h Phase Response
Curve.

Overlay: phase changes produced in LD 16:8 entrainment.
o denote values for males.
x denote values for females.
Figure: phase response curve to 16 h light pulses
(See Figure 3.12).
3.4 DISCUSSION

Dissociation between two or more circadian rhythms monitored simultaneously in an individual has been reported in rodents (Zucker and Stephan, 1973; Wolterink et al., 1977; Finkelstein et al., 1978), primates (Moore-Ede et al., 1976, 1977, 1978, 1979; Sulzman et al., 1977, 1978) and humans (Wever, 1975; Aschoff and Wever, 1976). This phenomenon indicates that there are a multiplicity of circadian pacemakers in individual mammals (see Chapter One). There is also considerable evidence that each circadian pacemaker in multicellular organisms consists of a population of interacting oscillating units (Vanden Driesche, 1973; Pavlidis, 1978b).

Under natural environmental conditions these unit oscillators are presumably forced into close synchrony by entrainment to the 24 h zeitgeber cycle, and effectively behave as a single oscillator (Winfrey, 1975). Single oscillator models of circadian pacemakers can thus accommodate adequately many of the functionally important aspects of the behaviour of overt circadian rhythms, particularly phase-dependent phase shifting and entrainment (e.g. Pittendrigh, 1965, 1974, 1976; Wever, 1965, 1972; Pavlidis, 1967a, b, 1968; Johnsson and Karlsson, 1972; Karlsson and Johnsson, 1972; Gander and Lewis, 1979).

In the absence of external synchronizing stimuli, the multi-oscillatory properties of the pacemaker population eventually become more apparent. Much information about the structure of the population is thus elicited from studies of the sources of free-running period lability (Eskin, 1971; Hoffmann, 1971; Pittendrigh, 1974; Pittendrigh and Daan, 1976a; Christensen and Lewis a, in prep.). This rationale determined the experimental approach taken in this study, which included particular emphasis on the properties of the free-running locomotor activity rhythm, as well as the examination of its functionally important characteristics such as the mechanism of entrainment. R. exulans activity rhythms exhibit a range of behaviours which are most consistent with the properties of a pacemaker consisting of a population of weakly interacting circadian oscillators (Pavlidis 1978b). These are considered in detail below.
3.4.1 ACTIVITY PATTERNS IN RATTUS EXULANS

Before discussing the properties of the circadian time-keeping system revealed by the R. exulans aktograms, it is pertinent to clarify what types of activity actually contribute to the observed patterns. Typically, in both free-running and entrained rhythms, activity occurs in a series of bouts which become shorter and less intense towards the end of the active phase. Peak activity occurs around activity onset which, in entrainment, is associated with the onset of the dark phase. Particularly in free-running rhythms, brief intermittent bouts of activity commonly occur throughout the inactive phase. The amount of this activity varies considerably between individuals and tends to increase with age, but to decrease during entrainment. This pattern of activity distribution is very similar to that reported for wild and first generation laboratory Rattus rattus recorded in LD 12:12 in residential plus-mazes, and for first generation laboratory animals in running wheels (Barnett et al., 1975). Rats in the plus-mazes made irregular brief excursions out of the dark central nest box into each of the four arms of the maze during the light phase of the LD cycle, but peak activity occurred 2 to 3 h after the beginning of the dark phase. Two of the arms contained food and these were visited throughout the LD cycle. Barnett et al. (1975) also report unpublished observations on other members of the genus. Laboratory Rattus norvegicus and wild R. fuscipes and R. villosissimus in the same mazes exhibit a bimodal nocturnal activity pattern. In contrast, the activity of laboratory R. norvegicus in pressure sensitive cages does not appear to be bimodal (Honma et al., 1978).

It is not possible to distinguish from the R. exulans activity records which types of activity (feeding, drinking, grooming, general locomotor activity) were being performed, or if different types predominated during different parts of the circadian cycle. Both R. rattus (Barnett et al., 1975) and R. norvegicus (Richter, 1965; Levitsky, 1970; Terman and Terman, 1975) feed throughout 24 h in artificial light cycles in the laboratory, although peak feeding occurs during the dark phase. Food and water consumption are apparently controlled by a common circadian mechanism which also regulates a rhythm in brain self-stimulation in laboratory R. norvegicus (Boulos and Terman, 1979). Terman and Terman (1975) report informal observations that bouts of grooming and sleeping also occur throughout 24 h. The
brief bouts of "diurnal" activity in *R. exulans* may well represent feeding and/or drinking excursions from the nest box, in which the animals always slept. The limited amount of grooming activity observed during feeding and rehousing always occurred just outside the nest box or in the corners of the cage. If this observation is indicative of the general pattern of grooming, then this activity would probably have remained largely undetected. It has not yet been established which of these variously motivated types of gross locomotor activity are under circadian control in *R. exulans* or what possible interactions there might be between their controlling mechanisms (see Chapter One).

3.4.2 THE FREE-RUNNING RHYTHM

The mean free-running period of the locomotor activity rhythms of male *R. exulans* (23.7 h) is significantly shorter (*P* < 0.001) than the mean value for females (24.5 h). Several factors may have contributed to this difference. The value for males is derived from 16 *T* estimates from the activity rhythms of only 6 individuals, 5 of which were first generation laboratory animals, whereas the value for females comes from a much larger sample (31 rats, 1 of which was a first generation laboratory animal). Females were recorded in constant dim light of intensities ranging from 0.02 to 0.07 lux while males were recorded in constant darkness. Such a small intensity difference would appear however, to be insufficient to account for the observation that the mean period for females is 3.4% longer than that for males. Limited evidence indicates that only about 5% period lengthening occurs in female activity rhythms in response to much larger increases in light intensity (0.02 - 0.07 lux to 520 lux). Further data for wild males would be necessary to establish if there is a sexual dimorphism in free-running period, as has been previously reported in some birds (Aschoff, 1979). There is apparently also sexual dimorphism in the circadian system controlling locomotor activity in the golden hamster *Mesocricetus auratus* (Alvis et al., 1978). Males and females exhibit different phase angles in entrainment and different ranges of entrainment, both of which could be due to sexual dimorphism in free-running period.

In Table 3.6 the species mean period (\( \bar{T} \)) and mean standard deviation (S.D.) for female *R. exulans* is compared with these values
for running wheel activity rhythms of four other species of nocturnal rodents for which comparable information is available (from Pittendrigh and Daan, 1976 b, Table 2).

<table>
<thead>
<tr>
<th>SPECIES</th>
<th>$\bar{T}$</th>
<th>S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rattus exulans</td>
<td>24.51</td>
<td>0.47</td>
</tr>
<tr>
<td>Mesocricetus auratus</td>
<td>24.07</td>
<td>0.08</td>
</tr>
<tr>
<td>Peromyscus leucopus</td>
<td>24.01</td>
<td>0.09</td>
</tr>
<tr>
<td>Mus musculus</td>
<td>23.50</td>
<td>0.15</td>
</tr>
<tr>
<td>Peromyscus maniculatus</td>
<td>23.36</td>
<td>0.30</td>
</tr>
</tbody>
</table>

Table 3.6
Interspecific Comparison of $\bar{T}$ and S.D.

It can be seen that *R. exulans* conforms to the generalization proposed by Pittendrigh and Daan (1976a) that as the species mean period gets further away from 24 h, the standard deviation of $\bar{T}$ increases. This trend is considered to reflect a functional requirement for stabilization of the phase angle $\Psi$ between a circadian rhythm and the 24 h environmental zeitgeber cycle (Pittendrigh and Daan, 1976 a). Recognition of local time through the maintenance of a stable $\Psi$ is proposed to be one of the primary functions of circadian time-keeping systems (Pittendrigh and Daan, 1976 b). These authors demonstrated by simulation that $\Psi$ between a simple harmonic oscillator of period $\tau$ and a driving oscillation of period $T$ is most sensitive to fluctuations in $\Psi$ when $\tau/T$ is close to unity. This implies that for circadian pacemakers, selection pressure for the homeostasis of $\tau$ is greatest when $\tau$ is closest to 24 h. If this hypothesis is correct then the trend illustrated in Table 3.6 should extend to conspecific interindividual $\tau$ variability and to intraindividual $\tau$ variability. Individuals with free-running periods close to 24 h would be expected to show greater day-to-day stability of period than individuals whose rhythms have $\tau$ values further from 24 h. It is thus predicted that there should be a significant negative correlation between $\bar{T}$ and S.D.$\bar{T}$ for rhythms with $\bar{T} \leq 24$ h, and a significant positive correlation for rhythms with $\bar{T} > 24$ h. (Fig. 3.4). Neither correlation is significant at the 95% level for *R. exulans* activity rhythms ($\bar{T} \leq 24$ h, $0.9 > Pt > 0.5$; $\bar{T} > 24$ h, $0.1 > Pt > 0.05$). Similarly, no significant
correlation between \( \bar{T} \) and S.D. \( \bar{T} \) is found in activity records of individual *R. exulans* (Table 3.1). This finding is in contrast to observation of the expected relationship in the activity rhythms of man (Aschoff, Gurecke *et al.*, 1971), the rodents *Peromyscus maniculatus* and *Mus musculus* (Pittendrigh and Daan, 1976 a), chaffinches (Aschoff, Gurecke *et al.*, 1971) and the orthopteran *Hemideina thoracica* (Gander, 1976).

No significant changes in \( \bar{T} \) or in its day-to-day variability were evident in the locomotor activity rhythms of 7 rats monitored for durations equal to or greater than their expected natural life span (Table 3.2). Nor was there any indication of an endogenous annual variation in the free-running period. The only consistent change in free-running rhythms with age was a trend to decreasing amplitude. These results contrast with the systematic shortening observed with age in the period of running wheel activity in male *Mesocricetus auratus*, *Peromyscus maniculatus* and *P. leucopus* (Pittendrigh and Daan 1974, 1976 a). In these species the length of the active phase also tends to decline with age, probably due to the well-known general decline in running wheel activity with age. Male laboratory *Rattus norvegicus* also show a clear decline in the total amount of running wheel activity with age in both DD and LD 12:12, with a simultaneous tendency to decreasing amplitude of the entrained rhythm (Finger, 1979). These rats usually exhibit a decrease in free-running period with age, although there are individual exceptions. Figure 17a in Richter (1965) shows gradual period lengthening in the activity rhythm of a blinded wild *R. norvegicus* recorded for over 24 months. On the other hand no conspicuous age-related changes in \( \bar{T} \) are observed in the diurnal rodents *Ammospermophilus leucurus* (Kramm, 1976; Kenagy, 1978) and *Tamiasciurus hudsonicus* (Kramm, 1975 b) or in the nocturnal kangaroo rat *Dipodomys merriami* (Kenagy, 1978). Eskin (1971) concludes that there is no aging effect on \( \bar{T} \) in *Passer domesticus*.

The lack of variation in \( \bar{T} \) with age in female *R. exulans* provides indirect evidence that the activity rhythm is probably not sensitive to estrogen feedback (Chapter Five). As they get older, mature laboratory rats progress from regular to irregular estrous cycles, then to constant estrous or pseudopregnancies of irregular length and finally to anoestrous, probably due to age-related alterations in anterior hypothalamo-pituitary function (Huang *et al.*, 1978).
Limited observations suggest that in R. exulans death is preceded by several days of total arrhythmicity, which may be the end point of a progressive decline in amplitude of the activity rhythm. This is consistent with the suggestion (Pittendrigh and Daan, 1974) that decay in circadian organization accompanies, and may even be a possible cause of, the general physiological deterioration associated with aging and eventual death. If the circadian pacemaker controlling locomotor activity is envisaged as a single oscillator, then this observation can be explained in terms of gradual loss of pacemaker control of the overt rhythm. Alternatively, it may represent increasing desynchronization among a population of weakly interacting circadian oscillators controlling activity.

Spontaneous period changes (Eskin, 1971; Pittendrigh, 1974; Kramm, 1975a; Christensen and Lewis a, in prep.) are not a feature of R. exulans activity rhythms. A variety of types of induced period instability (after-effects) have been observed however, and these constitute the major evidence in support of a multioscillator model for the controlling circadian pacemaker mechanism.

Preliminary observations suggest that the free-running period of the R. exulans activity rhythm is longer in LL (520 lux) than in DD (0.02 to 0.07 lux), i.e. that it conforms to Aschoff's rule for a nocturnal organism (see Aschoff, 1979 for a review). The occurrence of prolonged period shortening following LL exposure (e.g. Fig. 3.5) stands in contrast to the after-effects of the treatment reported for rhythms in Peromyscus leucopus and Drosophila pseudoobscura, which both show period lengthening, and to the observation that running wheel activity in Mesocricetus auratus shows no after-effects of LL exposure (Pittendrigh and Daan, 1976a, Table 3). Christensen and Lewis (a, in prep.) found that in the locomotor activity rhythm of the weta Hemideina thoracica, the direction of the after-effect (period lengthening or shortening) observed following dim LL exposure depends on the magnitude of the period increase in LL over that in DD. This is obviously a complex phenomenon and considerably more data are required before generalizations about the behaviour of the R. exulans rhythm would be justified.
Period shortening is observed following phase advances produced by 8 h and 16 h light pulses. The magnitude of the period change is significantly correlated with the magnitude of the phase change for 8 h pulses. The combined mean period after phase advances is significantly shorter than the combined mean period before, for 8 h but not for 16 h pulses. Period lengthening is observed following 16 h light pulses that produce phase delays and the combined mean period after such pulses is significantly longer than the combined mean period before. Again the magnitude of the period change is significantly correlated with the magnitude of the phase change. These data conform to the most commonly observed pattern of after-effects following light pulses, which are summarized in Table 3.7 (an expansion of Table 3, Part I, Pittendrigh and Daan, 1976 a).

### TABLE 3.7
Summary of After-Effects of Single Light Pulses

<table>
<thead>
<tr>
<th>SPECIES</th>
<th>SIGN OF EFFECT</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rodents</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rattus exulans</td>
<td>+Δφ</td>
<td>this study</td>
</tr>
<tr>
<td>Ammospermophilus leucurus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>large Δφ</td>
<td>-</td>
<td>Kramm, 1976.</td>
</tr>
<tr>
<td>small Δφ</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Tamiasciurus hudsonicus</td>
<td>-Δφ</td>
<td>Kramm, 1975 b.</td>
</tr>
<tr>
<td>Glauciums volans</td>
<td>0</td>
<td>De Coursey, 1960.</td>
</tr>
<tr>
<td>Birds</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Taeniopygia guttata</td>
<td>+Δφ</td>
<td>Pittendrigh, 1960.</td>
</tr>
<tr>
<td>Insects</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemideina thoracica</td>
<td>+Δφ</td>
<td>Christensen and Lewis a (in prep.).</td>
</tr>
</tbody>
</table>

**KEY:** - after-effect indicates period shortening.  
+ after-effect indicates period lengthening.
Three different types of after-effects of entrainment have been examined in the *R. exulans* activity rhythm: 1) after-effects following entrainment to light cycles of various periods $T$ and containing 8 h light pulses; 2) after-effects of entrainment to $T = 24$ h cycles with 8 h or 16 h photoperiods; 3) after-effects following exposures of various durations to LD 8:16 and 16:8.

Following entrainment to cycles of 8 h light pulses with $T$ ranging from 24 h to 28 h, period shortening was observed if $\bar{T} > T$, while the period remained unchanged or lengthened if $\bar{T} - T \leq -0.2$.

There is a significant negative correlation between the magnitude and sign of after-effects produced by entrainment to LD 16:8 and 8:16, and $\bar{T}$ before entrainment. Whereas the after-effects of LD 8:16 range from period shortening, through no change, to period lengthening, entrainment to LD 16:8 always results in subsequent period shortening (Figs. 3.8 and 3.9). The combined mean period after LD 16:8 entrainment is significantly shorter than the combined mean period before. Period shortening following entrainment to long photoperiods has also been reported for *Peromyscus leucopus* (Pittendrigh and Daan, 1976 a), the bat *Myotis lucifugus* (Menaker, 1961) and two species of birds (*Passer domesticus*, Eskin, 1971; and *Carduelis flammea*, Pohl, 1972 b).

Preliminary observations indicate that there may be a positive correlation between the duration of exposure of *R. exulans* to an entraining cycle and the duration of the subsequent after-effect. A similar trend occurs in the locomotor activity rhythm of *Hemideina thoracica* (Christensen and Lewis b, in prep.).

3.4.3 A MODEL FOR THE CIRCADIAN PACEMAKER CONTROLLING THE *RATTUS EXULANS* LOCOMOTOR ACTIVITY RHYTHM

From these experiments on the sources of free-running period lability, a number of inferences can be drawn about the nature of the population of oscillators controlling the *R. exulans* activity rhythm, based on the description of the general behaviour of populations of coupled oscillators given in Pavlidis (1978 b).
The lack of spontaneous period changes and the absence of age-related effects on $\tau$ indicate that the unit oscillators of the population have relatively stable periods through time and/or that there is strong, steady coupling between them. The sensitivity of the rhythm to induced period instability (indicated by the occurrence of after-effects following LL, single pulses, or entrainment) argues against strong coupling, as does the comparatively large day-to-day instability of the rhythm (Table 3.6). On this basis the frequency demultiplication model of Pavlidis (1969) can be rejected. This model is based on the observation that when similar oscillating units are very strongly coupled, the system may cease to oscillate, or alternatively it may produce an oscillation at a frequency quite different to that of the constituent units (Pavlidis, 1978b). The model was invoked as a possible mechanism for producing low frequency circadian oscillations from higher frequency biochemical ones.

The opposite extreme in multisecillator circadian pacemaker models is represented by the Winfree (1975) "Clockshop" model in which the oscillators are proposed to be independent. *R. exulans* rhythms persist for years in the absence of external synchronizing agents and repeatedly return, following perturbations, to a specific period length. These observations place unrealistic constraints on the long-term stability of independent oscillators and would also require that they be identical.

It is thus concluded that the oscillators controlling the rhythm have similar, circadian periods and that they are weakly coupled. To account for the occurrence of after-effects following entrainment, the strength of coupling must be sufficient to ensure that when all the units are brought near synchrony by a zeitgeber, they will tend to stay together even after the zeitgeber is removed. Coupling strength in a population of oscillators is a function not only of any specific coupling mechanism but also of the similarity of the unit oscillators. Similarity between the units acts as a coupling strengtheners so that a specific coupling mechanism must be stronger to induce a given degree of synchrony between dissimilar oscillators than it needs to be to induce the same degree of synchrony between more similar oscillators (Pavlidis, 1978b).
The decline in amplitude of free-running rhythms with age associated with increasing activity outside the main active phase may be due to a decrease in coupling strength with age. Alternatively it could be the result of progressively increasing instability of the individual oscillating units such that the prevailing coupling strength is insufficient to retain all of them in synchrony at any given time. The concomitant maintenance of an unchanged $\tau$ value would imply that the unit oscillators have a fairly narrow distribution of period values, since desynchronization of oscillators from the main group does not significantly alter its period.

An alternative model has been proposed for the circadian pacemakers controlling activity in nocturnal rodents (Pittendrigh, 1974; Pittendrigh and Daan, 1976c; Daan and Berde, 1978), which is a limiting case of the more general class of models in which the pacemaker is envisaged as a population of weakly interacting circadian oscillators (Pavlidis, 1978b). In this model it is postulated that the pacemaker consists of two separate oscillators or principle groups of oscillators, E and M, coupled to each other in a stable phase relationship $\psi_{E,M}$. This phase relationship depends on the spontaneous frequencies, i.e. the frequencies that would be displayed in the absence of coupling, of E and M, which are postulated to have opposite dependence on light intensity. In turn, the period $\tau$ of the coupled system varies with $\psi_{E,M}$ which also determines the activity time $\alpha$, since onset and end of activity are each under the control of one of the oscillators (E and M respectively in nocturnal organisms). The system tends to preserve any established $\psi_{E,M}$. Simulations of the model (Daan and Berde, 1978) indicate that the period of the coupled system need not be intermediate between the natural periods of the two oscillators separately. It is possible to simulate splitting and refusion of rhythms and long term frequency changes by assuming either that the oscillators have different dependence on light intensity or that light affects the strength of coupling between them. Simulations indicate that weak coupling between similar oscillators is the best model, which implies that each of the oscillators is itself a precise and stable circadian pacemaker. Two stable states (synchrony and $180^\circ$ antiphase) occur only when coupling is weak and nearly equal in both directions. The model is thus able to account qualitatively for rhythm splitting, the history dependence of $\tau$ and $\alpha$ seen in after-effects, and the
interdependence of \( \tau \), \( \alpha \) and phase response curves found in the four species of nocturnal rodents examined. It can also accommodate the interdependence of \( \tau \) and \( \alpha \) described in Aschoff's rule, and the commonly observed bimodality of daily activity patterns. If it is postulated that the M oscillator is coupled to sunrise while the E oscillator is coupled to sunset, then the model can be extended to accommodate adjustment to seasonal changes in photoperiod. Such a system could also serve as a mechanism for the daylength discrimination known to be involved (in most instances) in photoperiodic induction. It is suggested that the two oscillators may relate anatomically to the suprachiasmatic nuclei of the hypothalamus, which have been implicated in circadian control in rodents (Chapter Five).

While the bimodal daily activity pattern apparently occurs widely in rodents (Marten, 1973; Barnett et al., 1975) and other vertebrates and invertebrates (Aschoff, 1966), there is no evidence in either entrained or free-running \textit{R. exulans} activity rhythms of a second activity peak towards the end of the active phase. The occurrence of a second minor peak in activity at \( 180^\circ \) antiphase to the main active phase is not strictly similar to reported cases of rhythm splitting (Pittendrigh, 1960; Hoffmann, 1971; Gwinner, 1974; Pittendrigh and Daan, 1976 c; Rusak, 1977; Underwood, 1977; Boulos and Terman, 1979; Christensen and Lewis c, in prep.) in which activity splits into two approximately equal components. It is difficult to envisage the "asymmetric split" observed in \textit{R. exulans} rhythms resulting from the interaction of two oscillators, unless one contributes very much less to observed activity than the other (which might also explain its apparent absence in unsplit activity patterns). However, for two stable states to occur, coupling between the two oscillators must be nearly equal in both directions, and slight dominance of one oscillator over the other removes the possibility of transfer from one state to the other (Daan and Berde, 1978). Thus the oscillators would need to be of approximately equal strength, but unequally expressed in the overt rhythm. Clearly this phenomenon would require the addition of new assumptions to be explained in terms of the two oscillator model. A population of weakly coupled oscillators has two stable states - synchrony and in groups \( 180^\circ \) apart (Pavlidis, 1978 b).

Occasionally, following perturbations or in prolonged free-runs, \textit{R. exulans} activity rhythms temporarily disintegrate into more than two components. Decay of the activity rhythm into ultradian components
with 4 - 6 h periods has been observed in laboratory rats after prolonged exposure to LL (Honma and Hiroshige, 1978 b). These observations are difficult to reconcile with a two oscillator model, although they may be explicable if two populations of oscillators are postulated.

While none of these observations actively precludes a two oscillator model, there is no specific evidence in support of the hypothesis that such a system controls the *R. exulans* activity rhythm. Moreover, all the available data are consistent with the more general model of a population of weakly interacting circadian oscillators, which has therefore been adopted as a working hypothesis.

### 3.4.4 PHASE RESPONSE CURVES

Many of the characteristics of circadian phase response curves can be simulated equally well by single or multiple oscillator pacemaker models (Pavlidis, 1978 a,b). Phase-dependent phase shifting by zeitgebers is a requisite for non-parametric entrainment and is thus functionally important in circadian time-keeping systems in nature. This common functional requirement is probably the cause of the major similarities among phase response curves of very diverse organisms (Aschoff, 1965; Pittendrigh, 1965, 1974). The three phase response curves derived for the *R. exulans* activity rhythm all conform to the generalization that phase delays are effected by light pulses falling late in the subjective day and early in the subjective night, while phase advances are effected by pulses falling late in the subjective night and early in the subjective day. (Pittendrigh and Daan, 1976 b). Although synchronization of circadian controlled behaviour with the natural environment is undoubtedly more complex (Section 3.4.5), the phase-dependent light sensitivity described in this generalization can be understood in broad functional terms. A nocturnal animal which begins activity too soon before sunset and perceives unaccustomed light will tend to be phase delayed to subsequently regain its appropriate phase relationship with the day/night cycle, while if it continues activity for too long into the dawn it will be phase advanced so that subsequent nightly activity starts and finishes earlier.

Information about the structure of circadian clocks can, however, be best elicited from experiments involving stimuli that are very different from those seen by the organism in its natural environment.
Light pulses which coincide with the region of transition from delays to advances in strong phase response curves (the region of the singularity (Pavlidis, 1967 b)) produce effects which aid discrimination between some single and multiple oscillator clock models (Pavlidis 1978 a,b). From the above generalization it is evident that this region corresponds to the middle of the subjective night and therefore it is highly improbable that these behaviours have any relevance in the natural environment or that they have ever been subject to selection pressures. They are incidental attributes of the time-keeping mechanism which may provide information about its structural nature.

Several workers have sought functional relationships between the free-running period of a circadian rhythm and the shape of its phase response curves (which varies with the intensity and duration of the stimulus used to produce the phase changes). The three phase response curves for the activity rhythms of female R. exulans are derived from the records of animals with $\tau$ values ranging from 23.7 h to 25.5 h. While there is some evidence of interindividual variability in phase shifts, the data do not concur with the finding of Natalini (1972) that in the kangaroo rat Dipodomys merriami, the phase response curves of animals with $\tau > 24$ h are mirror images of those with $\tau < 24$ h. Natalini measured phase shifts in the first cycle following 1 h light pulses (4 lux background to 35 lux during the pulse). The phase changes produced by these pulses were apparently only transient.

Daan and Pittendrigh (1976 a) attribute functional significance to the generalization that animals with $\tau < 24$ h have larger delaying (D) than advancing (A) sections under their phase response curves, while the reverse occurs in animals with $\tau > 24$ h. The generalization is based on comparative data for five species of rodents, for conspecific individuals, and within individual Mus musculus following period changes induced as entrainment after-effects. Female R. exulans have a species mean period of 24.5 h and yet in all 3 phase response curves, the area under the delay section of the curve is considerably greater than the area under the advance section (Table 3.5). There are several differences in experimental procedure which might have contributed to this discrepancy. Firstly, female rats were subjected to continuous low level background light (0.02 to 0.07 lux) whereas the nocturnal rodents examined by Pittendrigh and Daan were maintained in DD between light
pulses. This probably tended to lengthen $\tau$ in the rats, however if the generalization holds, there should have been a compensatory change in shape of the phase response curves. The second difference in procedure, which probably had a more significant effect, is that the phase response curves in the experiments of Pittendrigh and Daan are responses to 15 min light pulses and phase advances never exceed 3 h. In the 4 h phase response curve for R. exulans activity rhythms, the maximum phase advance is 2.4 h and the maximum delay 5.9 h. It is possible that the R. exulans phase response curve to 15 min light pulses might show the expected $D - A < 0$. Honma et al. (1978) describe a phase response curve to 1 h light pulses for laboratory Rattus norvegicus (800 lux on a background intensity of 5 lux). The 4 animals studied had free-running periods ranging from 24.4 h to 23.9 h and the phase response curve shows the expected relationship with the area under the advance section being greater than that under the delay section. On the other hand $D - A$ is more positive for the 4 h than for the 8 h R. exulans phase response curve so there does not appear to be a systematic decrease in $D - A$ with decreasing pulse length.

Further experimental investigation would be desirable to clarify this issue. Nevertheless, on the basis of available evidence, the R. exulans activity rhythm does not appear to conform to the generalization that animals with free-running periods greater than 24 h have larger advancing than delaying sections under their phase response curves. This generalization forms the basis of the definitions of nocturnal and diurnal entrainment strategies elaborated in Pittendrigh and Daan (1976 b,c) which would thus appear to be inapplicable to R. exulans. (The data of Honma et al. (1978) indicate that the activity rhythm of nocturnal Rattus norvegicus conforms to the optimal diurnal strategy, having $\tau > 24$ h and larger advancing than delaying sections under the phase response curve.) Presumably the functional requirement for an animal with $\tau > 24$ h is only that a sufficiently large advance, in response to the natural zeitgeber cycle, should be possible in order for entrainment to occur.

The existence of a singularity is accommodated by a number of single oscillator models including the limit cycle oscillator model of Pavlidis (1967 b), and the feedback oscillator models of Johnsson and Karlsson (1972), Karlsson and Johnsson (1972), and Gander and Lewis, (1979).
A corollary of the existence of a singularity is the prediction of a qualitative change in shape of phase response curves from continuous to discontinuous, the former leaving the limit cycle above the singularity and the latter below it (Pavlidis, 1978a). A transition from weak to strong (continuous to discontinuous) phase response curves with increasing pulse duration has previously been reported in response to light and temperature pulses for the eclosion rhythm of *Drosophila pseudoobscura* (Winfree, 1970; Chandrashekaran, 1974) and the petal movement rhythm of *Kalanchoe blossfeldiana* (Engelmann et al., 1973, 1974). An analogous transition is observed for the *R. exulans* activity rhythm. The phase response curve for 4 h light pulses is continuous i.e. weak. In response to 8 h light pulses at around CT 8-9, some rats show (temporary) arrhythmicity or splitting of the rhythm into several components, (e.g. Figs. 3.13 and 3.14). Since such effects cannot be plotted as phase shifts, there is a region of discontinuity in the phase response curve for these animals i.e. it constitutes a Winfree (1970,1971) Type 0 or strong phase response curve. Rat 31 however, shows measurable phase shifts in response to pulses in this region, indicating that its phase response curve (points joined by the dotted line in Fig. 3.11) is in fact Type 1 or weak. These inter-individual differences suggest that the 8 h pulse duration may be close to the critical length for the transition from weak to strong phase response curves. The 16 h phase response curve is considered to be of the strong type, since a region of discontinuity exists around CT 14 and there is no significant correlation between CT before and CT after (Fig. 3.15). This phase response curve is unusual in that both the maximum phase advance and the area under the advance section of the curve are smaller than in the 8 h phase response curve, whereas generally the amplitude of phase response curves increases with increasing pulse duration. The 16 h curve is, however, unlikely to be of functional importance since the maximum duration of daylight on Tiritiri is about 15.7 h (Section 3.4.5). Also, although in the experimental apparatus an animal could retreat to its nest box and close its eyes, it probably perceived on average, much higher light intensities than it would in a natural nest (Dr J. Craig, M. Nicholas pers. comm.). The 16 h phase response curve does, however, reveal non-functional behaviour of the pacemaker which is of interest in considerations of the entrainment mechanism (Section 3.4.5).
Following 15 min light pulses at the singularity (Winfree, 1970, 1971) or near the critical stimulus (Winfree, 1973, 1975), there is no indication that the *Drosophila* eclosion rhythm is returning to its limit cycle after two days. These observations indicate at least a slow return to rhythmicity, if not a stable singularity. Arrhythmicity has been observed to persist in the locomotor activity rhythm of *Hemideina thoracica* for at least 25 days following a pulse at the singularity (Christensen and Lewis c, in prep.). The petal rhythms of individual *Kalanchoe* flowers can be damped or stopped completely for at least one week by critical pulses (Engelmann et al., 1978). In *Rattus exulans* only one 8 h pulse was sufficiently close to the "singular point" to produce arrhythmicity (Fig. 3.13) which persisted for about 9 days. The single limit cycle model of Pavlidis (1967 b, 1968) would require careful selection of parameters to accommodate this slow return to rhythmicity (Pavlidis, 1978 a). It would likewise require considerable modification to simulate the various types of free-running period lability described in section 3.4.2 (Pavlidis 1978 b). In contrast, only minor adjustment is required for the single feedback oscillator model of Johnsson and Karlsson (1972), Karlsson and Johnsson (1972), to accommodate a stable or an unstable singularity (Engelmann et al., 1978). A critical stimulus striking a nearly synchronous population of weakly coupled oscillators may cause damping, or stop some of the unit oscillators and will also increase the relative spread of phases of the remainder. Because of the weak coupling, resynchronization may be delayed, if not inhibited (Pavlidis, 1978 b). This model also predicts that organisms which present significant free-running period lability will also be easily brought to an arrhythmic state by critical pulse experiments (and the converse). While various treatments produce period after-effects in *R. exulans* activity rhythms, spontaneous period changes are not observed, which suggests that the rhythm is neither highly stable nor highly labile. It is hard to assess how comparatively difficult or easy it is to produce arrhythmicity with critical pulses, particularly when comparing the behaviour of different individuals. The observation of a number of discrete phase shifts in response to 8 h pulses at around the same CT times as those which produce arrhythmicity and splitting up of rhythms (Fig. 3.11) suggests that the timing and duration of pulses producing arrhythmicity may be fairly critical.
3.4.5 THE ENTRAINMENT MECHANISM

Entrainment and phase shifting are the main types of behaviour of circadian rhythms that are successfully simulated by single oscillator pacemaker models (Pavlidis, 1978a, b). There are several factors contributing to this success. Firstly, as previously noted, synchronization by the environment is crucial to the functioning of circadian time-keeping systems in nature. Investigations of their behaviour in response to perturbations resembling natural zeitgebers may provide information on the mechanism of synchronization but generally reveal little about the structure of the underlying system (Pavlidis, 1971). Secondly, imposition of an exogenous synchronizing cycle enhances synchrony between a population of weakly interacting circadian oscillators (Pavlidis, 1978b). Thus in steady-state entrainment, the behaviour of such a population most closely resembles that of one of its unit oscillators (Winfree, 1975). (This enhanced synchrony may be an explanation of the increased amplitude of *R. exulans* activity rhythms in entrained by comparison with free-running conditions.)

The mechanism of synchronization of *R. exulans* activity rhythms by light cycles has been investigated by testing the applicability of a purely non-parametric entrainment model. In this model, derived from investigations of entrainment of pupal eclosion rhythms in insects (Pittendrigh and Minis, 1964; Pittendrigh, 1965, 1966), the circadian pacemaker is viewed as a single oscillator. The model assumes known values of $\tau$, phase response curve shape and the phase angle $\psi$ between the rhythm and the light cycle in the entrained steady state. Any single estimate of a parameter of the *Drosophila* eclosion pacemaker is an average value for a large number of pupae and such estimates are highly reproducible among batches of pupae. The free-running period, phase response curve shape and $\psi$ of the *Drosophila* eclosion rhythm can thus be estimated with a high degree of precision. When rhythms of individual animals are monitored, as in the work of Pittendrigh and Daan (1976b) and this study, the criteria of known $\tau$, phase response curve shape and $\psi$ pose some practical difficulties. In *R. exulans* experiments, animals were allowed to free-run until the period was apparently free of transients and after-effects of previous treatment, before $\bar{\tau}$ for each animal was estimated (Tables III.7 and III.8). On the other hand, phase response curves for individual rats are not known so the combined curves must be used in testing model predictions. The major source of error in the individual $\psi$ values is the measurement
error of \( \pm 0.05 \) h. Taking these restrictions into account, there is reasonable agreement between \( \varPsi \) values predicted from the 8 h and 16 h phase response curves and the observed \( \varPsi \) values, as indicated by the circadian time of entraining pulses and the phase changes \( \Delta \varphi = \tau - T \) that they produce (Figs. 3.21 and 3.22). From Fig. 3.17 it is evident that in LD 8:16 entrainment, as \( \tau - T \) decreases, activity begins progressively earlier, although the relationship is not linear. If there is a real sexual dimorphism in \( \tau \), with males having shorter free-running periods than females, then it would be predicted that males begin activity earlier than females. This prediction could be tested by field observation.

In non-parametric entrainment, by definition, it is the rapid transitions in light intensity at the beginning and end of a pulse which are primarily responsible for its phase-shifting effect. The effects of a long pulse can therefore be simulated by 2 brief pulses corresponding to its DL and LD transitions (skeleton photoperiods). Laboratory experiments on entrainment to skeleton photoperiods indicate that an exclusively non-parametric entrainment mechanism has the inherent complication of a phase jump – activity tends to coincide with the longer interval between two short light pulses in a 24 h cycle (Pittendrigh, 1966; Pittendrigh and Daan, 1976 b). It has been suggested that a number of nocturnal rodents effectively experience skeleton photoperiods in nature, since during daylight they retreat to dark nests and thus only perceive light briefly at the beginning and end of activity (Kenagy, 1976; Pittendrigh and Daan, 1976 b). If such animals relied on an exclusively non-parametric entrainment mechanism, then they would tend to switch to being diurnal in long photoperiods.

Field observations (Dr. J. Craig, M. Nicholas pers comm.) suggest that R. exulans on Tiritiri generally spend the day in dark or semi-dark nests. It thus seems likely that, unless animals venture out from cover during the day, in the natural environment the entrainment mechanism must be primarily non-parametric, resulting from interaction of light seen as activity begins around sunset and before it ceases around sunrise. (Ten minutes of light per 24 h is sufficient for entrainment in the laboratory). The longest day length on Tiritiri is about 15 h 40 min.* This is about

the skeleton photoperiod length found to produce phase jumps in *Mesocricetus auratus* running wheel activity, but is somewhat shorter than that which produced phase jumps in *Peromyscus leucopus* running wheel activity (Pittendrigh and Daan, 1976 b).

The likelihood of predation on Tiritiri is maximal during daylight and probably non-existent during darkness (Moller, 1977). Diurnal activity is thus likely to represent a sub-optimal temporal niche in this environment (see Chapter Six). There are several possible ways in which *R. exulans* in the field might perceive sufficient light during the day to avoid the phase jump. Firstly, it is possible that animals may perceive dim light throughout the day in their burrows. Light affecting mammalian circadian time-keeping systems is, however, perceived via the retina (Chapter Five), so that closing the eyes and covering them in the curled up sleeping position alters the pattern of light affecting the circadian pacemaker(s). Secondly, if the bouts of activity observed in the laboratory during the light phase of entraining LD cycles represent a natural field behaviour, then rats may make brief excursions from the nest throughout the day. Detailed field observations would be necessary to confirm or refute these speculations.

On the other hand, laboratory data do not exclude the possibility that there is also a parametric action of light involved in entrainment of *R. exulans* activity. Both diurnal and nocturnal rodents can be entrained by sinusoidal light cycles (Swade and Pittendrigh, 1967). The importance of a parametric effect in natural entrainment would again depend on the duration of light exposure that an animal normally receives. It is worthy of note that in laboratory studies to date, the action of light in entrainment of nocturnal rodents appears to be primarily non-parametric (De Coursey, 1972; Pittendrigh and Daan, 1976 c), while in diurnal rodents parametric effects have been shown to be important (Swade, 1969; De Coursey, 1972; Kramm, 1974, 1975 a, b, 1976).
4.1 THE ESTROUS CYCLE
OF RATTUS

4.1.1 INTRODUCTION

The second hypothesis examined in this thesis concerns the possible role of circadian time-keeping in the control of the estrous cycle in Rattus exulans. This cycle does not appear to have been previously examined in any detail. It was firstly necessary therefore, to establish its basic pattern and duration. In view of the considerable similarity among the estrous cycles of muroid rodents (e.g. Dewsbury et al., 1977), the estrous cycle of R. exulans was not expected to be markedly different from that of R. norvegicus.

The hypothesis of circadian involvement in the control of estrous in Rattus derives from a considerable body of literature on laboratory rats. Before examining the proposed mechanism of this control, it is pertinent to outline briefly the neuroendocrine dynamics of the estrous cycle of Rattus norvegicus, as they are presently understood. A detailed analysis of this highly complex field is well beyond the scope of this thesis, but a basic understanding is necessary to appreciate the evidence for circadian involvement discussed in Section 4.1.4.

Rats are spontaneous ovulators. Non-pregnant mature female laboratory R. norvegicus undergo repeated estrous cycles in which the interval between successive occurrences of behavioural receptivity and ovulation is typically either 4 or 5 days (hence the descriptions "4-day" and "5-day" rats). Although wild rats in the field probably seldom experience estrous without mating, a similar estrous cycle is also observed in captive wild R. norvegicus (Perry, 1971).

Four stages are generally distinguished in the estrous cycle. During the 2 or 3 days comprising metestrous and diestrous, follicular growth and development take place in the ovaries. On the afternoon of proestrous, an ovulatory surge of gonadotropins is released from the pituitary and ovulation occurs 10 to 12 h later, on the morning of estrous. Behavioural receptivity is exhibited from the evening of proestrous through to the morning of estrous (Everett, 1964).
4.1.2 METESTROUS AND DIESTROUS

Shortly after ovulation, a new cohort of follicles starts to grow in the ovaries. This growth is stimulated by the presence of high plasma levels of follicle-stimulating hormone (FSH) which result from enhanced pituitary secretion of this gonadotropin around the time of ovulation. During diestrous, the developing follicles begin to secrete estrogen under the influence of basal levels of luteinizing hormone (LH) secreted by the pituitary (Freeman et al., 1976). In the presence of basal serum LH and FSH concentrations during diestrous, the follicles become increasingly sensitive to LH (Uilenbroek and Richards, 1979). The neural mechanism regulating this basal LH secretion is apparently different to that controlling the preovulatory LH surge (Lawton and Sawyer, 1968).

The slow rise in circulating estrogen on the last day of diestrous initiates uterine ballooning on proestrous and vaginal cornification at estrous (Lawton and Sawyer, 1968; Freeman et al., 1976). A rapid increase in ovarian estrogen secretion occurs very late on the last day of diestrous and into the first hours of proestrous. This raises the systemic estrogen concentration above a threshold level necessary to facilitate the neural "trigger" of the proestrous LH surge (Kalra, 1975; and see below).

4.1.3 PROESTROUS AND ESTROUS

On the afternoon of proestrous there is an abrupt rise in serum LH, FSH and prolactin levels and ovulation occurs 10 - 12 h later (Everett, 1964; Gay et al., 1970; McCormack and Benin, 1970; Daane and Parlow, 1971). Each of these pituitary gonadotropins is apparently controlled by a different releasing or regulatory mechanism and only the LH surge is essential for ovulation to proceed (Kimura and Kawakami, 1978).

Prior to the proestrous LH surge, elevated circulating estrogen levels apparently enhance pituitary responsiveness to luteinizing hormone-releasing hormone (LH - RH). Once the LH surge begins, however, high estrogen levels apparently have an inhibitory effect on pituitary secretion of LH in response to LH - RH. As serum gonadotropin levels rise, estrogen levels decline (Turgeon and Barraclough, 1977; Turgeon, 1979). There is also an accompanying increase in the activity of the pituitary - adrenocortical system and the thyroid gland, the function of which is not clear (Buckingham et al., 1978).
The Proestrous Luteinizing Hormone Surge

The precise timing of the LH surge on the afternoon of proestrous, and of subsequent ovulation, is dependent on the light regime (Everett, 1964; Hoffmann, 1969; McCormack, 1976; Sridaran and McCormack, 1977 a, 1979) and on an adrenal factor, probably progesterone (Mann et al., 1976).

There is a "critical period" on the afternoon of proestrous, during which centrally active drugs can block LH release from the pituitary, and subsequent ovulation (Everett, 1964; Blake, 1974, 1978). The critical period lasts 20 to 35 min in each animal, with variation between individuals resulting in an overall critical period lasting about 2 h in 4-day rats (Everett, 1964; Blake, 1974) and somewhat longer in 5-day rats (Lawton and Smith, 1970).

The "critical" neural activity taking place during this period is apparently the activation of the neuronal mechanism controlling the release of luteinizing hormone-releasing hormone (LH - RH) from the medial basal hypothalamus into the hypophyseal portal system (and thence to the pituitary). It is thought that LH - RH is synthesized in the preoptic area, which in rodents contains the majority of LH-RH containing cell bodies, while only a few are found in the medial basal hypothalamus itself. From the preoptic area LH - RH apparently passes down axons to nerve terminals in the median eminence (Fraser, 1979). Evidence reviewed in Section 4.1.4 suggests that somewhere en route the LH - RH releasing mechanism is regulated by a circadian pacemaker.

It would appear that LH - RH may be released from the medial basal hypothalamus as a series of pulses, beginning slightly before the proestrous LH surge. The pituitary shows a marked increase in responsiveness to LH - RH prior to and during the LH surge and is apparently presensitized by LH - RH released before the surge (Barr and Barraclough, 1978). LH - RH stimulates the release of both LH and FSH. The concentration of LH - RH in the pituitary stalk blood increases prior to the preovulatory LH surge and shows a second peak just after the LH surge which might be responsible for the elevated FSH levels found at this time (Fraser, 1979).

In Section 4.1.4, evidence is reviewed which supports the hypothesis, originally formulated by Everett and Sawyer (1950), that the neural trigger controlling LH release in the rat is regulated by a circadian pacemaker. In the presence of sufficiently high circulating estrogen levels, the ovulatory LH surge is triggered by the pacemaker which thus controls the timing of ovulation and the duration of the estrous cycle.
In rats (and hamsters) (Rusak and Zucker, 1979)), estrogen (estradiol - 17β) from the ovaries thus determines the day of the LH surge while the actual time of its onset is governed by a circadian time-keeping system. There is no evidence, however, of involvement of a circadian rhythm in the timing of LH release in sheep or monkeys. In these species the ovaries determine the timing of onset of the LH surge (Fraser, 1979).

After the critical period, the action of one or several steroids of ovarian and/or adrenal origin is apparently necessary for LH release to proceed (Wilson et al., 1978).

The Proestrous Follicle-Stimulating Hormone Surge

In the rat estrous cycle there is a biphasic increase in serum FSH around the time of ovulation. The first rise occurs in association with the LH surge on the afternoon of proestrous and the second starts during late proestrous and continues into estrous after serum LH levels have declined (Daane and Parlow, 1971; Ashiru and Blake, 1979).

FSH can induce ovulation in the absence of LH (Siegel et al., 1976) but ovulation can proceed in the absence of the proestrous FSH surge (Schwartz et al., 1973; Hoffmann et al., 1979). The proestrous FSH surge is apparently important in the early development of follicles destined to grow and ovulate in the next cycle (Schwartz et al., 1973).

Both FSH and LH are released from the pituitary in response to LH - RH and on the basis of present evidence, the existence of a separate FSH releasing hormone seems unlikely (Fraser, 1979). Nevertheless, results from a variety of types of experimental manipulations suggest that the mechanisms regulating FSH and LH release are at least partially separate (Daane and Parlow, 1971; Schwartz et al., 1973; Kimura and Kawakami, 1978; Hoffmann et al., 1979). There must be a mechanism allowing a differential response of the pituitary to the common releasing hormone (Fraser, 1979).

The Proestrous Prolactin Surge

The rise in plasma prolactin levels on the afternoon of proestrous is not necessarily simultaneous with the LH surge (Gay et al., 1970). It does, however, have a number of characteristics in common with the LH surge including proestrous sensitivity to centrally active drugs and to estrogen and progesterone levels. The neural mechanism controlling pituitary discharge of prolactin would seem however, to be less strictly
synchronized by LD cycles than that controlling LH release (Mann et al., 1976). This lesser sensitivity to light may be associated with the observation (Kimura and Kawakami, 1978) that the pathway from the preoptic area to the medial basal hypothalamus associated with prolactin release apparently passes outside the suprachiasmatic nuclei. These nuclei are implicated in the light sensitivity of a number of other circadian rhythms in mammals (see Chapter Five).

The hypothalamus releases a prolactin inhibiting factor, although several hypothalamic compounds capable of causing pituitary release of prolactin have also been isolated. The physiological significance of the latter compounds remains unclear (Fraser, 1979). Prolactin has a luteotrophic function in rats i.e. it enhances the persistence of the corpora lutea, and their ability to secrete progesterone (Perry, 1971).

The 2 daily prolactin peaks in pseudopregnant rats, and the daily peak in ovariectomized animals (which resembles the proestrous peak in intact females) have both been demonstrated to be under circadian clock control (Pieper and Gala, 1979).

The Proestrous Progesterone Peak

After a follicle ruptures and releases its oocyte at ovulation, it undergoes luteinization to form a corpus luteum and begins to secrete progesterone (Perry, 1971). Generally follicular maturation in mammals is inhibited until the corpora lutea cease to secrete progesterone and begin to regress. In non-pregnant muroid rodents the corpora lutea produce only minimal amounts of progesterone and therefore development of the next batch of follicles begins shortly after the previous ovulation. This so-called "inactive" luteal phase is the reason for the characteristic very short estrous cycle of muroid rodents (e.g. Dewsbury et al., 1977).

On the day of proestrous, plasma levels of progesterone increase slowly prior to the LH surge due to adrenal secretion of progesterone, which facilitates LH release. The adrenals are not, however, essential for ovulation to proceed (Feder et al., 1971; Buckingham et al., 1978; Hoffmann, 1978). The major function of this adrenal progesterone seems to be in regulating the timing of the proestrous LH surge (Everett, 1964; Nequin and Schwartz, 1971), although the LD cycle is apparently a more powerful synchronizing agent (Mann et al., 1976).

There is a rapid increase in serum progesterone levels during and after the LH surge due to LH stimulation of ovarian progesterone secretion
(Feder et al., 1971). One of the functions of this progesterone surge may be to abolish expression of the circadian neural timing mechanism for LH release. Another signal is not then expressed until a new cohort of follicles has attained steroidogenic capacity and secreted sufficient quantities of estrogen to again approach the threshold level for activation of the LH release mechanism (Freeman et al., 1976; Banks and Freeman, 1978, McPherson and Mahesh, 1979).

The proestrous progesterone surge also acts in synergism with estrogen to produce full behavioural receptivity (see below).

**Behavioural Estrous**

The onset of behavioural receptivity occurs several hours after the LH surge and after the major progesterone rise on the early evening of proestrous (Everett, 1964; Feder et al., 1971; Nequin and Schwartz, 1971).

Estradiol must be continuously available throughout the 18 - 24 h prior to the onset of behavioural estrous for full sexual receptivity to occur. Progesterone facilitates sexual receptivity in ovariectomized rats only if estradiol has been administered 18 - 24 h previously (Moreiness and Powers, 1977). If a sufficiently low dose of progesterone is used, another period of sexual receptivity can be induced with a second injection of progesterone given 24 h after the first. If however, the first dose is sufficiently large, there is a refractory period during which a second injection fails to induce estrous behaviour (sequential inhibition). This inhibitory action of high doses of progesterone may be related to its extinguishing effect on the circadian LH release mechanism. Its inhibitory action on lordosis is apparently due to a reduction in available cytoplasmic progesterin receptors in the brain, rather than to an interaction of progesterone with neural estrogen receptors (Moguilewsky and Raynaud, 1979; Schwartz et al., 1979).

**4.1.4 THE HYPOTHESIS OF CIRCADIAN CONTROL OF THE ESTROUS CYCLE IN RATTUS NORVEGICUS.**

Evidence for the circadian nature of the neurogenic stimulus controlling the ovulatory LH surge in female laboratory rats has been observed in three experimental preparations: intact rats, ovariectomized rats primed with estrogen, and immature rats treated with a gonadotropin derived from pregnant mare's serum (PMS).
Intact Rats

The 24 h nature of the LH release apparatus in intact rats was first recognized from experiments in which centrally active drugs (e.g. barbiturates) were administered prior to the proestrous critical period. This procedure results in a 24 h delay in the plasma LH surge and ovulation (Everett and Sawyer, 1950; Blake, 1974). A second injection of barbiturate 24 h after the first, delays the LH surge a further 24 h (i.e. a total delay of 48 h) in 4-day rats (Everett and Sawyer, 1950; Butcher et al., 1974), but results in a failure of ovulation in that cycle in the majority of 5-day rats. The latter have older preovulatory follicles which probably have less estrogen secreting capability remaining than those of 4-day animals (Van der Schoot, 1978).

A third injection of barbiturate 24 h after the second causes failure of ovulation in that cycle in most 4-day rats (Everett and Sawyer, 1950). These centrally active compounds appear to exert their effect on the LH - RH mechanism at the hypothalamic level, rather than by altering pituitary responsiveness to LH - RH. The above data suggest that the blocking effect is not at the level of the circadian mechanism, which apparently continues its 24 h periodicity without any change in phase, as indicated by the timing of the subsequent LH release. This interpretation is, however, difficult to equate with the observation that when sub-blocking doses of such compounds are administered at the onset of the critical period, the LH surge is delayed by 1 - 6 h and is reduced in amplitude and duration (Blake, 1974).

If the LH surge has been blocked by appropriate injection of sodium pentobarbital, then subsequent administration of progesterone for a critical duration prevents the expected LH surge the following day (Freeman et al., 1976; Banks and Freeman, 1978).

There is also evidence of daily fluctuations throughout the estrous cycle in hypothalamic neural activity related to the gonadal axis (Suzuki et al., 1974) and of daily fluctuations in serum LH levels, on which the proestrous LH surge is apparently superimposed (Gay et al., 1970).

The proposed model for the mechanism of circadian control of the estrous cycle in the laboratory rat can be broadly summarized thus: in the intact non-pregnant animal, the daily neural trigger for LH release is expressed only in the presence of supra-threshold levels of circulating estradiol, which are attained by the end of diestrous or proestrous. The expression of the neural trigger, in the proestrous release from the
pituitary of the LH surge, causes the ripe follicles in the ovary to ovulate and then luteinize and secrete progesterone. This progesterone in turn prevents repeated expression of the neural trigger (i.e. LH release) on subsequent days.

A circadian rhythm, by definition, persists in the absence of cyclic environmental input with a period close to 24 h, but can be entrained by zeitgebers (e.g. natural temperature and/or light cycles) to an exactly 24 h period. To demonstrate that the timing of ovulation, and hence the duration of the estrous cycle of *R. norvegicus* is controlled by a circadian clock, albeit expressed only every fourth or fifth day, it must be demonstrated that the estrous cycle conforms to these criteria.

Most laboratory strains of *R. norvegicus* continue to exhibit estrous cycles when blinded or in DD (Hoffmann, 1967, 1970; Suzuki *et al.*., 1974; McCormack and Sridaran, 1978). Estrous cycles also persist in dim LL (0.2 lux), but the time of ovulation becomes progressively later compared with that in DD or LD 14:10 i.e. the period of the estrous cycle in dim light is longer than the period in constant darkness. The estrous cycle thus obeys Aschoff's rule for a nocturnal organism (McCormack, 1976; McCormack and Sridaran, 1978).

When rats are maintained in constant bright light, estrous cycles become irregular and eventually cease, with animals displaying persistent vaginal estrous (Hoffmann, 1970; Suzuki *et al.*, 1974; Lambert, 1975; McCormack and Sridaran, 1978). This inhibitory effect of constant bright light is a common feature of circadian rhythms of nocturnal organisms (Aschoff, 1979).

The estrous cycle of *R. norvegicus* is entrained by LD cycles and phase advances or delays in the lighting regime produce corresponding advances or delays in the timing of ovulation. There is evidence that light perceived around dawn advances ovulation while light perceived around dusk delays it (Sridaran and McCormack, 1977 a, 1979). The estrous cycle would thus appear to conform to the characteristic pattern of phase-dependent light sensitivity described for other circadian rhythms (Section 3.2.2). Suzuki *et al.* (1974) review evidence which indicates that temperature cycles can also entrain the estrous cycle in rats in DD, however light cycles apparently dominate when both types of zeitgeber are applied simultaneously.
Ovariectomized Rats

A second type of experimental approach has provided evidence in support of the hypothesis that release of the preovulatory LH surge is in fact controlled by a circadian mechanism which is expressed only in the presence of sufficiently high circulating estrogen levels. This approach involves rats which are ovariectomized (thus removing the natural estrogen source and the ovulating mechanism) and then primed with exogenous estrogen.

In long-term ovariectomized rats in LD cycles, a daily proestrous-like LH surge occurs for at least three days following a single injection of estradiol (Legan et al., 1975; Freeman et al., 1976) or for longer than nine days following implantation of a solid estrogen source (Chazal et al., 1977). This daily LH peak coincides with the timing of the proestrous LH surge in intact rats in the same light regime and is similarly inhibited by sodium pentobarbital. It is apparently due to an oscillating LH – RH releasing mechanism of neural origin rather than to a daily fluctuation in pituitary sensitivity to LH – RH (Chazal et al., 1977). It continues for four days after the removal of the estrogen implant which suggests that high circulating estradiol levels are necessary for the initiation but not for the continuation of daily LH surges in long-term ovariectomized rats. In terms of the estrogen threshold model this implies some sort of "memory" that estrogen levels have been suprathreshold (Legan et al., 1975). In contrast, in acutely ovariectomized rats, elevated serum estradiol levels must be maintained for daily LH surges to persist (Legan and Karsch, 1975).

The daily LH peaks in long-term ovariectomized rats implanted with solid estradiol sources are entrained by LD cycles, and phase shifts in the light regime produce corresponding phase shifts in the timing of the LH peaks (Chazal et al., 1977).

PMS-Treated Immature Rats

Precocious puberty can be induced in immature female rats by administration of pregnant mare's serum gonadotropin (PMS). PMS administered at 0930 h to 30 day old rats in LD 14:10 (lights on from 0900 to 1500 h) produces ovulation around 0135 h on day 33, following a surge of pituitary gonadotropins 12 – 14 h earlier. The timing of the PMS injection determines the day, but not the time of day, of gonadotropin release.

Gonadotropin release and subsequent ovulation can be blocked by barbiturate administration during a critical period which corresponds precisely in timing to the proestrous critical period for LH release in
mature rats in the same light regime. Barbiturate blocking of PMS-treated rats entrained and then released into DD indicates that the critical period continues at approximately the same time of day for at least a week i.e. it has a period close to 24 h in DD. The timing of gonadotropin release and ovulation is delayed in LL by comparison with DD or LD 14:10, the delay being proportional to the duration of the LL exposure and intensity dependent. Reversal of an LD 12:12 light regime causes a 12 h phase change in the time of the critical period (McCormack and Benin, 1970; McCormack, 1974).

PMS-treated immature female R. norvegicus thus have an endogenous circadian rhythm controlling LH release, which is entrained by LD cycles but free-runs in DD and dim LL and obeys Aschoff's rule for a nocturnal organism.

4.1.5 CLOCK LOCATION AND PATHWAYS OF CONTROL

In the proposed model for circadian control of the estrous cycle of Rattus norvegicus it is postulated that this control is exerted through circadian regulation of the release of LH - RH from the medial basal hypothalamus. The sites of LH - RH secretion and the pathway(s) via which it reaches the medial basal hypothalamus are thus key factors for an understanding of the mechanism of circadian control of estrous.

LH - RH is apparently released from the medial basal hypothalamus in response to neuronal input from the preoptic area (POA). Transection experiments on the morning of proestrus (Kimura and Kawakami, 1978) indicate that this signal does not arise in the POA itself, but is dependent on neural inputs from areas anterior and superior to the POA. Some of these areas are also implicated as positive feedback sites of estradiol involved in the induction of LH release and ovulation. These findings conflict with those of Koves and Halasz (1970) who report that severing all but the posterior connections to the POA does not prevent ovulation. Kimura and Kawakami (1978) suggest that this observation may be attributable to the POA becoming responsive to the stimulatory action of estradiol after prolonged deafferentation.

Experiments involving damage to the POA do not present a cohesive picture. This is at least partially attributable to considerable variation in experimental procedures and to the use of different endpoints (e.g. occurrence of spontaneous ovulation; ability to elicit an LH surge with
exogenous steroids) to determine their effects (see Rusak and Zucker (1979) for a review). The POA contains the majority of LH - RH containing cell bodies in the rat brain (Fraser, 1979) and daily fluctuations in its LH - RH content have been reported (Kalra, 1976). However, as Rusak and Zucker (1979) point out, there is little basis for supposing a high correlation between LH - RH concentrations in the brain and the rate of its release into systemic or portal circulation (the latter being important in stimulation of LH - RH release). The pattern of change in the concentration of LH - RH in the hypophyseal portal blood during the stages of the estrous cycle is not yet known in detail (Rusak and Zucker, 1979), although LH - RH concentration apparently increases prior to the proestrous LH surge (Fraser, 1979).

LH - RH is apparently delivered by an axonal route from the POA to the median eminence (Kalra, 1976; Fraser, 1979). The actual pathways involved are the subject of continuing debate. Much work implicates the suprachiasmatic nuclei (SCN) as a crucial element (see Rusak and Zucker, 1979 for a review). In particular, anterior hypothalamic deafferentation either through the SCN, or caudal to them, decreases the LH - RH content of the medial basal hypothalamus and increases LH - RH content in the POA (Kalra, 1976). Destruction or isolation of the SCN before the proestrous critical period blocks the LH surge and ovulation, but not the FSH or prolactin surges. Stimulation of the POA can induce ovulation in intact pentobarbital-blocked proestrous rats, but not in animals in which the SCN have been isolated or destroyed. These findings strongly suggest that the neural inputs responsible for the ovulatory surge of LH pass through the suprachiasmatic area and/or the SCN (Kimura and Kawakami, 1978).

In contrast, Wiegand et al., (1979) maintain that damage to the SCN alone fails to eliminate progesterone induced LH release in ovariectomized estrogen-primed rats. Their work, and that of Sampson and McCann (1979), implicates structures anterior to the SCN in control of this response. The latter authors report that the response is blocked by lesions of the organum vasculosum lamina terminalis (OVLT), which presumably do not disturb general circadian organization (Rusak and Zucker, 1979). The OVLT contains most of the rostrally located LH - RH in the rat hypothalamus. (It is not, however, a crucial source of LH - RH to the median eminence for the maintenance of tonic LH secretion, which is probably controlled by a different neural mechanism to that controlling the proestrous LH surge (Lawton and Sawyer, 1968).) Sampson and McCann (1979) suggest that
the positive feedback action of gonadal steroids and the preovulatory surge of gonadotropins may be mediated by rostral LH - RH secreting neurons whose axons pass through the OVLT and perhaps caudally through the medial preoptic nucleus (the structure implicated by the work of Wiegand et al., 1979).

Wiegand et al., (1979) describe two possible roles for the SCN in the control of the rat estrous cycle, consistent with the above information. The positive feedback effect of estrogen may be dependent upon circadian signals generated by the SCN while the facilitatory influence of progesterone is not. Alternatively, since the SCN receives a direct retino-hypothalamic projection which is implicated in the LD entrainment of other circadian rhythms (Chapter Five), they may be necessary only for synchronization of circadian rhythms governing the estrous cycle, not for their generation. This latter hypothesis offers an explanation for the appearance of persistent estrous or irregular estrous cycles, and the variable magnitude of the progesterone induced LH surge observed after SCN lesions. These phenomena may represent desynchronization of the rhythms regulating the reproductive cycle in the rat. A similar "coupling" role has been suggested for the SCN in the control of the circadian rhythms of feeding, drinking and brain self-stimulation in laboratory rats (Boulos and Terman, 1979) and of running wheel activity in the golden hamster Mesocricetus auratus (Rusak, 1977).

A different role for the SCN is envisaged in a model for the rat estrous cycle outlined by Rusak and Zucker (1979) in which it is postulated that neurons of the SCN generate circadian rhythms which regulate both the release of LH - RH from the median eminence into the hypophyseal portal system, and the diurnal rhythm of estradiol secretion.
4.1.6 CHANGES IN VAGINAL CYTOLOGY DURING THE ESTROUS CYCLE.

The neuroendocrine events occurring during the estrous cycle of laboratory Rattus norvegicus are reflected in changes in the vaginal epithelial cells. These changes are readily monitored by taking regular vaginal smears. Six stages in the cycle have been identified on the basis of vaginal cytology (Mandl, 1951; Perry, 1971).

1. Early estrous or proestrus (about 18 h); a thick smear, mainly of basophilic nucleated epithelial cells.
2. Estrous (about 25 h); epithelial cells have lost their nuclei and become cornified. Towards the end of this phase the smear becomes "cheesy". Cornified cells are acidophilic.
3. Late estrous or metestrous (about 5 h); many cornified cells but also some large basophilic "Shorr" cells and some small basophilic epithelial cells.
4. Early diestrous (about 24 h); a thick smear consisting almost entirely of leucocytes; some cornified cells, Shorr cells and a few basophilic epithelial cells.
5. Diestrous (about 28 h); a thin smear, mainly leucocytes, practically no cornified cells, diminishing numbers of Shorr cells.
6. Late diestrous (about 7 h); leucocytes and some clearly nucleated basophilic epithelial cells, no cornified cells, no Shorr cells.

The duration of the diestrous part of the cycle is generally more variable than either proestrus or estrous. Ovulation occurs during late estrous (metestrous). Persistent estrus is indicated by the continued presence of cornified epithelial cells in vaginal smears (Lambert, 1975).

This pattern of changes in the vaginal cytology of laboratory rats was used as the basis for identification of the stages in the R. exulans estrous cycle from vaginal smears. (When smears are taken only at daily intervals, all six stages noted above are not necessarily sampled in each cycle.)
4.2 METHODS

Vaginal smears were taken daily between 0800 h and 1100 h using a small wire loop sterilized in an alcohol flame and cooled in distilled water. The vaginal debris thus obtained was smeared on a standard microscope slide and fixed and stained as described in Appendix IV. This procedure stained cornified cells yellowish-pink and nucleated epithelial cells and leucocytes purplish-blue. Three different groups of animals were examined in this way to investigate the estrous cycle of *Rattus exulans*.

The first experiment was designed to complement and extend the experiments on photoperiodic control of estrous described in Chapter Six. Ten perforate females (Experimental Group I) were collected in May 1979 i.e. during the non-breeding period (Moller, 1977; Bunn, 1979). Five were maintained in LD 8:16 (lights on 0900 h to 1700 h) and 5 in LD 16:8 (lights on 0500 h to 2100 h) for a total of 125 days. (Day 1 was 13/5/79). The animals were allowed 36 days to adjust to the housing conditions and for possible differential effects of the light regimes to begin to act. (No minimum time for the induction and/or inhibition of estrous cycling in these regimes was established (Chapter Six), however Bunn (1979) estimates that on Tiritiri female *R. exulans* take 4 - 6 weeks to achieve the "physiological readiness necessary prior to breeding"). Beginning on day 37, daily vaginal smears were taken for 20 days, followed by a break of 20 days, then for a further 20 days, followed by a similar break, and finally for 10 days. On day 126 the surviving 8 animals were transferred to dim LL (0.02 to 0.07 lux) and on day 168,6 were ovariectomized and 1 was sham operated (Chapter Five).

Experimental Group II consisted of 6 females collected as imperforate juveniles in May 1979. (Day 1 was 13/5/79.) These animals were housed in pairs in a separate room in natural light cycles at 20 ± 1°C. On day 130 one rat was transferred to the experimental cabinets and housed in dim LL until day 169, after which it experienced the same treatment as the remaining animals. On day 169 (18/10/79) all 6 rats were rehoused singly in the experimental cabinets in LD 16:8, at which time they were all found to be perforate. Daily vaginal smears were taken from day 180 to day 226 (47 days). The animals were transferred to LL dim on day 233. Between days 300 and 302, 2 of the surviving 5 animals were ovariectomized and 3 were sham operated (Chapter Five).
The third group of rats examined for estrous cycles (Experimental Group III) were 9 perforate females collected between 20th and 22nd November, 1979 i.e. probably early in the 1979-1980 breeding season (Moller, 1977; Bunn, 1979). These animals were maintained in LD 16:8 for 28 days (day 1 was 23/11/79) and daily vaginal smears were taken for 20 days starting on day 4. From day 29 onwards all animals were exposed to LL dim. Between days 96 and 98, 6 of the surviving 7 animals were ovariectomized and the seventh was sham operated (Chapter Five).
4.3 RESULTS

Three cell types were readily identifiable in *R. exulans* vaginal smears - cornified and nucleated epithelial cells and leucocytes. Estrous smears were distinctive in that they contained only cornified epithelial cells. Smears were considered to be metestrus when leucocytes appeared among the cornified cells. The stages of diestrus were categorized by the presence of leucocytes and a progressive increase in the number of nucleated epithelial cells. Smears in which nucleated epithelial cells predominated were considered to be prooestrus (Dewsbury et al., 1977).

On the basis of the experiments reported in Chapter Six, it was expected that the majority of the Group I animals housed in LD 16:8 would have been exhibiting estrous cycles by the end of the observation period, while those in LD 8:16 would not. However none of the rats in Experimental Group I exhibited estrous cycles during the two 20-day sequences and one 10-day sequence of smears taken over a period of 125 days. Each animal retained throughout a fairly stable ratio of the three cell types with leucocytes predominating. There was variability among individuals in the relative proportions of the cell types. The total absence of fully cornified smears indicates that high preovulatory estrogen levels were never attained and that ovulation did not occur (Lawton and Sawyer, 1968; Freeman et al., 1976). When the 5 animals that had been housed in LD 16:8 were subsequently ovariectomized (after 42 days in constant conditions), 3 had luteal tissue present in the ovaries, indicating recent ovulation.

The 6 animals in Experimental Group II were collected as juveniles in winter (May) and were housed in natural light cycles and constant temperature. By mid-October (near the expected beginning of the breeding season on Tiritiri) they had all become perforate. It was anticipated that they would have begun estrous cycling at around this time (Bunn, 1979) but in fact no animals showed regular estrous cycles during the 47 consecutive days that vaginal smears were taken. As in Group I, the smears mainly consisted of fairly stable proportions of leucocytes and cornified and nucleated epithelial cells. Three animals showed occasional increases in the proportion of cornified cells and one fully cornified smear was obtained but these instances were not part of any
regular sequence. Neither of the 2 animals subsequently ovariectomized (after 67 to 69 days in constant conditions) had luteal tissue present in the ovaries, indicating that neither had ovulated for some time prior to ovariectomy. (The "inactive" corpus luteum of the unmated laboratory rat persists for as long as the active corpus luteum of pseudopregnancy which lasts for about 14 days (Perry, 1971)).

The 9 perforate females in Experimental Group III were collected early in the breeding season when they would have been expected either to be undergoing estrous cycles or to be pregnant. On the first day of sampling (the fourth day after transfer to LD 16:8) 3 of these animals had fully cornified, "cheesy", estrous smears, one was metestrous and the remainder were diestrous. The 4 animals showing estrous or metestrous smears exhibited one complete estrous cycle (two 6-day and two 5-day cycles) and then reverted to the characteristic anoestrous smears dominated by leucocytes with (interindividually) varying proportions of nucleated and cornified epithelial cells. The remaining 5 animals showed no evidence of estrous cycling. All 6 animals subsequently ovariectomized (after 67 to 69 days in constant conditions) had luteal tissue present in the ovaries, indicating recent ovulation.

Daily handling apparently did not have any synchronizing effect on the activity rhythm. There was no significant difference between the mean phase angle in entrainment measured during the daily vaginal smear programme and the mean phase angle observed when the same animals were left undisturbed in an identical light regime (Table 4.1). Nor did handling, which occurred during the inactive phase, induce any abnormal subsequent activity (e.g. Fig. 3.16).
### TABLE 4.1
Effects of Daily Vaginal Smears on $\psi$
Between the Activity Rhythm and The Light Cycle.

<table>
<thead>
<tr>
<th>RAT NUMBER</th>
<th>$\psi_{vs}$</th>
<th>$\psi_{nvs}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>72</td>
<td>-0.3</td>
<td>-0.4</td>
</tr>
<tr>
<td>74</td>
<td>0.5</td>
<td>0.7</td>
</tr>
<tr>
<td>75</td>
<td>-0.3</td>
<td>-0.2</td>
</tr>
<tr>
<td>76</td>
<td>-0.3</td>
<td>-0.4</td>
</tr>
<tr>
<td>77</td>
<td>-0.4</td>
<td>-0.4</td>
</tr>
<tr>
<td>81</td>
<td>-0.2</td>
<td>-0.1</td>
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<tr>
<td>83</td>
<td>0.1</td>
<td>-0.1</td>
</tr>
<tr>
<td>84</td>
<td>-0.4</td>
<td>-0.4</td>
</tr>
<tr>
<td>86</td>
<td>-0.4</td>
<td>-0.2</td>
</tr>
<tr>
<td>87</td>
<td>-0.4</td>
<td>-0.4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>RAT NUMBER</th>
<th>$\psi_{vs}$</th>
<th>$\psi_{nvs}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>65</td>
<td>-1.0</td>
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<tr>
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<td>69</td>
<td>-1.1</td>
<td>-1.1</td>
</tr>
<tr>
<td>70</td>
<td>-1.3</td>
<td>-1.4</td>
</tr>
</tbody>
</table>

$t_i = 0.055$

$Pt > 0.9$

$t_i = -0.426$

$0.9 > Pt > 0.5$

**KEY:**

$\psi_{vs}$ phase angle between activity onset and onset of darkness, measured during the time that daily vaginal smears were being taken.

$\psi_{nvs}$ phase angle between activity onset and onset of darkness, measured when animals were not disturbed daily.
4.4 DISCUSSION

From the limited available evidence it appears that the sequence of smear types of the Rattus exulans estrous cycle resembles the characteristic pattern for muroid rodents (Dewsbury et al., 1977). It seems unlikely that R. exulans is an induced ovulator since data presented here and in subsequent Chapters indicate that ovulation occurs in animals maintained in isolation for prolonged periods. Although the data are minimal, the 5-6 day cycle length observed is well within the characteristic range for muroid rodents (Mandl, 1951; Dewsbury et al., 1977).

Experiments designed to examine the effects of photoperiod on the estrous cycle of R. exulans (Chapter Six) suggest that ovulation continues in LD 16:8 but not in LD 8:16. It was therefore expected that the majority of Group I animals in LD 16:8 would have begun estrous cycling in the course of the 125 day study, while those in LD 8:16 would have remained anoestrous (the non-breeding condition). The failure of all 10 rats to show estrous cycles prompted the third set of experiments, designed to establish whether or not this failure was indeed in direct contradiction to the experiments in Chapter Six, or if it might be attributable to other causes. That 3 out of 5 of the Group I animals in LD 16:8 subsequently showed evidence of recent ovulation (after 42 days in constant conditions) indicates that these animals at least were physiologically capable of ovulating.

The aim of the second set of experiments was to provide a group of estrous cycling, non-pregnant females. Juveniles collected in winter were allowed to mature in natural light cycles and constant temperature until around the beginning of the natural breeding season, at which time they were perforate. No animals showed regular estrous cycles and neither of the 2 rats subsequently ovariectomized showed any indication of recent ovulation. This suggests that these animals may not have reached reproductive maturity. (Perforate R. exulans females are not necessarily sexually mature (Wirtz, 1972; Moller, 1977).)

It is possible that some environmental factor (physical or social) critical for reproductive development was absent in the laboratory environment. Since animals were exposed to the natural photoperiod, it seems unlikely that this contributed to the delay in maturation, although
background light intensities were undoubtedly higher in the laboratory (in a built-up area) than in the field. Temperature influences the onset of puberty in some rodents, although its effects vary among species and also with factors related to the depression of growth rate (Sadlier, 1969). The comparatively high, constant temperature to which the Group II animals were exposed may thus have influenced their reproductive development. There is also evidence that reduction in the plane of nutrition delays puberty in laboratory rats (Sadlier, *loc. cit.*). While the Group II rats had *ad libitum* access to food and water, their mean weight at the time of transfer from LD natural to LD 16:8 was 60.8 ± 5.36 g (standard deviation). This is somewhat less than the estimated mean weight at which 50% of females on Tiritiri are perforate (69 ± 1.0 g; Moller, 1979) and considerably less than the estimated mean female weight at the beginning of the natural breeding season (75 g; Bunn, 1979). Data derived from other experiments in the present study do not, however, support the hypothesis that attainment of reproductive maturity in female *R. exulans* is dependent on attainment of a threshold weight (Chapter Six). Stress induced by the daily handling involved in taking vaginal smears may well have contributed to the apparently delayed onset of puberty in Group II rats.

In view of the seemingly contradictory data obtained from Experimental Group I animals, 9 females (Experimental Group III) were collected from Tiritiri at a time anticipated to be early in the 1979-1980 breeding season, when they were expected to have been ovulating or pregnant. Four of these rats exhibited one complete estrous cycle before their vaginal cytology reverted to the steady diestrous condition described earlier. All 6 animals ovariectomized (72 to 74 days after vaginal smears were stopped) had luteal tissue present in the ovaries, indicating recent ovulation i.e. they were physiologically capable of ovulating. These observations, together with evidence that ovulation occurs in animals maintained in LD 16:8 for over 100 days (Chapter Six) suggest that both the housing conditions and the light cycle were permissive to ovulation and presumably to estrous cycling. The most likely explanation for the failure of estrous cycling in the Group III animals is therefore some stress-related effect associated with daily handling of these wild rodents. Such a response could also well be a factor in the failure of Group I rats in LD 16:8 to show estrous cycles.
Pseudopregnancy can be induced in laboratory rats by a variety of types of stress which are unrelated to reproductive function, as well as by mechanical stimulation of the cervix (Richter, 1965). Daily handling and/or taking of vaginal smears might thus have induced pseudopregnancies in R. exulans, however several observations argue against this interpretation. Pseudopregnancy in laboratory rats is characterized by marked inactivity, greatly increased food intake, increased body weight, diestrous vaginal smears, persistent corpora lutea, and the ability of the uterus to form deciduomata (Richter, 1965). While most R. exulans exhibited continuous diestrous smears, there was no conspicuous increase in food intake and the amplitude of the activity rhythm remained unaltered throughout the daily vaginal smear programme. In between pseudopregnancies in laboratory rats, i.e. at approximately 14 day intervals, all the functions noted above quickly return to their normal state. R. exulans were observed to retain diestrous smears for 20 days or more without any indication of ovulation and there were no changes in the total amount or distribution of activity during this time. Assuming that the duration of pseudopregnancy in R. exulans would be similar to that in R. norvegicus (the estrous cycle and pregnancy are of similar durations (Perry, 1971; Wirtz, 1973; this study)), it thus seems unlikely that the continuous diestrous smears observed in R. exulans were due to pseudopregnancies. It is therefore concluded that this persistent diestrous condition represents an anoestrous (non-breeding) state.

The difficulties encountered in monitoring estrous cycles precluded any definite experiments to test the hypothesis of circadian clock involvement in the control of estrous in R. exulans. (Facilities for LH assay were not available during this study but this alternative method of monitoring estrous cycles and ovulation may be pursued in the future.) A number of observations from the experiments described in this Chapter do, however, provide indirect evidence in support of this hypothesis. The close similarities between the estrous cycles of R. exulans and R. norvegicus may well indicate similarities in mechanism. The ovariectomy experiments mentioned here and described fully in the following Chapter indicate that animals continued to ovulate for prolonged periods in constant conditions prior to surgery. This suggests that ovulation is under endogenous control in R. exulans as it is in R. norvegicus.
The data discussed in Chapter Six also provide indirect evidence which supports the hypothesis that the estrous cycle is controlled by a circadian time-keeping system.
CHAPTER FIVE: INTERACTIONS BETWEEN THE ACTIVITY RHYTHM AND THE ESTROUS CYCLE
5.1 INTRODUCTION

It has long been recognized that the pattern of locomotor activity of female laboratory rats varies with the estrous cycle (Richter, 1965 cites Wang, 1923). In both free-running (blinded) and entrained 4-day animals, the total amount of wheel-running activity is very much greater on the evening of proestrous through to the morning of estrous, than on the intervening three nights (Richter, 1965, 1970; Raisman and Brown-Grant, 1977). This increased running activity is associated with high circulating estrogen levels (Young and Fish, 1945). Both the activity rhythm (Chapter Three) and the estrous cycle (Chapter Four) of laboratory Rattus norvegicus are under circadian control. In LD entrainment a consistent phase relationship is observed between activity onset on the afternoon of proestrous and ovulation, as determined by laparotomy. This relationship is conserved when animals are released into constant conditions (Carter, 1972; Sridaran and McCormack, 1977 b). These observations suggest that the two rhythms may be controlled by a common circadian pacemaker. Further support for this hypothesis comes from experiments which indicate that destruction of the suprachiasmatic nuclei (SCN) in adults disrupts both rhythms (Raisman and Brown-Grant, 1977). Neonatal ablation of the SCN produces persistent vaginal estrous in adult females and the circadian rhythms of locomotor activity and drinking are also absent (Mosko and Moore, 1978).

On the other hand, it has been claimed that the circadian mechanisms controlling estrous cyclicity and wheel-running activity are separable (Weber and Adler, 1978). This claim is based on the absence of a correlation between the onset of persistent estrous and "free-running" activity rhythms in a range of long photoperiods (in 24 h cycles) and in non-24 h light cycles. There are a number of criticisms of this experimental protocol. In the proposed model for circadian control of estrous in R. norvegicus it is envisaged that a circadian pacemaker regulates hypothalamic LH – RH release. In the persistent estrous condition this rhythm is not expressed i.e. an ovulatory surge of LH is never released by the pituitary and ovulation does not occur. This stationary state of the overt rhythm may be due either to the pacemaker being stopped, or to the overt rhythm becoming uncoupled from it, while the pacemaker continues to oscillate. In either case, this is not an
analogous phenomenon to the failure of entrainment of an overt rhythm, which reflects the inability of the zeitgeber regime to synchronize the pacemaker. Whereas the estrous cycle stops in persistent estrous, the activity rhythm does not cease outside its range of entrainment. Persistent estrous also develops in laboratory rats in constant light. It would therefore be more appropriate to compare the onset of persistent estrous and of arrhythmic activity in LL to see if the circadian control of these rhythms is separable. To establish that the circadian mechanism(s) stop in LL, it would be necessary to demonstrate that the rhythms always restart at a specific phase, regardless of the (solar day) time of release from LL. Available evidence is therefore considered to be consistent with the hypothesis that a common circadian mechanism regulates both the activity rhythm and the estrous cycle of *Rattus norvegicus*, although critical experiments to test whether or not the control of these rhythms is separable have not yet been published.

A similar hypothesis would appear to pertain to the estrous cycle and the activity rhythm of the hamster *Mesocricetus auratus*. There is a consistent phase relationship in constant conditions between the onset of wheel-running and the onset of behavioural receptivity (Fitzgerald and Zucker, 1976). There is also a fixed phase relationship between activity onset and peak preovulatory gonadotropin levels in LD cycles (Stetson et al., 1977b). Damage to the SCN disrupts both rhythms (Stetson and Watson – Whitmyre, 1976; Watson-Whitmyre and Stetson, 1977), and the locomotor activity rhythm breaks down at the same time as females develop persistent estrous after prolonged exposure to LL (Stetson and Watson-Whitmyre, 1976). On the other hand, the estrous cycle (as indicated by times of onset of behavioural receptivity) re-entrains more rapidly than the activity rhythm following LD 12:12 photoperiod reversal (Finkelstein et al., 1978). These two rhythms can thus be temporarily dissociated. This does not necessarily indicate, however, that they are controlled by separate circadian pacemakers. If a common pacemaker phase shifted instantaneously (e.g. Pittendrigh, 1965), the different rates of re-entrainment might represent the two overt rhythms regaining their normal phase relationships to it at different rates.

The locomotor activity rhythm of the female hamster, whether entrained or free-running, exhibits a characteristic "scalloped" pattern when the animal is undergoing estrous cycles. Activity onset occurs comparatively
earlier on days when estradiol secretion is highest (Morin et al., 1977). Estradiol benzoate administered by subcutaneous implants to ovariectomized hamsters advances activity onset relative to the light cycle in LD 12:12 and shortens the period of the activity rhythm in DD (blinded animals). After-effects of this estradiol-induced period shortening are in evidence up to 65 days after administration ceases. These observations indicate unequivocally that estradiol must act on the circadian time-keeping mechanism regulating the locomotor activity rhythm. Estradiol does not, however, appear to influence the suprachiasmatic nuclei directly, unless its effect is via some non-classical binding system.

Although comparable experiments do not appear to have been performed with laboratory rats, the onset of the most intensive locomotor activity phase during the estrous cycle also coincides with the highest circulating estradiol levels in this species (Carter, 1972). Experiments were therefore designed to examine the hypothesis that estradiol has a feedback action on the circadian mechanism regulating activity in Rattus exulans.
5.2 METHODS

To determine whether or not linkage mediated by the ovaries exists between the control mechanisms of the estrous cycle and the locomotor activity rhythm of Rattus exulans, the effects of ovariectomy (OVX) or sham ovariectomy (SHAM) on the free-running period of the activity rhythm were examined.

Prior to the first series of operations (see below) animals were maintained in dim LL (0.02 to 0.07 lux) for 27 - 33 days and \( T \) before was determined for the 10 cycles immediately preceding surgery. Surgical procedures involved removal of the rats from the experimental cabinets (Chapter Two) and exposure to about 8 h of natural daylight. The animals were subsequently returned to dim LL and \( T \) after was measured over 10 consecutive cycles, beginning at least 10 days after surgery. This interval was allowed for recovery and for possible phase shifts and transients resulting from the light exposure to be completed. Prior to the second series of operations (see below) animals free-ran for 68 - 70 days in dim LL and \( T \) before was determined over the 10 cycles immediately preceding surgery in 8 animals and 25 - 35 days earlier in 5 animals. (There were insufficient recording channels available at this time to monitor all animals simultaneously.) The free-running periods of the activity rhythms after OVX or SHAM were measured as in the first set of experiments.

Because of a lack of any surgical facilities in the Zoology Department, and of facilities in which feral rats were permitted in the University of Auckland School of Medicine, the first ovariectomies of Rattus exulans were performed by veterinarians of the Middlemiss Clinic, 1127 New North Road, Auckland. I am indebted to all the staff of the clinic for their co-operation and interest. Basal anaesthesia was achieved using an intramuscular injection of Saffan and deep anaesthesia maintained with halothane (2 bromo-2-chloro-1,1,1-trifluoroethane). Ovariectomy was performed by drawing each ovary in turn out through a medial ventral incision approximately 1.5 cm long and severing the uterus at the base of the coiled oviduct. The body wall and skin were then sutured. Sham ovariectomy was performed by making the incision through the skin and the body wall, drawing out and then replacing the ovaries, and finally suturing the body wall and skin.
R. exulans proved a difficult anaesthetic subject (also see below). Of the 8 animals anaesthetized, 3 died during surgery and 2 shortly after without regaining consciousness. Of the remaining 3 animals, one SHAM died 5 days later and the remaining SHAM and OVX survived.

Subsequently a further series of ovariectomies and sham operations was performed in improvised facilities in the Zoology Department by Professor C.R. Austin, University of Cambridge, whose skill and generous use of his sabbatical time I gratefully acknowledge. Equipment was loaned, and technical advice given by the Department of Anatomy, University of Auckland School of Medicine, to whom I am also indebted. Initially ether anaesthesia was attempted (Ether U.S.P. for anaesthesia, J.T. Baker Chemical Co. Phillipsburg N.J. 08865). The subject was placed in a 2 litre jar containing ether-soaked cotton wool and induction of basal anaesthesia occurred very rapidly (5 - 10 sec). The animal was then removed and deep anaesthesia maintained by periodically placing over the nose a 20 ml jar containing a small swab of ether-soaked cotton wool. Even without this improvised mask, in both females tested breathing rapidly became shallow and death resulted within 5 - 10 min, without any attempt at surgery. To investigate the generality and possible causes of this surprising sensitivity to ether, four male R. exulans were subsequently anaesthetized using the same technique. They were successfully maintained at operative level anaesthesia for 20 min and all survived. The only obvious explanation for this difference might be the greater body weight of the males, which may have resulted in lower initial ether concentrations.

Nembutal (sodium pentobarbital, 3 mg/100 g body weight, intraperitoneally) was found to induce basal anaesthesia in 10 - 15 min and with infrequent applications of the improvised ether mask, deep anaesthesia could be maintained for prolonged periods. Animals took 4 - 5 h to become fully conscious after this dosage of Nembutal. This technique proved entirely successful and no animals died during surgery or recovery. (Animals were transferred to a recovery room in natural light cycles at about 30°C for at least 18 h after surgery.)

Ovariectomy was performed through a mid-dorsal incision (about 1.5 cm in length) through the skin. The surrounding connective tissue between skin and body wall was broken and the skin incision then
manoeuvred to a more lateral position and an incision made through the body wall on each side in turn. Body wall incisions were sutured, while skin was either sutured or closed using Michel metal clips. The same procedure was followed in sham operations except that the ovaries were drawn out and then replaced, not removed.

All ovaries removed were fixed, sectioned and stained as described in Appendix V. The presence or absence of luteal tissue was subsequently ascertained.
### 5.3 RESULTS

The results of these experiments are summarized in Table 5.1.

**TABLE 5.1**

Effects of OVX and SHAM on $\bar{T}$ of the Activity Rhythm.

<table>
<thead>
<tr>
<th>RAT NO.</th>
<th>$\bar{T}$ before</th>
<th>S.D.</th>
<th>OVX</th>
<th>SHAM</th>
<th>$\bar{T}$ after</th>
<th>S.D.</th>
<th>LUTEAL TISSUE PRESENT</th>
<th>$t_i$</th>
<th>Pt</th>
</tr>
</thead>
<tbody>
<tr>
<td>66</td>
<td>24.5</td>
<td>0.38</td>
<td>✓</td>
<td>✓</td>
<td>24.9</td>
<td>0.37</td>
<td>-</td>
<td>-2.137</td>
<td>0.1&gt; P &gt; 0.05</td>
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<tr>
<td>84</td>
<td>23.7</td>
<td>0.25</td>
<td>✓</td>
<td>✓</td>
<td>23.9</td>
<td>0.37</td>
<td>-</td>
<td>-1.479</td>
<td>0.2&gt; P &gt; 0.1</td>
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<tr>
<td>85</td>
<td>25.0</td>
<td>0.53</td>
<td>✓</td>
<td>✓</td>
<td>24.8</td>
<td>0.30</td>
<td>-</td>
<td>-1.117</td>
<td>0.4&gt; P &gt; 0.2</td>
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<tr>
<td>86</td>
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<td>✓</td>
<td>✓</td>
<td>23.9</td>
<td>0.22</td>
<td>-</td>
<td>2.616</td>
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<tr>
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<td>✓</td>
<td>24.8</td>
<td>0.30</td>
<td>-</td>
<td>-1.282</td>
<td>0.4&gt; P &gt; 0.2</td>
</tr>
<tr>
<td>66</td>
<td>24.9</td>
<td>0.16</td>
<td>✓</td>
<td>✓</td>
<td>24.3</td>
<td>0.18</td>
<td>NO</td>
<td>3.214</td>
<td>0.02&gt; P &gt; 0.01*</td>
</tr>
<tr>
<td>81</td>
<td>24.4</td>
<td>0.40</td>
<td>✓</td>
<td>✓</td>
<td>24.8</td>
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<td>NO</td>
<td>-2.785</td>
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<td>✓</td>
<td>24.3</td>
<td>0.23</td>
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<tr>
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<td>✓</td>
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</tr>
<tr>
<td>88</td>
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<td>0.32</td>
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<td>✓</td>
<td>24.6</td>
<td>0.30</td>
<td>YES</td>
<td>-0.058</td>
<td>P &gt; 0.9</td>
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<td>✓</td>
<td>✓</td>
<td>24.3</td>
<td>0.30</td>
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<td>0.743</td>
<td>0.5&gt; P &gt; 0.4</td>
</tr>
<tr>
<td>91</td>
<td>24.3</td>
<td>0.25</td>
<td>✓</td>
<td>✓</td>
<td>24.4</td>
<td>0.24</td>
<td>YES</td>
<td>-1.023</td>
<td>0.4&gt; P &gt; 0.2</td>
</tr>
<tr>
<td>94</td>
<td>24.2</td>
<td>0.32</td>
<td>✓</td>
<td>✓</td>
<td>24.3</td>
<td>0.21</td>
<td>YES</td>
<td>-0.523</td>
<td>0.9&gt; P &gt; 0.5</td>
</tr>
</tbody>
</table>

* Significant at the 95% level.

Comparison of mean $\bar{T}$ before SHAM with mean $\bar{T}$ after SHAM.

$t_i = -0.316$

0.9 > Pt > 0.5

Comparison of mean $\bar{T}$ before OVX with mean $\bar{T}$ after OVX in rats without luteal tissue.

$t_i = -0.111$

Pt > 0.9
Comparison of mean $\bar{T}$ before OVX with mean $\bar{T}$ after OVX in rats with luteal tissue.

$t_i = -0.225$

$0.9 > P_t > 0.5$

No differences in the overall amount or distribution of activity were evident before and after either SHAM or OVX (Fig. 5.1). The combined mean period before sham ovariectomy is not significantly different from that after. This finding indicates that the operative procedures did not have any long-term effects on the free-running period of the locomotor activity rhythm. (All animals showed phase changes in their activity rhythms before and after SHAM or OVX. The extent to which these are attributable to actual surgery cannot however, be ascertained, since animals were also exposed to natural light during and after operations - see Section 5.2)

The combined mean period before ovariectomy (with or without luteal tissue present) is not significantly different from that after. Three of the four animals which had not ovulated prior to ovariectomy showed significant changes in $\bar{T}$ before and after surgery. Since either period lengthening or period shortening was observed, it is concluded that these period changes were not systematic responses to the treatment, but rather were attributable to other causes. In both cases where $\bar{T}$ lengthened significantly after OVX, there is evidence that when $\bar{T}$ before was measured (45 to 35 days before surgery), the rhythm was still undergoing after-effects of previous LD 16:8 entrainment. This would result in an abnormally short estimate of $\bar{T}$ before (Section 3.3.1.2).

The combined data support the hypothesis that in Rattus exulans there is no significant interaction between the ovaries and the circadian pacemaker controlling locomotor activity.
FIGURE 5.1

Activity Record of a Rat Before and
After Ovariection.

Part of an activity record in constant conditions (LL 0.02 to
0.09 lux, 21 ± 1°C).

\bar{T} \text{ for the 10 cycles immediately preceding ovariection} = 24.6 \text{ h}

\bar{T} \text{ for the last 10 cycles illustrated} = 24.6 \text{ h}

t_1 = -0.058 \quad P > 0.9
5.4 DISCUSSION

The locomotor activity rhythms of female *Rattus exulans* do not exhibit regular 5 - 6 day fluctuations in either period or in intensity of activity in association with the estrous cycle. The rat whose activity record is illustrated in Fig. 5.1, for example, was ovulating prior to ovariectomy but there is no indication of associated fluctuations in the activity rhythm. These observations contrast with the occurrence of exceptionally intense activity around the time of ovulation in laboratory rats and the systematic fluctuation of female hamster activity rhythms with estradiol levels during the estrous cycle (Section 5.1).

The observed lack of interaction between the activity rhythm and the estrous cycle in intact *R. exulans* is reinforced by the finding that ovariectomy has no significant effect on the period of the activity rhythm (Table 5.1). Nor are there any observable differences in the patterns of distribution, or intensity of activity before and after ovariectomy (e.g. Fig. 5.1). It is therefore concluded that there is no feedback action of the ovaries or estradiol on the circadian pacemaker controlling the locomotor activity rhythm in this species. This proposition is supported indirectly by the observation that there are no significant changes in either period or variability of activity rhythms (Table 3.2), associated with the degenerative changes that occur in the reproductive system with old age (Huang, et al., 1978).

Experiments reported by Richter (1970) imply that the circadian mechanism regulating locomotor activity in *R. norvegicus* may be involved in synchronizing mating behaviour. Blinded male and female laboratory rats were housed in pairs, one of each pair having an activity rhythm with a free-running period longer than 24 h and the other a period shorter than 24 h. Mating apparently occurred only when the female came into behavioural estrous at times when there was also sufficient overlap between the active phases of the two animals. Under the influence of natural zeitgebers, the circadian systems of wild rats never free-run. Nevertheless the results of these experiments in constant conditions suggest that the circadian mechanism regulating activity may have a role in synchronizing mating. In field conditions, behavioural receptivity and the period during which the ova remain fertile must coincide with the time when the female has the maximum
probability of encountering a mate. This presumably corresponds to the time when both males and (receptive) females are most active. The increased activity of female laboratory rats on the night of ovulation may thus reflect a mechanism moulded by selection to enhance the probability of finding a mate. A similar interpretation may apply to the early onset of activity on the night of ovulation in female hamsters (Section 5.1). Sensitivity of the circadian mechanism regulating activity to high preovulatory circulating estradiol levels has been demonstrated in hamsters (Morin et al., 1977). This sensitivity would ensure appropriate synchronization of enhanced activity with ovulation.

The experiments reported in this Chapter indicate however, that R. exulans does not exhibit earlier or more intense activity in association with ovulation. The probability of a receptive female R. exulans finding a mate must therefore be sufficiently high without any modification of the normal timing and intensity of activity. This may reflect differences in social organization among the species. Alternatively or in addition, the timing of activity relative to the day/night cycle in wild R. exulans may be sufficiently critical that selection has acted against early onset of activity in receptive females. Changes in activity patterns associated with the estrous cycle have apparently been reported only in laboratory rats and hamsters, which have been removed from such selection pressures for many generations.

The hypothesis that the estrous cycle and the activity rhythm are under common circadian control could not be directly examined in R. exulans because of the difficulty of identifying specific phase points in the estrous cycle (Chapter Four). This hypothesis is supported, however, by available evidence for laboratory rats and hamsters (Section 5.1). Experiments involving neonatal ablation of the suprachiasmatic nuclei (SCN) indicate an absence of functional and morphological plasticity in the development of the circadian system of laboratory rats (Mosko and Moore, 1978). In view of their findings, Mosko and Moore suggest that the central neural mechanisms necessary for circadian rhythm generation are highly genetically specified and have a very limited representation in the mammalian brain. It would therefore seem unlikely that there are major differences in the physiology of circadian pacemaker mechanisms between R. exulans and R. norvegicus. The experiments reported in this Chapter suggest, however, that there may be differences
between closely related species in interactions among the various circadian rhythms within an individual.

Two major lines of evidence have focused attention on the retina-hypothalamic tract (RHT) and the suprachiasmatic nuclei (SCN) of the hypothalamus which appear to play a crucial role in temporal organization of rodents and possibly other mammals (Kawamura and Ibuka, 1978; Menaker et al., 1978; Rusak and Zucker, 1979). The existence in the rat of the RHT with terminals in the SCN was suggested by the work of Stephan and Zucker (1972a,b) and confirmed by Moore and Lenn (1972). Comparable retino-hypothalamic projections have since been found in a diversity of mammals (reviewed in Kawamura and Ibuka, 1978; Rusak and Zucker, 1979). The RHT appears to be the major route mediating the entraining effects of environmental light cycles on rodent circadian rhythms (Moore and Eichler, 1972; Stephan and Zucker, 1972 a,b; Moore and Klein, 1974; Mosko and Moore, 1978; Stephan and Nunez, 1978).

Groos and Mason (1979) have investigated the functional properties of the RHT and conclude that these properties are in accordance with the known parametric and non-parametric effects of light on circadian rhythms. Some recent evidence suggests, however, that photic information may also have access to the circadian system through other pathways (Rusak, 1977b; Menaker et al., 1978; Rusak and Zucker, 1979).

The growing list of circadian rhythmic functions which in rodents have been found to be disrupted by damage to the SCN and environs is summarized in Table 5.2.
### TABLE 5.2
Circadian Rhythms Disrupted by SCN Damage

<table>
<thead>
<tr>
<th>RHYTHM</th>
<th>ANIMAL</th>
<th>REFERENCE</th>
</tr>
</thead>
</table>
| Ovulation                     | laboratory rat
t (Rattus norvegicus)             | Koves and Halasz, 1970,
Brown-Grant and Raisman, 1977,
Raisman and Brown-Grant, 1977,
Kimura and Kawakami, 1978,
| Drinking                      |                                      | Stephan and Zucker, 1972 a.                    |
|                               |                                      | Stephan and Nunez, 1977,                      |
|                               |                                      | Mosko and Moore, 1978,                        |
|                               |                                      | Nagai et al., 1978.                           |
| Eating                        |                                      | Nagai et al., 1978.                           |
|                               |                                      | Raisman and Brown-Grant, 1977,                |
|                               |                                      | Stephan and Nunez, 1977,                      |
| Adrenal corticosterone content|                                      | Moore and Eichler, 1972.                      |
| Pineal n-acetyl transferase   |                                      | Raisman and Brown-Grant, 1977.                |
| activity                      |                                      | Moore and Klein, 1974,                        |
|                               |                                      | Raisman and Brown-Grant, 1977,                |
|                               |                                      | Klein and Moore, 1979.                        |
| Sleep-wakefulness             |                                      | Ibuka and Kawamura, 1975,                     |
|                               |                                      | Ibuka et al., 1977,                           |
|                               |                                      | Mouret et al., 1978,                          |
|                               |                                      | Stephan and Nunez, 1977.                      |
| Brain temperature             |                                      | Stephan and Nunez, 1977.                      |
| Heart rate                    |                                      | Saleh and Winget, 1977.                       |
| Locomotor activity            | hamster (Mesocricetus auratus)       | Stetson and Watson-Whitmyre,1976,
|                               |                                      | Rusak, 1977a.                                |
| Drinking                      |                                      | Stetson and Watson-Whitmyre,1976,
| Estrous cyclicity             |                                      | Rusak and Morin, 1976,                        |
| Photoperiodic time measurement|                                      | Stetson and Watson-Whitmyre,1976.             |
There are numerous problems associated with lesioning and sectioning techniques, and experimental design and interpretation of results in this field (Rusak and Zucker, 1979). Nevertheless, the involvement of the SCN in regulation of such a diversity of rhythms indicates that it has a major role in circadian organization in rodents.

Regional brain functional activity is closely coupled to regional brain energy utilization (Schwartz and Gainer, 1977). Glucose consumption of the SCN, in contrast to other brain structures, is a function of both time of day and environmental lighting conditions, although this cycle has not yet been shown to be endogenous. Recent data suggest that the rat SCN may have a circadian rhythm of firing rate in vitro and in vivo (Groos, 1979).

The actual role of the SCN in the circadian organization of rodents remains unresolved. In several recent studies, residual circadian or ultradian rhythmicity has been found to persist in a variety of rhythms following SCN ablation (Rusak, 1977 a; Boulos and Terman, 1979; Powell et al., 1979; Stephan et al., 1979; Wiegand et al., 1979). These observations suggest that the SCN may normally function as a master oscillator that influences a population of self-sustained sub-oscillators and mediates the entraining effects of environmental lighting via the RHT. Alternatively the SCN may serve some as yet unknown coupling function among the other oscillators.
6.1 INTRODUCTION

Among temperate zone mammals, timing of the annual breeding season is often regulated predominantly by seasonal changes in photoperiod (Sadlier, 1969; Farner et al., 1973; Elliot, 1976). There is now extensive evidence to support the hypothesis, originally proposed by Bunning in 1936 (cited Bunning, 1960), that circadian time-keeping systems underly photoperiodic mechanisms in many organisms (Elliot, 1976; Farner et al., 1977; Saunders, 1978; Bunning, 1979).

*Rattus exulans* breeds seasonally on Tiritiri Island and its annual breeding cycle has been monitored from 1974 to 1979 (Moller, 1977; Bunn, 1979). A number of field observations on the breeding patterns of this species (detailed below) from these and other studies, suggest that a photoperiodic mechanism may be involved in controlling the timing of the breeding season in *R. exulans*. In view of these observations, and of the likelihood of circadian involvement in the control of estrous (Chapter Four), experiments were designed to examine the possible role of photoperiod in the control of estrous cycling and therefore in the timing of the breeding season.

6.1.1. REGULATION OF BREEDING SEASON IN TEMPERATE ZONE MAMMALS.

The timing of breeding in many temperate zone mammals can be viewed as a complex compromise synchronizing the most vulnerable stages from conception to weaning with a fixed optimal season (Sadlier, 1969). In most non-hibernating rodents the length of time from conception to weaning is sufficiently short that more than one conception occurs during the fixed optimal season. The mechanisms regulating the timing of the transitions from the non-breeding to the breeding condition and vice versa are of particular interest for chronobiological studies. The expression of the first estrous and the cessation of reproduction after the last lactation period (or after the last parturition in animals such as rats which exhibit post-partum estrous), are temporally distinct events presumably related to specific physiological changes and influenced by various environmental variables.
The importance of seasonal effects in the control of breeding in *Rattus exulans* is indicated (Sadlier, *loc. cit.* ) by the finding that the length of the breeding season shows a significant negative correlation with latitude. This generalization derives from data on populations in Malaya and throughout the Pacific (Table 6.1, after Moller (1977) Table 5.3).

**TABLE 6.1**

Variation in Length of Breeding Season with Latitude.

<table>
<thead>
<tr>
<th>LOCALITY</th>
<th>LATITUDE</th>
<th>LENGTH OF BREEDING SEASON (MONTHS)</th>
<th>REFERENCES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tiritiri, N.Z.</td>
<td>36.6°S</td>
<td>3 - 4</td>
<td>Moller (1977)</td>
</tr>
<tr>
<td>Kure Atoll</td>
<td>28°N</td>
<td>8</td>
<td>Wirtz (1972)</td>
</tr>
<tr>
<td>Oahu, Hawaiian Islands</td>
<td>21°N</td>
<td>12</td>
<td>Tamarin and Malecha (1972)</td>
</tr>
<tr>
<td>Ponope</td>
<td>7°</td>
<td>12</td>
<td>Jackson (1962)</td>
</tr>
<tr>
<td>Malaya</td>
<td>3°</td>
<td>12</td>
<td>Harrison (1951)</td>
</tr>
</tbody>
</table>

\[
r = -0.87 \quad p < 0.05
\]

Breeding peaks are apparently correlated with rainfall in Fiji. A seasonal pattern of reproduction is suggested on Oahu, with low reproduction in winter, and on Ponope. Only in Malaya has no marked seasonal variation in breeding been found.

These observations do not, however, indicate conclusively which seasonally varying aspects of the environment serve to cue physiological preparation for, and onset of, breeding and which factors influence its cessation. Four major environmental parameters have been most commonly implicated in regulation of breeding in mammals: daylength, temperature, precipitation and the nutritional status of the environment (Sadlier, 1969). The annual pattern of variation in daylength is the most reliable indicator of season in temperate latitudes.
Detailed understanding of the mechanisms controlling the timing of breeding in mammals is hampered by several methodological difficulties. Firstly, correlation between annually occurring environmental and physiological events can only be established by many years of observation to confirm its consistency, and observation alone cannot establish if the relationship is causal. Secondly, there is a danger of emphasising obvious and easily monitored environmental factors without any understanding of their relative causal importance (Sadlier loc. cit.). On the other hand, manipulation of single variables in a controlled laboratory environment does not give a comprehensive picture of the responses of the animal in the complex of environmental factors that it normally experiences (Ferry, 1971). These approaches obviously complement each other. The experiments described in this Chapter were thus designed to complement the available field observations and experimentation on breeding patterns of *R. exulans* on Tiritiri.

**Photoperiod**

The seasonal pattern of change in daylength is implicated as a major factor controlling the timing of breeding in many temperate zone mammals (Sadlier, 1969; Farner et al., 1973; Elliot, 1976). The onset of breeding is effectively the culmination of a series of physiological changes which bring the animal into breeding condition. These changes must be initiated in advance of the optimum environmental conditions for survival of the vulnerable stages from conception to weaning. Photoperiod is the most reliable cue for synchronizing this preparation. Other factors such as temperature and food availability may serve as fine tuning agents, modifying the actual timing of onset of breeding within an interval determined by the photoperiodically induced physiological readiness of the animal.

Sadlier (1969) discusses several observations on non-experimental mammals which indicate that seasonal photoperiod changes may be involved in the control of breeding. They are: relatively constant onset of breeding from year to year in a given location, variation in the duration of the breeding season with latitude, and alteration of the breeding season by transfer of animals across the equator. Information on all three of these lines of indirect evidence is available for *R. exulans*.

The first conceptions in the Tiritiri population apparently occur consistently from year to year in early to mid-November. (Data are
available for the breeding seasons beginning in November 1974, 1975, 1976 (Moller, 1977) and 1977, 1978 (Bunn, 1979).) Females seem to mature in synchrony at about the same time each year, whereas males appear to mature at varying times and weights (Moller, Bunn loc. cit.). These observations suggest that the regularity of onset of breeding is probably the result of year-to-year regularity in the timing of the first behavioural estrous in females. On Tiritiri, adults breeding in one season seldom, if ever, survive to the next. The onset of breeding is therefore probably determined by the attainment of reproductive maturity and breeding condition in females born in the previous season, rather than by mature females coming back into breeding condition. (This is not true of all R. exulans populations (Wirtz, 1972; J. Craig pers. comm.).) During the early part of the 1974/75 breeding season on Tiritiri there was a distinctly bimodal distribution of weights of juveniles, which suggests a high degree of synchrony of pregnancy (Moller, 1977). The relatively constant time of onset of breeding and the regular, synchronous maturation of females both suggest the involvement of an environmental cue which is highly consistent from year to year. The most consistent cue available is the seasonal change in photoperiod.

The trend to decreasing length of breeding season with increasing latitude in R. exulans populations has already been noted. Similar patterns have been observed in ungulates, lagomorphs, mustelids, and some other rodents (Sadlier, 1969).

Information is not available for R. exulans on the effects of transequatorial displacement on the breeding season of individuals from the same population. The breeding season of a Northern Hemisphere population (that on Kure Atoll, 28.4°N) can, however, be compared with that of the Tiritiri population (36.6°S). Whereas breeding on Tiritiri begins in November and continues for 3 - 4 months, on Kure it occurs from January until September, with most litters being born between May and August (Wirtz, 1972). The time of peak breeding in one population thus coincides with the non-breeding period of the other. The termination of breeding on Tiritiri is apparently largely determined by food availability (Bunn, 1979). This may well contribute (in addition to the difference in latitude) to the very short breeding season on Tiritiri by comparison with that on Kure.
Further indirect evidence implicating photoperiod in the control of the timing of breeding in *R. exulans* comes from two instances where laboratory colonies of this species have been established with individuals from seasonally breeding populations. As noted above, *R. exulans* on Kure Atoll have an 8 month breeding season. Laboratory colonies in natural light cycles on Kure and later transferred to Washington maintained this breeding season (Wirtz, 1972). When the colony was transferred to LD 12:12 at Cornell, this breeding pattern was lost (Wirtz, 1973). Similarly, a colony of *R. exulans* founded with animals from Cuvier Island, New Zealand and maintained in the Wellington Zoological Park in an LD 12:12 regime, showed a winter nadir and spring upsurge in birth rate in the first year after transfer, but has subsequently bred continuously (Averill and Wodzicki, 1975; Davis, 1976).

**Temperature**

Seasonal temperature changes have been proposed to have a modifying effect on the onset of breeding in a number of temperate zone mammals. Reviewing available data from field observations, Sadlier (1969) concludes that although photoperiod commonly stimulates mammals into a state of physiological readiness, temperature variations can alter, within limits, the actual onset of breeding. There are, as Sadlier notes, exceptions to such broad generalizations. The overall picture in rodents is complicated by hibernation in some species and by the possibility that temperature sometimes acts in combination with photoperiod.

**Nutrition and Rainfall.**

There is evidence that nutrition and rainfall may influence breeding in some mammals. Rodents in desert regions tend to breed immediately vegetation starts to improve after rain has fallen, and synchronization of breeding with respect to the rainy season is observed in some tropical species (Sadlier, 1969). If rainfall and vegetation fluctuations follow a seasonal pattern however, then photoperiod changes cannot be precluded as a possible factor in the regulation of breeding. Nutrition has been demonstrated to be a major factor in the control of breeding in some lagomorphs (Sadlier, *op. cit.*). Moller (1977) reviews evidence indicating that occurrence of winter breeding in some New Zealand populations of *Rattus rattus* and *Mus musculus* may be correlated with the availability of certain food sources. Food availability has been postulated to be a major factor in the regulation of the breeding season of *R. exulans* on Tiritiri (Bunn, 1979). While data concerning its
effects on breeding onset are inconclusive, enhanced food availability does appear to defer the termination of breeding (also see Section 6.4).
6.2 METHODS

To examine the possible role of photoperiod in control of reproductive activity in female *Rattus exulans*, mixed groups of perforate and non-perforate females were collected at approximately three monthly intervals from February 1978 through to January 1979. (Three additional animals were collected in May 1979 to supplement the May 1978 sample). The February and May 1978 samples each consisted of 15 animals. These were housed individually and distributed evenly among three different lighting regimes (LD 16:8, LD 8:16 or LL dim (0.02 to 0.07 lux)). The September 1978 and January 1979 samples consisted of 10 and 9 animals respectively which were divided between LD 16:8 and LD 8:16. (The final sample was delayed until early January 1979 because of an unsuccessful trapping trip in December 1978. Female *R. exulans* have previously proved difficult to capture early in the breeding season on Tiritiri (Moller, 1977; Bunn, 1979) when they are commonly pregnant or have pre-weaning litters.) After a minimum of 91 days (maximum 115 days) in the experimental light regimes, animals were weighed and then rapidly killed by being admitted to a 2 l jar containing chloroform-soaked cotton wool. Both ovaries were removed and then fixed, mounted and stained as described in Appendix V.

The ovarian sections thus obtained were examined for the presence or absence of luteal tissue. At ovulation the mature follicle discharges its oocyte and then undergoes a series of morphological and functional changes, described collectively as luteinization, to form a corpus luteum. The "inactive" (minimal progesterone secretion) corpus luteum of the unmated laboratory rat persists in the ovary about as long as the active (progesterone secreting) corpus luteum of pseudopregnancy which lasts about 14 days. Several sets of corpora lutea may thus be present in the ovary at one time, since laboratory rats usually have a 4 or 5 day estrous cycle. In histological preparations, however, it is usually only possible to identify the most recent set with tolerable certainty (Perry, 1971). The presence of luteal tissue in the ovary of non-pregnant rats is thus an unequivocal indication of recent ovulation.
6.3 RESULTS

The results of these experiments are summarized in Table 6.2. Several generalizations emerge from these data. Firstly, no animals that were non-perforate at the beginning of the experimental programme had luteal tissue present in their ovaries by the end of it. Secondly, with the exception of the January 1979 sample, no perforate females housed in LD 8:16 had luteal tissue present in their ovaries after 91 or more days of exposure. All perforate females in the January 1979 sample showed evidence of recent ovulation at the end of the experiment, regardless of the light regime they had experienced. This sample (collected in the middle of the 1978-79 breeding season) does not exhibit the differential effects of LD 8:16 and 16:8 evident in the other 3 samples (collected during the non-breeding part of the year), and is therefore excluded from the combined analyses described below.

Fisher's exact probability test was used firstly to determine whether or not the proportions of perforate and non-perforate females possessing luteal tissue were significantly different in each light regime (Table 6.3 a,b,c). Secondly it was used to determine whether or not the proportions of perforate animals possessing luteal tissue were significantly different among the light regimes (Table 6.3 d,e,f).

In LD 16:8 and LL dim the difference between the number of perforate and imperforate animals possessing luteal tissue is significant at the 95% level. There is no significant difference between the proportion of perforate females in LD 16:8 and LL dim possessing luteal tissue. There is, however, a significant difference between the proportions of perforate rats in either of these two regimes possessing luteal tissue, and the proportion of perforate animals in LD 8:16 possessing luteal tissue. These results indicate that while LD 16:8 and LL dim either induce or do not suppress ovulation, LD 8:16 either suppresses or does not induce ovulation. While the experimental photoperiods selected are somewhat extreme, the data support the hypothesis that ovulation occurs in 24 h light cycles with long photoperiods but not with short photoperiods.

Data from the January 1979 sample, in which all perforate animals continued to ovulate irrespective of the light regime, suggest that female *R. exulans* may become insensitive to photoperiod during the breeding season.

Bunn (1979) has postulated that females must attain a threshold weight (75 g) to reach breeding condition. To examine this hypothesis, animals were weighed before admittance to the experimental cabinets (Chapter Two) and immediately prior to being killed. The combined results
TABLE 6.2
Effects on Ovulation of Exposure to Different Photoperiods.


<table>
<thead>
<tr>
<th>RAT NO.</th>
<th>PERFORATE?</th>
<th>LUTEAL TISSUE PRESENT?</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>59d</td>
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</tr>
<tr>
<td>3</td>
<td>78d</td>
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</tr>
<tr>
<td>2</td>
<td>NO</td>
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</tr>
<tr>
<td>9</td>
<td>NO</td>
<td>NO</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>RAT NO.</th>
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<th>LUTEAL TISSUE PRESENT?</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>56</td>
<td>NO</td>
</tr>
<tr>
<td>5</td>
<td>80</td>
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</tr>
<tr>
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</tr>
<tr>
<td>11</td>
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<td>NO</td>
</tr>
</tbody>
</table>

LL dim (0.02 to 0.07 lux)

<table>
<thead>
<tr>
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<th>LUTEAL TISSUE PRESENT?</th>
</tr>
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<tbody>
<tr>
<td>7</td>
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</tr>
<tr>
<td>12</td>
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<td>YES</td>
</tr>
<tr>
<td>14</td>
<td>YES</td>
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</tr>
<tr>
<td>15</td>
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<td>YES</td>
</tr>
<tr>
<td>13</td>
<td>NO</td>
<td>NO</td>
</tr>
</tbody>
</table>


<table>
<thead>
<tr>
<th>RAT NO.</th>
<th>PERFORATE?</th>
<th>LUTEAL TISSUE PRESENT?</th>
</tr>
</thead>
<tbody>
<tr>
<td>34</td>
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<td>YES</td>
</tr>
<tr>
<td>36</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>32</td>
<td>NO</td>
<td>NO</td>
</tr>
<tr>
<td>33</td>
<td>NO</td>
<td>NO</td>
</tr>
<tr>
<td>35</td>
<td>NO</td>
<td>NO</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>RAT NO.</th>
<th>PERFORATE?</th>
<th>LUTEAL TISSUE PRESENT?</th>
</tr>
</thead>
<tbody>
<tr>
<td>*67</td>
<td>52</td>
<td>NO</td>
</tr>
<tr>
<td>*68</td>
<td>70</td>
<td>NO</td>
</tr>
<tr>
<td>*71</td>
<td>70</td>
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</tr>
<tr>
<td>24</td>
<td>NO</td>
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</tr>
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</tr>
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<td>27</td>
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</tr>
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<td>28</td>
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</tr>
<tr>
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<td>NO</td>
<td>NO</td>
</tr>
</tbody>
</table>

TABLE 6.2 cont.


<table>
<thead>
<tr>
<th>RAT NO.</th>
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</tr>
</thead>
<tbody>
<tr>
<td>16</td>
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<td>YES</td>
</tr>
<tr>
<td>17</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>22</td>
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<td>19</td>
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<td>NO</td>
</tr>
<tr>
<td>21</td>
<td>NO</td>
<td>NO</td>
</tr>
</tbody>
</table>

LL dim (0.02 to 0.07 lux)

Collected 30 August - 7 September, 1978

<table>
<thead>
<tr>
<th>RAT NO.</th>
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</tr>
</thead>
<tbody>
<tr>
<td>45%</td>
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</tr>
<tr>
<td>47%</td>
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<td>YES</td>
</tr>
<tr>
<td>46</td>
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<td>50</td>
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<td>NO</td>
</tr>
<tr>
<td>51</td>
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<td>NO</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

LD 16:8

<table>
<thead>
<tr>
<th>RAT NO.</th>
<th>PERFORATE?</th>
<th>LUTEAL TISSUE PRESENT?</th>
</tr>
</thead>
<tbody>
<tr>
<td>40 66</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>42 52</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>44 71</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>41</td>
<td>NO</td>
<td>NO</td>
</tr>
<tr>
<td>43</td>
<td>NO</td>
<td>NO</td>
</tr>
</tbody>
</table>

LD 8:16

<table>
<thead>
<tr>
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<th>LUTEAL TISSUE PRESENT?</th>
</tr>
</thead>
<tbody>
<tr>
<td>53</td>
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</tr>
<tr>
<td>55</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>56</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>57</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>61</td>
<td>NO</td>
<td>NO</td>
</tr>
</tbody>
</table>


<table>
<thead>
<tr>
<th>RAT NO.</th>
<th>PERFORATE?</th>
<th>LUTEAL TISSUE PRESENT?</th>
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</thead>
<tbody>
<tr>
<td>54</td>
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<td>YES</td>
</tr>
<tr>
<td>59</td>
<td>*YES</td>
<td>YES</td>
</tr>
<tr>
<td>60</td>
<td>*YES</td>
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<td>58</td>
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LD 16:8

<table>
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<tr>
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</thead>
<tbody>
<tr>
<td>53</td>
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<td>YES</td>
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<td>56</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>57</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>61</td>
<td>NO</td>
<td>NO</td>
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</table>

LD 8:16

<table>
<thead>
<tr>
<th>RAT NO.</th>
<th>PERFORATE?</th>
<th>LUTEAL TISSUE PRESENT?</th>
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</thead>
<tbody>
<tr>
<td>53</td>
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<td>YES</td>
</tr>
<tr>
<td>55</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>56</td>
<td>YES</td>
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<tr>
<td>57</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>61</td>
<td>NO</td>
<td>NO</td>
</tr>
</tbody>
</table>

* Gave birth to and weaned a litter during the experiment.

NB: Perforate animals are not necessarily reproductively mature.

All rats in the samples were immature at the time of collection, although
the data indicate that perforate animals were more mature than non-
perforate ones (see Section 6.4).
TABLE 6.3

Testing the Significance of the Different Effects of 3 Light Regimes on Ovulation in Perforate and Non-Perforate Rats.

<table>
<thead>
<tr>
<th></th>
<th>LD 16:8</th>
<th></th>
<th>LD 8:16</th>
<th></th>
<th>LL dim</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>YES</td>
<td>NO</td>
<td>Total</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>PF</td>
<td>5</td>
<td>1</td>
<td>6</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>NP</td>
<td>0</td>
<td>9</td>
<td>9</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td>Total</td>
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<td>10</td>
<td>15</td>
<td>0</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P = 0.002</td>
</tr>
<tr>
<td>PF</td>
<td>6</td>
<td>1</td>
<td>7</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>NP</td>
<td>0</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
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<td>4</td>
<td>10</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>P = 1.000</td>
<td></td>
<td>P = 0.033</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Perforate</th>
<th></th>
<th>Perforate</th>
<th></th>
<th>Perforate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>YES</td>
<td>NO</td>
<td>Total</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>LD 16:8</td>
<td>5</td>
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<td>6</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>LD 8:16</td>
<td>0</td>
<td>8</td>
<td>8</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>Total</td>
<td>5</td>
<td>9</td>
<td>14</td>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>P = 0.003</td>
<td></td>
<td># P &gt; 0.05</td>
<td></td>
<td>P = 0.001</td>
</tr>
</tbody>
</table>

KEY:
PF  Perforate  YES  number of animals with luteal tissue
NP  Non-perforate  NO  number of animals without luteal tissue

# P determined from Table 1, Critical Values of D (or C) in the Fisher Test  (Siegel, 1956).
for perforate animals in LD 16:8 and 8:16 (excluding the January 1979 sample) are summarized in Table 6.4.

**TABLE 6.4**
Weights of Perforate Animals in LD 8:16 and LD 16:8

<table>
<thead>
<tr>
<th>RAT NO.</th>
<th><strong>LD 8:16</strong></th>
<th></th>
<th><strong>LD 16:8</strong></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>INITIAL WEIGHT (g)</td>
<td>FINAL WEIGHT (g)</td>
<td>INITIAL WEIGHT (g)</td>
<td>FINAL WEIGHT (g)</td>
</tr>
<tr>
<td>4</td>
<td>56</td>
<td>58</td>
<td>1</td>
<td>59</td>
</tr>
<tr>
<td>5</td>
<td>86</td>
<td>97</td>
<td>3</td>
<td>78</td>
</tr>
<tr>
<td>40</td>
<td>66</td>
<td>56</td>
<td>34</td>
<td>-</td>
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<tr>
<td>42</td>
<td>52</td>
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<td>36</td>
<td>84</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>71</td>
<td>70</td>
<td>68</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

mean initial weight = 63.6± 11.88g (S.D.)
mean initial weight = 74.6± 9.32g
mean final weight = 69.1 ± 14.13g
mean final weight = 75.3 ± 16.92g

$t_i$ (initial:final) = - 0.843

0.5 > P > 0.4

$t_i$ (16:8 initial : 8:16 initial) = 1.747

0.2 > P > 0.1

$t_i$ (16:8 final : 8:16 final) = 0.749

0.5 > P > 0.4

Although animals in LD 8:16 were on average lighter than those in LD 16:8, both before and after the experiments, these differences are not significant. Differences in weight between the two groups cannot therefore account adequately for the highly significant differences in their reproductive condition as judged by the presence or absence of luteal tissue.
6.4 DISCUSSION

In these experiments female rats were collected at approximately three monthly intervals and maintained in one of three light regimes (LL 0.02 to 0.07 lux, LD 16:8 or LD 8:16) for at least 91 days. Results from similar experiments on antelope ground squirrels (Ammospermophilus leucurus, a non-hibernator) have recently been published (Kenagy and Bartholomew, 1978). Male ground squirrels were collected at four times throughout the year and transferred to either LD 8:16 or LD 16:8 for 50 days. A sample of females was also collected in winter. Since none of the samples showed any differential effects on the reproductive system of the two regimes, the authors concluded that the breeding cycle of this species is controlled by an endogenous annual rhythm.

In contrast, perforate female Rattus exulans collected during the non-breeding part of the year showed differential responses to these light regimes. Animals maintained in LL dim and LD 16:8 showed evidence of recent ovulation at the end of the experiments, whereas none of the animals in LD 8:16 were ovulating. (The differences between LL dim and LD 8:16, and between LD 16:8 and LD 8:16 are significant, but the difference between LL dim and LD 16:8 is not - Table 6.3). Perforate animals collected during the breeding season continued to ovulate irrespective of the light regime. Rats could not have been pregnant at the time of sacrifice and it seems unlikely that they were undergoing pseudopregnant cycles, since their levels of activity and food intake were not abnormal (Section 4.4). It is therefore concluded that the presence of luteal tissue in the ovaries of rats at the end of these experiments indicates that they had been undergoing estrous cycles. Since the majority (6/7) of perforate animals in LL dim had luteal tissue present, it is concluded that the estrous cycle persists in constant conditions. This is consistent with the hypothesis that it is under circadian control (Section 4.1.4).
6.4.1 EFFECTS OF PHOTOPERIOD ON THE ONSET OF BREEDING

On Tiritiri adults breeding in one season seldom, if ever, survive to the next. The population undergoes a dramatic slump around the onset of winter and apparently only juveniles survive. All perforate females collected outside the breeding season were immature (judged by the absence of uterine scars at the time of sacrifice). The data suggest, however, that perforate animals were consistently closer to maturity than non-perforate animals, none of which ovulated in any of the light regimes. The occurrence of ovulation after exposure to LD 16:8 thus represents an accelerated attainment of sexual maturity and breeding condition.

At the end of the experiments, mean weights of perforate animals in LD 8:16 and LD 16:8 were not significantly different, and animals lighter than the mean for the alternative regime were found in both cases. These data do not support the proposition (Bunn, 1979) that the attainment of sexual maturity in female R. exulans is dependent on attainment of a threshold weight.

To examine the importance of food availability in the regulation of the timing of breeding onset on Tiritiri, Bunn (1979) added wheat to an experimental live-trapping grid at fairly regular intervals throughout the winter of 1977. Animals on this grid had faster growth rates, and females apparently matured somewhat earlier than rats on a control grid. Data on the timing of onset of the breeding season were, however, inconclusive, although enhanced food availability would appear to defer the termination of breeding (see below).

In the experiments reported in this Chapter, perforate juvenile females collected in February and May 1978 and housed in LD 16:8 were found to be ovulating by May and August respectively, i.e. 6 months and 3 months earlier than the expected onset of the breeding season in November. On the basis of these observations it is proposed that photoperiod is the major factor involved in initiating the series of physiological changes which bring female R. exulans into breeding condition. Food availability may well be important in regulating the precise timing of the onset of breeding once the animals attain a sufficient level of physiological readiness.

The hypothesis of photoperiodic control is supported indirectly by the observation that females on Tiritiri tend to mature in synchrony at approximately the same time each year. Males, on the other hand,
tend to mature earlier than females and at more variable rates, both within years and between years (Moller, 1977; Bunn, 1979). It has been suggested (Bunn, 1979) that selection favours early maturation in males, since adults tend to predominate over juveniles in terms of social dominance and other behavioural interactions. Differences in microhabitat and/or foraging ability are proposed to account for localized variability in maturation rates. While similar selection pressures may be envisaged acting on females, very strong selection presumably operates to restrict the production of young to the optimal time of year. The timing of the first behavioural estrous in females would therefore be expected to be more strictly synchronized with respect to the seasonal cycle than onset of spermatogenesis in males. This proposition is consistent with observations on the Kure Atoll population of *R. exulans* which breeds seasonally, but in which a significant proportion of animals breed in more than one season (Wirtz, 1972). (In both populations animals do not generally breed in the season in which they are born). Once males on Kure attain sexual maturity, they remain fertile throughout the year, whereas the reproductive activity of adult females shows seasonal trends.

6.4.2. EFFECTS OF PHOTOPERIOD DURING THE BREEDING SEASON

All perforate females collected from Tiritiri during the breeding season (the January 1979 sample) were reproductively mature. These animals continued to ovulate regardless of the light regime to which they were exposed (Table 6.2). This may not necessarily be due to insensitivity to photoperiod however, if LD 16:8 has a stimulatory effect on the reproductive system and LD 8:16 has no effect. If this is the case, once the reproductive system has become active further stimulation may be ineffective.

Conversely, if LD 8:16 has an inhibitory effect on the reproductive system, then mature females must be insensitive to this effect during the breeding season. Since animals only breed for one season on Tiritiri, the sensitivity or insensitivity of the mature female to seasonal photoperiod changes would not affect the timing of onset of breeding since this is probably determined primarily by the time of attainment of reproductive maturity in females. On the other hand, in a population such as that on Kure Atoll, where mature females cease to breed for about
4 months of the year, the timing of onset of the breeding season will be determined by young females reaching maturity and/or by old females coming back into reproductive condition. The observations on Kure (Wirtz, 1972) are not sufficiently long term to comment on the regularity of onset of the breeding season. Nevertheless the available data do not preclude the possibility that a photoperiodic mechanism is involved in regulation of the timing of breeding onset. It would be interesting to repeat the experiments reported here on females from the Kure population captured in the non-breeding state after their first breeding season. Such experiments could establish whether or not photoperiod is involved in the return to reproductive condition of adult females.

6.4.3 FACTORS AFFECTING THE END OF THE BREEDING SEASON

The timing of the end of the breeding season on Tiritiri seems to be rather more variable than the timing of breeding onset. The last juveniles were estimated to have been born in middle to late January 1975, but pregnant females were still being caught in early February 1976 (Moller, 1977). Last births apparently occurred around the first week in February 1978 (Bunn, 1979). These estimated times of cessation of breeding cover a range of photoperiods * from about 15 h 15 min (January 21) to 14 h 11 min (February 20). As noted previously, a laboratory population of R. exulans established from nearby Cuvier Island breeds continuously in LD 12:12 (Averill and Wodzicki, 1975; Davis, 1976). It therefore seems unlikely that daylength is the critical environmental factor regulating cessation of breeding in the Tiritiri population of R. exulans.

Available data support the hypothesis that termination of the breeding season in this population is determined largely by food availability. On the food addition grid described earlier Bunn (1979) found that breeding was still occurring as late as April 1978. This represents a 2 - 3 month extension of the breeding season presumably attributable to the enhanced food supply available to these animals. The survival of

*Footnote: Daylength as measured from sunrise to sunset plus twice the duration of civil twilight (Sadlier, 1969). Sunrise and sunset data from the New Zealand Nautical Almanac and Tide Tables 1979, Marine Division, Ministry of Transport, Wellington. Duration of civil twilight extrapolated from northern hemisphere data in the Smithsonian Meterological Tables (prepared by R.J. List). Smithsonian Institute Press, Washington D.C.
juveniles through to maturity would appear to be at least partially dependent on attainment of a sufficient weight prior to declining food availability in winter. (There is a major drop in population density at the onset of winter and a progressive loss of body weight in animals that do survive. Parallel changes occur in the major dietary items as the summer grass seed crop finishes and rats convert to a predominantly invertebrate diet). It is estimated (Bunn, 1979) that for juveniles to attain the required weight, they must be born no later than about the third week in January. Animals born after this time presumably have a reduced probability of survival.

In summary, it is concluded that the onset of the breeding season of R. exulans on Tiritiri is primarily regulated via a photoperiodic mechanism controlling the timing of maturation in females. Superimposed on this "coarse" control there are probably "fine tuning" adjustments by factors such as food availability. These factors determine the actual time of breeding onset within a range defined by the photoperiodically induced physiological readiness of the females. The question of the sensitivity of the mature female reproductive system to photoperiod during the breeding season has not been resolved. Termination of the breeding season is probably determined largely by food availability, since reproduction ceases in photoperiods considerably longer than 12 h, which is sufficient to permit continuous breeding in laboratory populations.

6.4.4 THEORIES OF PHOTOPERIODIC TIME MEASUREMENT

There are two general classes of hypotheses which have been proposed as mechanisms for photoperiodic time measurement. In the first it is envisaged that time measurement is based on some form of "hourglass" or "interval timer". A hypothetical reaction product is postulated to accumulate during the light (or dark) phase of the LD cycle. If the accumulation phase is long enough then sufficient of the product is produced to trigger the photoperiodic response. The time measuring process begins anew with each DL (or LD) transition and only continues in the presence of environmental light cycles. This type of mechanism has been demonstrated to occur in the aphid Megoura vicieae (Lees, 1972), but it is apparently much less common than was originally proposed (Elliot, 1976).

In the second general class of hypotheses it is considered that
photoperiodic time measurement is based on an endogenous circadian time-keeping system. In one model, (the external coincidence model (Pittendrigh, 1966, 1972)), photoperiodic induction is proposed to occur when environmental light coincides with the light-sensitive (photo-inducible) phase of an endogenous circadian rhythm of sensitivity to the photo-inductive effects of light. In this model light performs a dual role in entraining the sensitivity rhythm and in inducing the photoperiodic response (Pittendrigh and Minis, 1964). In the second model, (the internal coincidence model (Pittendrigh, 1972, 1974)), photoperiodic induction occurs when critical phase points of two endogenous circadian oscillators coincide. The phase relationship between the two oscillators is postulated to be dependent on the length of the photoperiod of the entraining LD cycle. In practice it is very difficult to distinguish unequivocally between these two mechanisms by experimentation (Saunders, 1978).

Photoperiodic time measurement has been shown to involve an endogenous circadian component (or components) in a number of plants, insects and birds (Farner et al., 1977; Saunders, 1978; Bunning, 1979). The possible role of circadian time-keeping in photoperiodic responses in mammals has, however, been investigated in only two species, the hamster *Mesocricetus auratus* (Elliot et al., 1972; Stetson et al., 1975, 1976; Elliot, 1976; Fitzgerald and Zucker, 1976; Rusak and Morin, 1976) and the field vole *Microtus agrestis* (Grocock and Clark 1974). In both species available evidence is consistent with the hypothesis of a circadian rhythm of sensitivity to the photo-inductive effects of light underlying the photoperiodic responses of the reproductive system (i.e. the external coincidence model).

Further experimentation (e.g. resonance experiments) is required to establish whether or not the photoperiodic regulation of the onset of estrous cycling in *R. exulans* is under circadian control. Several lines of indirect evidence however, support the involvement of a circadian-based time measuring system in this response. Firstly the estrous cycle itself is very probably under circadian control (Chapter Four). The only other seasonal breeder in which circadian control of estrous has been demonstrated is the hamster, *Mesocricetus auratus* (Alleva et al., 1971; Fitzgerald and Zucker, 1976; Stetson and Watson-Whitmyre, 1976). Reproduction in females of this species is probably regulated by a circadian-based photoperiodic mechanism (Sorrentino and Reiter, 1970;
Reiter, 1975; Seegal and Goldman, 1975; Tamarkin et al., 1976 a, b). Secondly photoperiodic mechanisms in the great majority of plants, insects, birds and mammals examined to date would seem to be circadian-based (Elliot, 1976; Farner et al., 1977; Saunders, 1978; Bunning, 1979).

By considering the available data for the female R. exulans photoperiodic response in terms of the external coincidence model, several testable hypotheses regarding this response can be formulated. There are a number of assumptions underlying applications of this model (Elliot et al., 1972). The proposed circadian rhythm of sensitivity to the photo-inductive effects of light cannot be assayed independently of induction. It is therefore common practice to use another readily monitored overt rhythm, generally activity, as an indicator of the circadian system of the organism. In the case of the hamster, there is evidence that a common circadian time-keeping mechanism controls the rhythms of locomotor activity, estrous cyclicity and photoperiodic sensitivity. Destruction of the suprachiasmatic nuclei abolishes all three rhythms (Stetson and Watson-Whitmyre, 1976). It is not, however, essential to assume a common clock to use one rhythm as an indicator of another. The necessary criterion is that the two rhythms have a fixed phase relationship, as occurs in steady-state entrainment (Chapter One).

With this assumption, the position of the photo-inducible phase of the proposed photoperiodic photosensitivity rhythm of R. exulans; relative to the locomotor activity rhythm, can be approximately estimated. Taking activity onset as CT 0, the mean circadian times of lights on and lights off in entrained rhythms of females in LD 16:8 and 8:16 can be derived from the data in Tables III.7 and III.8. (Since $\Psi$ is measured from activity onset to lights off, the CT time of lights off is CT 0 (= CT 24) + $\Psi$.)
TABLE 6.5
Mean CT Times of the LD and DL Transitions in LD 16:8
and LD 8:16 Entrainment.

<table>
<thead>
<tr>
<th>LD</th>
<th>MEAN CT LIGHTS ON</th>
<th>MEAN CT LIGHTS OFF</th>
<th>n</th>
<th>S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:8</td>
<td>7.8</td>
<td>23.8</td>
<td>15</td>
<td>0.47</td>
</tr>
<tr>
<td>8:16</td>
<td>13.2</td>
<td>21.2</td>
<td>6</td>
<td>1.84</td>
</tr>
</tbody>
</table>

KEY: n - number of animals
S.D.- standard deviation of mean CT lights on (or off).

Thus, by comparison with animals in LD 8:16, rats in LD 16:8 receive on average an additional 5.4 h of light around the end of the active phase (CT 7.8 to 13.2) and an additional 2.6 h of light around the time of activity onset (CT 21.2 to 23.8). The photoinducible phase must therefore occur between CT 7.8 and CT 13.2 and/or between CT 21.2 and CT 23.8.

A directly comparable situation is reported for the hamster photoperiodic responses. In photostimulatory photoperiods, light coincides with both the early and late portions of the active phase of the locomotor activity rhythm, while in non-stimulatory photoperiods it occurs only during the inactive phase (Elliot et al., 1972). It would thus appear that the phase relationship between the activity rhythm and the rhythm of photoperiodic photosensitivity may be similar in the two species. The photoinducible phase in the hamster begins shortly before activity onset and lasts for nearly 12 h (Elliot, 1976). This is consistent with the earlier finding of Gaston and Menaker (1967) that the minimum photoperiod length for induction is about 12.5 h. By comparison, in R. exulans 12 h of light per day is sufficient to permit breeding in laboratory populations (Wirtz, 1973; Averill and Wodzicki, 1975; Davis, 1976).

Rats in LD 8:16 always begin and end activity within the dark portion of the cycle. In LD 16:8 however, animals often show a build up of activity prior to lights off, although the main bulk of activity still occurs in the dark phase (Section 3.3.3.2). This light exposure around the beginning of the active phase would seem likely to be the
critical factor inducing the photoperiodic acceleration of the onset of reproductive maturity in LD 16:8. Presumably in constant dim light of the intensities used, animals received sufficient light throughout the photoinducible phase to produce the same response (Table 6.1). It would be necessary to perform resonance and "T" experiments (Elliot, 1976) to test the hypothesis that there is a circadian rhythm of sensitivity to the photo-inductive effects of light underlying this response, and to investigate the above predictions regarding its phase relationship to the locomotor activity rhythm.

To understand how such a photoperiodic mechanism might function in the field, very detailed information would be required on the times of emergence from, and return to, nests at different times of year. The patterns of activity in the field (including possible daytime emergence from nests) and the light intensities that rats typically receive while in nests would also influence a circadian-based photoperiodic mechanism. Although there are undoubtedly practical difficulties involved in gathering such information, the Tiritiri population of R. exulans could provide a unique opportunity to examine the functioning of a circadian-based photoperiodic mechanism in a mammal in a field situation.

6.5.5. PHYSIOLOGY OF MAMMALIAN PHOTOPERIODISM

It has been recognised for some time that the mammalian pineal influences reproductive function (e.g. Reiter and Sorrentino, 1970; Reiter, 1974, 1975; Hoffmann, 1975). Whether this effect is consistently either antagonistropic or progonadotropic remains unresolved. Similarly, the pineal compound (or compounds) responsible for its influence on the reproductive system have not yet been conclusively identified. Because its biosynthetic activity is light sensitive (see below), the pineal is generally considered the most likely organ to mediate the effects of photoperiod on the mammalian reproductive system. Recent work on hamsters suggests that photoperiod may affect the reproductive system by altering the sensitivity of the hypothalamic-hypophyseal system to steroid feedback, and that the pineal is involved in this effect (Turek, 1979 a).

A variety of data suggests that the pineal has a moderate anti-gonadotropic effect on the reproductive systems of juvenile and adult laboratory rats, but that this does not seriously affect the reproductive capability of adults (Reiter and Sorrentino, 1970; Hoffmann and Pomerantz,
The activity of the pineal gland of the female laboratory rat is highly sensitive to photoperiod, which suggests that it could still function to discriminate seasonal changes in photoperiod length. The estrous cycle persists, however, over a very broad range of photoperiods and laboratory *R. norvegicus* are no longer seasonal breeders, as are their wild conspecifics. There is evidence that the reproductive system of laboratory females has become less sensitive to variations in pineal activity (Shivers and Yochim, 1979). This reduced sensitivity could well explain the minimal or inconsistent effects of photoperiod and the pineal on reproduction in laboratory rats. In view of seasonal breeding patterns of *R. exulans* on Tiritiri, and of the sensitivity of females to photoperiod, the effects of pinealectomy on this species would provide an interesting comparison to the data from laboratory *R. norvegicus*.

More clear cut and reproducible effects of the pineal on reproduction are generally observed in seasonally breeding mammals (Hoffmann, 1975). The situation is not, however, simple. In the ferret, for example, the pineal appears to exert a progonadotrophic effect at some stages of the annual reproductive cycle, but to be antigonadotrophic at others (Thorpe and Herbert, 1976). The rates of development of juvenile hamsters are affected by the pineal in some species but not in others (Hoffmann, 1975). Although there are complications, the pineal is definitely implicated in photoperiodic effects on the reproductive systems of voles (Grocock and Clarke, 1974), hamsters (Reiter, 1969, 1975; Sorrentino and Reiter, 1970; Reiter et al., 1974; Hoffmann, 1975; Elliot, 1976; Stetson et al., 1977 a; Turek, 1979 a), ferrets (Herbert et al., 1975, Thorpe and Herbert, 1976) and sheep (Rollag et al., 1978).

In the absence of light, the pineal actively synthesizes and releases a substance which appears to act at the hypothalamic level to modify release of gonadotropin releasing factors and thus pituitary gonadotropin release (Hoffmann and Melvin, 1974). Whether the substance involved is melatonin, some other pineal indole-derivative, or a polypeptide remains unresolved. Both enzymes involved in the synthesis of melatonin from serotonin (n-acetyl transferase and hydroxyindole O-methyltransferase) in the rat pineal are regulated by a circadian rhythm generated in the suprachiasmatic nuclei (SCN) and entrained via the retino hypothalamic tract (Klein, 1974; Klein and Moore, 1979). Ablation of the SCN abolishes photoperiodic responses in hamsters. Progonadotropic and antigonadotropic effects of melatonin have been reported in different mammals and even within the same species (Reiter and Sorrentino, 1970; Herbet et al., 1973;
Reiter et al., 1974; Reiter, 1975; Brown et al., 1976 Elliot, 1976; Reiter and Banks, 1976; Thorpe and Herbet, 1976; Rudeen and Reiter, 1977; Stetson et al., 1977 a). In male hamsters exogenous melatonin has opposite effects at different stages of the annual reproductive cycle (Turek et al., 1975; Turek, 1979 b). Differences in experimental procedure probably account for some of the apparent contradictions in the literature. There is evidence, for example, that the effects of melatonin on the reproductive systems of male and female rats depend on the method of administration (Reiter and Banks, 1976). There is also a daily cycle in sensitivity of the hamster reproductive system to melatonin (Tamarkin et al., 1976 b). It remains to be determined whether melatonin exerts its effects directly on the hypothalamic mechanisms regulating gonadotropin release, or alternatively, acts on the pineal to trigger the release of some other pineal factor which then acts on the hypothalamus.
The role of the circadian time-keeping system in regulation of locomotor activity and certain aspects of reproduction has been investigated in Rattus exulans. This study had two primary objectives: to provide data on a wild species for comparison with available information on laboratory rodents, and to relate rhythmic phenomena observed in laboratory conditions to what is known of the behaviour of R. exulans in the field.

7.1 THE ACTIVITY RHYTHM

Rats adapt readily to the housing facilities designed and constructed for this research and are thereby amenable to long-term experimental programmes. In the activity monitoring apparatus developed, R. exulans exhibit precise circadian locomotor activity rhythms which may persist for periods well in excess of the expected natural lifespan of the animals. The properties of the free-running activity rhythm have been examined to enable discrimination between several current models of circadian pacemaker mechanisms. Age-related changes do not occur in the free-running period or in its day-to-day variability in females. There appears, however, to be sexual dimorphism in period with the mean value for males being significantly shorter than that for females. Rhythms either damp out in constant light (520 lux) or exhibit longer free-running periods than in constant darkness. This response is typical of circadian rhythms of nocturnal organisms (Aschoff, 1979).

After-effects on period occur in free-running activity rhythms following single light pulses or entrainment by light cycles. Significant phase-dependent period shortening occurs following 8 h phase advancing light pulses, while significant phase-dependent period lengthening is observed following 16 h delaying pulses. The after-effects produced by entrainment depend on the period of the light cycle, the duration of its photoperiod and on the number of entraining cycles to which the animal is exposed. The regularities evident in these after-effects indicate that the period changes observed are not spontaneous and unpredictable. Rather, they represent long-term responses of the pacemaker to the prior treatment and provide strong evidence in support of the hypothesis that the pacemaker is a multioscillator system (Pavlidis, 1978 a,b). All available data for the R. exulans activity rhythm are consistent with
a general model of the pacemaker as a population of weakly interacting circadian oscillators.

Phase response curves of the activity rhythm to 4 h, 8 h and 16 h light pulses have been derived. They conform to the typical circadian pattern with phase delays in occurring in response to light pulses falling late in the subjective day and early in the subjective night, and phase advances occurring in response to pulses late in the subjective night and early in the subjective day. The 4 h phase response curve is a weak or Type 1 curve (Winfree, 1970, 1971). Eight hour light pulses appear to be close to the critical duration for the transition from weak to strong phase response curves. Only pulses of this length produce the (temporary) arrhythmicity characteristic of pulses coinciding with the singularity (Winfree, 1970, 1971; Engelmann et al., 1978; Christensen and Lewis c, in prep.). Both the 8 h and 16 h curves are of the strong type (Type 0).

Observed phase angles between R. exulans activity rhythms and entraining light pulses are in reasonable agreement with predictions from the phase response curves for equivalent single light pulses. The activity rhythm therefore conforms to the assumption of the non-parametric entrainment model (Pittendrigh, 1965, 1966) that the underlying pacemaker phase shifts within one cycle following a light pulse. Observed transients thus represent the overt rhythm regaining its normal phase relationship to the reset pacemaker.

Field observations indicate that the action of light in entraining R. exulans activity rhythms under natural conditions must be predominantly non-parametric. Animals retreat to dark or semi-dark nests during daylight and presumably perceive full sunlight only briefly, around the beginning and end of their nocturnal activity. It is possible that animals perceive sufficient light while in nests during the day and/or make brief diurnal excursions from nests and thus avoid the phase jump which is predicted to occur when light has a purely non-parametric action in entrainment (Pittendrigh and Daan, 1976 b). A phase jump would result in animals becoming diurnal during long summer photoperiods. Available data do not, however, preclude an additional parametric action of light in entrainment of the activity rhythm.
7.2 THE ESTROUS CYCLE

Female *R. exulans* were found to be ovulating (Chapter Six) after more than 90 days in constant conditions (LL 0.02 to 0.07 lux, 21 ± 1°C). This observation supports the hypothesis that the estrous cycle is regulated by an endogenous mechanism. The pattern of change in vaginal cytology of *R. exulans* during the estrous cycle closely resembles that of laboratory *R. norvegicus*. In the latter species the timing of ovulation, and hence the duration of the estrous cycle, is regulated by a circadian time-keeping mechanism apparently controlling hypothalamic release of luteinizing hormone-releasing hormone. Available evidence is consistent with the hypothesis that the estrous cycle in *R. exulans* is under similar circadian control, although technical limitations precluded definitive experiments.

7.3 INTERACTIONS BETWEEN THE ACTIVITY RHYTHM AND THE ESTROUS CYCLE

Systematic changes in the activity rhythm, associated with the stages of the estrous cycle, have been reported in laboratory *R. norvegicus* (Richter, 1965, 1970; Raisman and Brown-Grant, 1977) and in the hamster *Mesocricetus auratus* (Stetson and Watson-Whitmyre, 1976; Morin et al., 1977). An increase in activity occurs in association with high circulating estradiol levels around the time of ovulation in these species. This may represent a mechanism moulded by natural selection to enhance the probability of a receptive female finding a mate.

In contrast, the activity rhythms of female *R. exulans* do not exhibit regular 5 - 6 day fluctuations in either period or intensity of activity in association with the estrous cycle. Ovariectomy has no significant effect on the period of the activity rhythm, and no observable effect on the distribution or intensity of activity. It is therefore concluded that there is no feedback action of the ovaries or estradiol on the circadian pacemaker controlling locomotor activity in this species. This proposition is supported indirectly by the observation that there are no significant changes in either period or variability of the activity rhythm in association with the degenerative changes that occur in the reproductive system in old age (Huang et al., 1978).
The probability of a receptive female *R. exulans* finding a mate must therefore be sufficiently high without any modification of the normal timing and intensity of activity. This may reflect differences in the social organization of *R. exulans* compared with *R. norvegicus* and *M. auratus*. Alternatively or in addition, the timing of activity relative to the day/night cycle in wild *R. exulans* may be sufficiently critical that selection has acted against early onset of activity in receptive females. Changes in the activity rhythm associated with the estrous cycle have apparently been reported only in laboratory rodents which have been removed from such selection pressures for many generations.

7.4 PHOTOPERIODISM AND THE BREEDING SEASON

*R. exulans* populations show a significant correlation between length of the breeding season and latitude throughout Malaya and the Pacific region. The population on Tiritiri Island at 36.6° S is the highest latitude population studied to date and has an approximately three month breeding season from November to January. The time of peak breeding in this population coincides with the non-breeding period (and vice versa) of the most northerly population examined, that on Kure Atoll (28.4° N; Wirtz, 1972). The onset of the breeding season on Tiritiri occurs consistently from year to year in early to mid-November, while the end of the breeding season is rather more variable (Moller, 1977; Bunn, 1979). These observations provide indirect evidence in support of the hypothesis that the onset of breeding in *R. exulans* may be regulated by seasonal changes in photoperiod (Sadlier, 1969).

This hypothesis is further supported by the finding that the majority of perforate juvenile females collected outside the breeding season and housed in LD 16:8 began ovulating within about 90 days. In contrast, none of the perforate juveniles collected at the same times but housed in LD 8:16 were found to be ovulating at the end of identical experimental periods. It is concluded that the longer photoperiod enhanced the onset of reproductive maturity, since perforate juveniles collected in February and May and maintained in LD 16:8 were found to be ovulating in May and August respectively. This is well in advance of the November onset of breeding in the wild population.
Mature females collected during the breeding season continued to ovulate regardless of the light regime to which they were exposed. Available data do not indicate whether this insensitivity to photoperiod is temporary or permanent. Females breed in one season only on Tiritiri and do not survive to the next. Mature females coming back into reproductive conditions are not therefore a factor in the timing of breeding onset in this population, as they are in the Kure population (Wirtz, 1972). On Tiritiri females tend to mature synchronously at about the same time each year whereas males tend to mature earlier and at more variable rates both within and between years (Moller, 1977; Bunn, 1979).

On the basis of field observation and laboratory experimentation it is therefore concluded that the onset of breeding in the Tiritiri population is controlled primarily by a photoperiodic mechanism regulating the attainment of reproductive maturity in females. Food availability may alter the actual time of breeding onset, within limits determined by the photoperiodically induced physiological readiness of the animal, and appears to be the major factor regulating the termination of breeding (Bunn, 1979).

Circadian time-keeping mechanisms underly photoperiodic responses in a diversity of organisms (Elliot, 1976; Farner et al., 1977; Saunders, 1978; Bunning, 1979). The external coincidence model has been found to be applicable to both mammalian species (the hamster Mesocricetus auratus and the vole Microtus agrestis) in which the circadian basis of photoperiodic responses of the reproductive system has been investigated. While it has not been conclusively established that the photoperiodic response in female R. exulans is circadian-based, consideration of the available data in terms of the external coincidence model enables the formulation of a number of testable predictions. In particular, this model would predict that light falling early and late in the active phase of the locomotor activity rhythm is critical for photoperiodic induction to occur. It is deduced that the phase relationship between the activity rhythm and the postulated circadian rhythm of sensitivity to the photoinductive effects of light should be similar to that in hamsters. In this species the photo-inducible phase begins shortly before activity onset and continues for nearly 12 h (Elliot, 1976). Data from laboratory colonies of R. exulans suggest that the critical daylength for photoperiodic
induction in these rats is somewhat shorter than that required for induction of photoperiodic responses in hamsters.

7.5 CIRCADIAN TIME-KEEPING AND TEMPORAL NICHE SEPARATION

The ability of *R. exulans* to associate with other species of *Rattus* apparently varies within its geographic range through a gradation from close association, as seen in Malaya, to apparent exclusion from a habitat as seen in New Zealand. Experiments reported here indicate that *R. exulans* from Tiritiri are predominantly nocturnal. In some areas of the Pacific however, where *R. exulans* is found in sympatry with other members of the genus, temporal niche separation is observed with *R. exulans* becoming diurnal (Williams, 1973). It would be of considerable interest to see if this temporal pattern persists in isolated individuals. Differential activity distributions in sympatric species, which persist in isolated individuals, have been reported for a number of other rodents (Kilduff and Dute, 1979). If diurnal *R. exulans* have endogenous circadian rhythms with a reversed phase relationship to LD cycles, then they could provide a useful starting point (in comparison with nocturnal forms) for genetic analyses of mammalian circadian time-keeping systems (Sargent, 1976).

7.6 CONCLUDING REMARKS

Wild *R. exulans* proved to be a very satisfactory animal for laboratory experimentation, once preliminary investigations into suitable caging and housing facilities had been carried out and appropriate equipment constructed. Although there are initial practical problems to overcome, there are a number of advantages to be derived from examining the circadian system in wild as opposed to laboratory rodents, particularly in species for which some ecological and demographic information is available.

Several important differences between *R. exulans* and laboratory rodents are evident from these studies. The lack of interaction between the estrous cycle and the activity rhythm in *R. exulans* indicates that comparatively major differences may exist in the circadian systems, even of closely related species. These differences presumably reflect the different selection pressures experienced by animals of each species.
Generations of laboratory breeding may effectively alter functionally important aspects of the circadian time-keeping system. For example, the pineal is implicated as the most likely organ to be mediating the effects of photoperiod on the reproductive systems of mammals. In both seasonally breeding rodents investigated, these photoperiodic responses are circadian-based (Chapter Six). It would appear however, that the reproductive system of female laboratory R. norvegicus has become less sensitive to variations in pineal function, which may explain why these animals are no longer seasonal breeders, as are their wild conspecifics (Shivers and Yochim, 1979). It has been demonstrated that the attainment of reproductive maturity in female R. exulans is regulated by photoperiod. Wild R. exulans might thus be a more appropriate animal than laboratory rats for investigation of interactions between environmental light cycles, the pineal and the reproductive system. Experience from this project suggests that they adapt more readily to laboratory conditions and are more manageable than other wild Rattus species.

The locomotor activity rhythm of R. exulans shows sufficient precision and persistence to be amenable to detailed analyses. Investigations to date have allowed discrimination between several current models for circadian pacemaker mechanisms. Future studies, particularly on the free-running rhythm, could help to further elucidate the multioscillator properties of pacemakers. The absence of age-related changes in the period and variability of free-running activity rhythms makes R. exulans particularly suitable for the long-term experimental programmes necessary in such research. It is also a species in which examination of temporal behaviour patterns seems feasible in some field situations. Together with laboratory studies, such information could greatly improve understanding of the mechanism of entrainment of circadian rhythms.

The potential value of wild R. exulans as an experimental animal is enhanced by the fact that there is a reasonable body of ecological information already available on the species throughout its range. This is probably a reflection of its economic importance as a crop pest in parts of the Pacific and South East Asia, and its importance in public health as a vector of the typhus carrying mite and the plague flea in some areas (Williams, 1973). Interactions of R. exulans with some of New Zealand’s more vulnerable native fauna are also of current concern in conservation programmes (Moller, 1977). Information about the ecology of a species
provides a functional perspective for physiological and behavioural investigations.

It is concluded that investigations into the circadian time-keeping system of *R. exulans* have provided, and could further provide, valuable comparisons with data from laboratory rodents which have received considerable attention in the literature. Studies on wild species for which some ecological information is available have the advantage that data obtained can be evaluated in a functional context.


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APPENDIX I:
FACILITIES FOR HOUSING INDIVIDUAL RATTUS EXULANS
IN A CONTROLLED LABORATORY ENVIRONMENT.

A. CAGES.

Since there are no previous accounts of the maintenance of wild R. exulans in controlled conditions, suitable cages were designed and constructed for the project. After preliminary observations of the behaviour of these rats in a variety of types of cages, the design illustrated in Fig. I.1 was selected.

The base consists of a white plastic stacking container approximately 29.5 cm square by 12.5 cm high (internal measurements). These bases have the advantages of being easily cleaned, having rounded rather than square corners, and of being economical in terms of storage space. Matched holes are drilled in opposite sides of the container to permit transmission of the deep red or infra-red activity-detecting beam.

Handling is facilitated by the provision of a white plastic jar approximately 7.5 cm in diameter by 9 cm deep, which serves as a nest-box in which the animal sleeps and to which it retreats when disturbed. The jar is anchored to the side of the cage so that the animal cannot deflect it into the activity-detecting beam. It can, however, be detached from outside the cage. The rat can thus be trapped in it and easily and safely transported.

The lid is constructed from 0.7 cm galvanized mesh and 26 gauge galvanized iron and is spot-welded. The central channel, triangular in cross-section, holds sufficient food to last the animal in excess of 3 weeks and also holds the water bottle at an appropriate angle to permit easy flow when the animal touches the nozzle. Although time-consuming to construct, these cages proved entirely satisfactory from an experimental point of view, as well as being durable and comparatively inexpensive.

B. THE CABINET COMPLEX.

As there were no facilities in the Zoology Department for housing small mammals in controlled environments, the cabinet complex illustrated in Fig. I.2 was designed and constructed with assistance from technical staff. Each cabinet is light-proof, having two baffled air-intake ducts
FIG I.1

Cage design - see text.
FIGURE I.2
The Animal Housing Complex.

A. Baffled air-intake ducts.
B. Baffled extractor duct containing a fan.
C. Fluorescent lighting.
D. Electronic timers.
E. Circuitry and relays for the activity monitoring system.
F. Event Recorder.
(A) on the sides and a baffled extractor duct (B) containing a fan, in
the roof. Shelves 43 cm deep and at 0.61 m intervals provide sufficient
surface area to house up to 15 animals per cabinet. The cabinets
form 3 sides of a central light-proof enclosure which serves as a light
trap so that any cabinet can be opened at any point in its lighting
regime without exposing its occupants to extraneous light.

Lighting in each cabinet is provided by two 20 watt 0.61 m Philips
cool white fluorescent tubes (C) mounted on the upper and lower doors.
To improve the evenness of illumination, all interior surfaces are coated
with high gloss white enamel paint. When the lights are on, the intensity
at the floor of the cages is about 520 lux.

Electronic timers (D) mounted on the exterior of the cabinet complex
enable lighting regimes to be manipulated without entering the complex.
Circuitry and relays (E) for the activity monitoring system are mounted
in the central enclosure, while the event recorder (F) is outside the
complex. This permits regular maintenance, chart replacement etc.
to be carried out without disturbance to the experimental animals.

The cabinet complex is situated in a room controlled to 21 ± 1°C.
Appendix II: The Infrared Activity Detecting System

Each IR emitting diode (Optron Inc. OP133) and its spectral matched phototransistor (OP805) were mounted in a rigid steel frame (Fig. II.1) so that they were permanently in alignment. A 1.5 cm opaque plastic tube (A) served as a shield to minimize ambient light interference at the detector. A 320 Ω load resistor at the negative terminal of the emitter was secured on a plastic insulating block on the mounting frame.

The prototype circuit board is depicted in Fig. II.2. The basis of the system is an LM339 integrated circuit from National Semiconductor, which is a "quad-comparator" i.e. there are four independent channels per chip. Each circuit compares two analogue inputs and gives an output in digital form - either high or low (Fig. II.3). Initially no hysteresis was provided for the LM339, however later versions have regulated reference levels and hysteresis to prevent spurious pulses caused by supply fluctuations and ambient light. Incorporation of input filters to counteract fluorescent lighting flicker was considered, however in practice this proved unnecessary as the OP805 failed to detect the flicker.

When the input voltage on the comparator is greater than or equal to the reference level, the output of the comparator switches to ground thus switching off the transistor and breaking contact in the relay. The switching of the relay deflects an event recorder pen.
### APPENDIX III:
ACTIVITY RHYTHM DATA TABLES

#### TABLE III.1

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After-Effects of LD 8:16, 8:18, 8:20, 8:22,

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<th>( \bar{T} ) after - ( \bar{T} ) before</th>
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# females
* \( \bar{T} \) before not available. \( \bar{T} \) measured subsequently when the rhythm is not undergoing after-effects or transients.
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# females

* $\bar{\tau}$ before not available. $\bar{\tau}$ measured subsequently when the rhythm is not undergoing after-effects or transients.

Mean $\bar{\tau}$ after and mean $\bar{\tau}$ before are significantly different ($0.01 > \text{Pr} > 0.001$).

$\bar{\tau}_{\text{after}} - \bar{\tau}_{\text{before}}$ is significantly correlated with $\bar{\tau}_{\text{before}}$.

$r_s = -0.713$

$0.01 > \text{Pr}_s > 0.001$
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<th>NORMALISED Δ Φ</th>
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CT before versus CT after (Fig. 3.15)

Spearman's rank correlation coefficient $r_s = 0.960$

$t_s = 18.767$

$P_{t_s} < 0.001$
TABLE III.5
Phase Response Curve to 8 h Light Pulses

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<th>NORMALISED Δ φ</th>
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CT before versus CT after (Fig. 3.15)

Spearman's rank correlation coefficient \( r_s = 0.137 \)

\( t_s = 0.651 \)

\( 0.9 > P_{ts} > 0.5 \)
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CT before versus CT after (Fig. 3.15)

Spearman's rank correlation coefficient

\[
\begin{align*}
    r_s &= -0.288 \\
    t_s &= -1.672 \\
    0.2 &< t_s < 0.1
\end{align*}
\]
### TABLE III.7

Effects of Entrainment by 8 h Light Pulses in Cycles of T = 24, 26, 28 and 30 h.

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<td>8:20</td>
<td>23.8</td>
<td>- 4.2</td>
<td>-</td>
<td>RC</td>
<td>15</td>
</tr>
<tr>
<td>54C</td>
<td>8:20</td>
<td>23.6</td>
<td>- 4.4</td>
<td>24.3</td>
<td>+ 0.3</td>
<td>10</td>
</tr>
<tr>
<td>60B</td>
<td>8:20</td>
<td>23.6</td>
<td>- 4.4</td>
<td>-</td>
<td>RC</td>
<td>10</td>
</tr>
<tr>
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<td>8:20</td>
<td>23.5</td>
<td>- 4.5</td>
<td>-</td>
<td>RC</td>
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</tr>
<tr>
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<td>8:22</td>
<td>23.6</td>
<td>- 4.4</td>
<td>-</td>
<td>RC</td>
<td>10</td>
</tr>
<tr>
<td>60B</td>
<td>8:22</td>
<td>23.7</td>
<td>- 4.3</td>
<td>-</td>
<td>RC</td>
<td>10</td>
</tr>
<tr>
<td>60F</td>
<td>8:22</td>
<td>23.7</td>
<td>- 4.3</td>
<td>-</td>
<td>RC</td>
<td>10</td>
</tr>
</tbody>
</table>

# females

* \(\tau\) before not available. \(\tau\) subsequently measured when the rhythm is not undergoing transients or after-effects.

RC - relative co-ordination.

Negative \(\Psi\) indicates that activity begins after the onset of the light pulse.
### TABLE III.8

**Effects of LD 16:8 Entrainment**

<table>
<thead>
<tr>
<th>RAT NUMBER</th>
<th>$\bar{T}$ before</th>
<th>$\Delta \phi$</th>
<th>CT Pulse</th>
<th>$\psi$</th>
<th>DURATION OF LD (CYCLES)</th>
</tr>
</thead>
<tbody>
<tr>
<td># 18</td>
<td>24.8</td>
<td>+ 0.8</td>
<td>15.4</td>
<td>- 0.6</td>
<td>42</td>
</tr>
<tr>
<td># 30</td>
<td>*24.1</td>
<td>+ 0.1</td>
<td>15.0</td>
<td>- 1.0</td>
<td>104</td>
</tr>
<tr>
<td># 31</td>
<td>*24.3</td>
<td>+ 0.3</td>
<td>15.5</td>
<td>- 0.5</td>
<td>104</td>
</tr>
<tr>
<td># 52</td>
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<td>+ 0.6</td>
<td>15.5</td>
<td>- 0.5</td>
<td>118</td>
</tr>
<tr>
<td># 59A</td>
<td>*23.8</td>
<td>- 0.2</td>
<td>16.6</td>
<td>+ 0.6</td>
<td>232</td>
</tr>
<tr>
<td># 72</td>
<td>*23.8</td>
<td>- 0.2</td>
<td>15.6</td>
<td>- 0.4</td>
<td>124</td>
</tr>
<tr>
<td># 74</td>
<td>*23.8</td>
<td>- 0.2</td>
<td>16.7</td>
<td>+ 0.7</td>
<td>124</td>
</tr>
<tr>
<td># 75</td>
<td>*23.6</td>
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<td>15.8</td>
<td>- 0.2</td>
<td>124</td>
</tr>
<tr>
<td># 76</td>
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<td>- 0.4</td>
<td>124</td>
</tr>
<tr>
<td># 77</td>
<td>*23.7</td>
<td>- 0.3</td>
<td>15.6</td>
<td>- 0.4</td>
<td>124</td>
</tr>
<tr>
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<td>15.9</td>
<td>- 0.1</td>
<td>64</td>
</tr>
<tr>
<td># 83</td>
<td>*24.0</td>
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<td>- 0.1</td>
<td>64</td>
</tr>
<tr>
<td># 84</td>
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<td>- 0.3</td>
<td>15.6</td>
<td>- 0.4</td>
<td>64</td>
</tr>
<tr>
<td># 85</td>
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<td>16.5</td>
<td>+ 0.5</td>
<td>64</td>
</tr>
<tr>
<td># 86</td>
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<td>+ 0.1</td>
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<td>- 0.2</td>
<td>64</td>
</tr>
<tr>
<td>53B</td>
<td>*23.6</td>
<td>- 0.4</td>
<td>15.8</td>
<td>- 0.2</td>
<td>27</td>
</tr>
<tr>
<td>54C</td>
<td>*23.6</td>
<td>- 0.4</td>
<td>15.9</td>
<td>- 0.1</td>
<td>27</td>
</tr>
<tr>
<td>60B</td>
<td>*23.7</td>
<td>- 0.3</td>
<td>16.4</td>
<td>+ 0.4</td>
<td>27</td>
</tr>
<tr>
<td>60F</td>
<td>*23.7</td>
<td>- 0.3</td>
<td>17.5</td>
<td>+ 1.5</td>
<td>27</td>
</tr>
</tbody>
</table>

* females

* $\bar{T}$ before not available. $\bar{T}$ subsequently measured when the rhythm is not undergoing transients or after-effects.

Negative $\psi$ indicates that activity begins after the onset of the light pulse.
APPENDIX IV:
FIXING AND STAINING OF VAGINAL SMEARS.

Vaginal smears were taken using a wire loop sterilized in an alcohol flame and cooled in distilled water. The vaginal debris thus obtained was smeared on a standard microscope slide (Section 4.2).

PROCEDURE (Gurr, 1962).
1. Fix wet smear in equal parts ether and 100% alcohol: 1 - 2 minutes.
2. Hydrate through 100% alcohol, 100% alcohol, 80% alcohol, 60% alcohol, then rinse in distilled water for 10 seconds.
3. Stain in Harris Haemotoxylin (see below): 2 minutes.
4. Wash in running water: 5 minutes.
5. Stain in Shorr Stain (see below): 2 minutes.
6. Dehydrate through 60% alcohol, 80% alcohol, 100% alcohol.
7. Clear in toluene and dry.

HARRIS HAEMOTOXYLIN
haemotoxylin 10% in absolute alcohol  5 ml
mercuric oxide  0.25 g
potash alum 10% aqueous  100 ml
glacial acetic acid  4 ml
Mix haemotoxylin and alum solutions. Raise to boiling point, then add mercuric oxide. When the solution turns deep purple, remove from heat. Cool, add acetic acid and then filter.

SHORR STAIN
Shorr Stain  1 g
50% alcohol  55 ml
glacial acetic acid  0.5 ml
Dissolve the stain by warming with alcohol, then allow to cool. Add acetic acid, shake well and then filter.
APPENDIX V:
FIXING, MOUNTING AND STAINING OF OVARIAN SECTIONS.

Both ovaries, together with the surrounding fatty tissue, were removed from each rat by severing the uteri just below the clearly visible, coiled oviducts. The tissue was fixed in Bouins solution (see below) for 48 h and then transferred to 50% alcohol.

Following fixation the ovaries were separated from the fatty tissue and then processed on a Histokinette Automatic Tissue Processor on a 17 h clock as follows:

50% alcohol 1 h
70% alcohol 1 h
80% alcohol 1 h
95% alcohol 1 h
95% alcohol 1 h
100% alcohol 2 h
100% alcohol 2 h
100% alcohol 1 h
toluene 1 h
toluene 1 h
wax 2 h
wax 1-2 h

The ovaries were embedded in Gurr’s pastillated paraffin wax (melting point 58°C). Blocks were cut on a Minot Rotary Microtome at 5 μm. Adhesion of sections to slides was achieved by incubation with a fresh egg albumin/glycerine mixture at 60°C for 1 hour.

Initially slides were stained with either Mallory–Heidenhain stain (see below) or Erlich's Haemotoxylin and Eosin (see below). The latter was found to make the luteal tissue more easily discernible and was subsequently used exclusively.

Slides were mounted in Harleco Coverbond (piccolyte and toluene).

BOUISN'S FIXATIVE.
15 parts (by volume) aqueous saturated picric acid.
5 parts 40% formaldehyde.
1 part glacial acetic acid.
MALLORY-HEIDENHAIN STAIN - RAPID ONE STEP METHOD (Humason, 1972).

Solution

distilled water 200 ml

Dissolve each of below before adding next stain:

phosphotungstic acid 1.0 g
orange G (C.I. 16230) 2.0 g
aniline blue, WS (C.I. 42780) 1.0 g
acid fuchsine (C.I. 42685) 3.0 g

This solution keeps for several months.

Procedure
1. Deparaffinize through 2 baths of toluene and hydrate through 100% alcohol, 100% alcohol, 95% alcohol, distilled H₂O.
2. Stain: 5 minutes.
3. Wash in running water: 3-5 seconds.
4. Dehydrate rapidly through 75% alcohol, 95% alcohol, 100% alcohol. Clear through 2 baths of toluene and mount.

ERLICH'S HAEMOTOXYLIN AND EOSIN (Humason, 1972).

Solution

haemotoxylin 2.0 g
alcohol 100 ml

Dissolve haemotoxylin in alcohol and then add each of the following:
ammonium alum 3.0 g
glycerine 100 ml
distilled water 100 ml
glacial acetic acid 10 ml

This solution ripens in 6-8 weeks and kept with a loose stopper in a warm, light place lasts several years.

Procedure
1. Hydrate as in 1. above.
2. Stain in haemotoxylin: 20 minutes.
3. Wash in running water until blue: about 10 minutes.
4. Differentiate in 1% HCl in 70% alcohol: 3 - 4 dips.
5. Wash in running water until blue again.
6. Stain in 1% A P Eosin: 5 minutes (1% CaCl₂ may be added to stop eosin bleeding out in alcohols).
7. Dehydrate, clear and mount as in 4. above.