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THE FEEDING ENTRAINABLE SYSTEM OF THE HERBIVOROUS RABBIT, *ORYCTOLAGUS CUNICULUS*

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PhD = 99% Perseverence + 1% Inspiration

LIST OF FIGURES

DECLARATION

The research reported in this thesis is entirely original. It has not been submitted for the award of any other degree or diploma. Except where due reference is made, all material contained in this thesis is the author's own work.

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Clara Stojanovska

PREFACE

The number of studies investigating the feeding entrainable circadian system has declined in the last decade. The reason for this is unclear, but a likely explanation is that the feeding entrainable circadian system is proving to be intractable. By comparing herbivores to omnivores and carnivores, the present thesis offers a new way of looking at an old problem and new avenues for research.

The majority of studies investigating the influence of feeding schedules on the circadian system have employed the omnivorous rat as subject; considerably fewer data are available on carnivores, and even less research has been done with herbivores. The extent to which the behaviour of these classes of species are similar remains to be established, but preliminary analysis of previous findings has yielded some inconsistencies. The limited data from herbivorous species emphasizes the need for the present research. The focus of this thesis was the rabbit, with the aim of developing a comprehensive model of the herbivore feeding entrainable circadian system.

This thesis is divided into three parts. Part one, consisting of chapters 1 to 4, provides a critical evaluation of published research on this topic. The general characteristics of the mammalian circadian system are detailed in chapter 1. Theoretical issues and empirical findings with regard to LD cycles are discussed with a view to providing a framework for the current thesis. In chapter 2, past research on feeding schedules is reviewed, in particular empirical findings supporting a zeitgeber role for feeding schedules in the omnivorous rat. Inconsistencies in empirical findings across omnivores, carnivores, and herbivores are highlighted in chapter 3 and are discussed with reference to methodological practices. This chapter focuses on the hamster because this is the most thoroughly studied herbivorous species. It is noted that the traditional paradigm used to study the feeding entrainable circadian system cannot be applied to the hamster, since the hamster is poorly adapted to time limited feeding schedules, and hence alternative paradigms have been adopted. It is argued that the methodology used in these studies is not adequate, since the paradigms are varied and hence the meaning and validity of research findings is difficult to evaluate. Chapter 4 addresses the need for more research with herbivores and considers the suitability of the rabbit as a herbivore model of the feeding entrainable circadian system. Because of its tolerance of food deprivation, it is argued that the rabbit provides an opportunity to investigate the feeding entrainable circadian system using traditional methodology involving time limited feeding schedules, thus avoiding the potential confounding effects associated with other methods.

Part two of this thesis consists of chapters 5 to 9. Chapter 5 describes the general methodology used in the present thesis and chapters 6 to 9 are the empirical research chapters. These chapters report five experiments which investigated the effects of feeding schedules on the activity rhythms in the rabbit. The first experiment provides basic information concerning the ability of the rabbit to tolerate feeding schedules. The results of this study indicated that the traditional methodology used to study the feeding entrainable circadian system can be applied to the rabbit. The next experiment examines the reentrainment process following a phase shift of the feeding schedule and yields evidence of transients. A subsequent experiment explores the limits of entrainment to cycles of food availability. It was found that entrainment occurs when the period of the feeding schedule does not deviate too far from 24h. In the final two experiments, rabbits were exposed to multiple meals and found to entrain to two daily meals, but not three daily meals.

Part three contains major conclusions based on the results of this thesis. The significance of current findings, as well as the suitability of the rabbit as a herbivore model is discussed in chapter 10. It is concluded that the rabbit provides a promising model for investigating the properties of the feeding entrainable circadian system. Comparisons are made between omnivores, carnivores, and herbivores; inconsistencies are noted and discussed with reference to ecological factors. It is concluded that these discrepancies appear sufficient to warrant the pursuit of an analysis by class approach.

PART ONE. INTRODUCTION

CHAPTER 1. AN INTRODUCTION TO THE MAMMALIAN CIRCADIAN SYSTEM

1.1. CIRCADIAN RHYTHMS

1.1.1. Definition

A circadian rhythm is an endogenous biological process that recurs at a regular interval of 24h. Circadian rhythms can be characterized by the following parameters: (i) period (tau), which is the time taken to complete a single cycle of a rhythm; (ii) amplitude, which is a measure of the intensity of the rhythm and refers to the distance from trough to peak; (iii) waveform, which describes the shape of the rhythm; and (iv) phase, which refers to a particular time point in a rhythm. Circadian rhythms have been reported to occur in a variety of behavioural, physiological, and biochemical variables, including the sleep-wake cycle, body temperature, and plasma cortisol (see Moore-Ede et al., 1982 for a review).

1.1.2. Free-running rhythms

In 1729 Jean Jacques d'Ortous de Mairan recognized that some biological rhythms with a period of approximately 24h persist under constant conditions, but it was not until 1832 that 'free-running rhythms' with periods that deviated slightly from 24h were first documented (Moore-Ede et al., 1982). More recently it was argued that this finding indicated that circadian rhythms are endogenously generated by a 'pacemaker(s)'. There are now hundreds of reports of free-running rhythms. Estimates of the free-running tau are varied, but usually range from 20h to 28h (Aschoff, 1960; Moore-Ede et al., 1982). Tau is reported to depend on a number of factors, including the particular species, the individual and its physiological state, including its age (Aschoff, 1979; Hofman, 2000; Illnerova et al., 1999; Kohler & Wollnik, 1997; Pittendrigh & Daan, 1974; Turek et al., 1995; Weinert, 2000; Zhang et al., 1996). Other relevant factors include light intensity (Aschoff, 1960, 1965; DeCoursey, 1961; Ferraro & McCormack, 1986; Lee & Labyak, 1997; Pittendrigh & Daan, 1976a; Summer et al., 1984; Swade & Pittendrigh, 1967), certain hormones and pharmacological agents (Bobbert & Riethoven, 1991; Hafen & Wollnik, 1994; Klemfuss, 1992; LeSauter & Silver, 1993a,b; Mistlberger & Nadeau, 1992; Van Reeth & Turek, 1990; Wirz-Justice, 1983; Wollnik, 1992; Yanielli et al., 1998), and changes in levels of activity and arousal (Aschoff, 1960; Edgar et al., 1991; Kohler & Wollnik, 1997; Mistlberger et al., 1998; Mrosovsky, 1993; Weisgerber et al., 1997).

1.1.3. Entrainment of circadian rhythms

It is well established that circadian rhythm(s) can be 'entrained' to environmental time cues. The term 'zeitgeber' has been adopted by researchers for the entraining agent. In a series of eloquent papers dating 1976, and again in 1981, Pittendrigh and Daan formally defined entrainment as follows:

> One oscillator or pacemaker whose free-running period tau can couple to and be entrained by another (z), sometimes called the zeitgeber, with different but similar period (T). In the entrained steady state the oscillator's period is changed from tau to tau* which is similar to T, and a unique phase relation is established between the entrained oscillator (o) and its zeitgeber (z) (Pittendrigh & Daan, 1981, pp. 95).

Enright (1981) argued that the criteria for establishing that an environmental agent acts as a zeitgeber should include: (i) under constant conditions, before the potential zeitgeber is imposed, the circadian rhythm must free-run; (ii) once the putative zeitgeber is imposed, tau must equal the period of the zeitgeber (T); and, (iii) when the potential zeitgeber is removed, the rhythm must free-run from a phase determined by the zeitgeber, and not by the rhythm prior to entrainment. Figure 1 illustrates the concept of entrainment.

It is well established that the light-dark (LD) cycle is the most dominant zeitgeber for almost all mammalian circadian rhythms (Aschoff, 1960; Zucker, 1976). While results from most studies are consistent with this finding, there is evidence to suggest that other periodic signals are also effective as zeitgebers, including cycles of food availability (see Mistlberger 1994 for review), temperature cycles (Aschoff & Tokura, 1986; Barrett & Takahashi, 1995; Francis & Coleman, 1989; Lindberg & Hayden, 1974; Rajaratnam et al., 1997; Tokura & Aschoff, 1983), social cues in the form of conspecific presence (Erkert & Schardt, 1991; Halberg et al., 1954; Marimuthu et al., 1981; Mrosovsky, 1988, 1996; Mrosovsky et al., 1989; Rajaratnam, unpublished; Reebs, 1989), electromagnetic fields (Dowse & Palmer, 1969), novelty-induced running (Janik & Mrosovsky, 1993; Mistlberger, 1991; Mrosovsky, 1989, 1995, 1996; Mrosovsky & Janik, 1993), and certain hormones and pharmacological agents (Armstrong et al., 1986; Brown, 1994; Gillette & McArthur, 1996; Honma et al., 1987a; Hyde & Underwood, 1995; Mrosovsky, 1996; Redman et al., 1983; Van Reeth & Turek, 1989). This chapter will consider theoretical issues and discuss empirical findings with regard to the LD cycle with a view to providing a framework for the current thesis. Chapter 2 will consider entrainment to cycles of food availability.

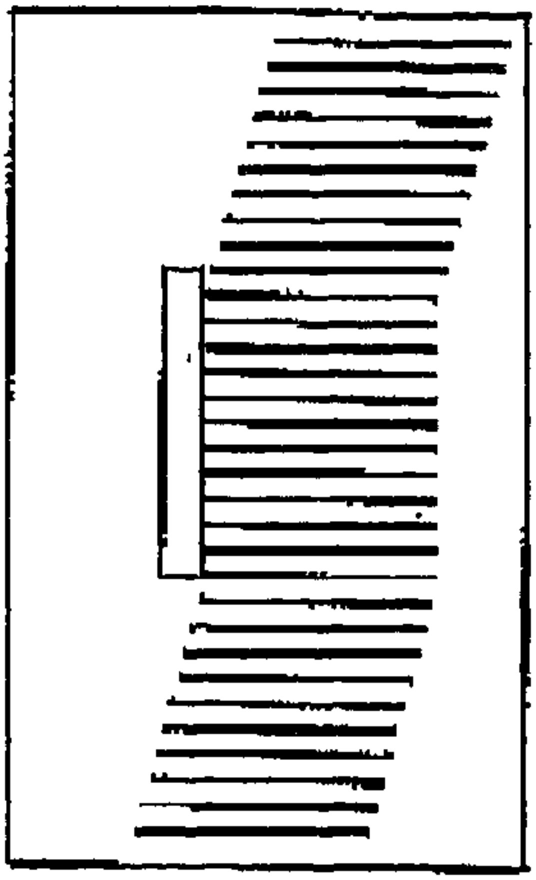


FIGURE 1. SCHEMATIC REPRESENTATION OF ACTIVITY RECORDS PRESENTED IN ACTOGRAM FORMAT, SHOWING AN ENTRAINED RHYTHM. EACH LINE REPRESENTS ONE DAY AND SUCCESSIVE DAYS ARE PLOTTED BENEATH EACH OTHER. HOLLOW VERTICAL LINE REPRESENTS THE ZEITGEBER. BLACK BANDS REPRESENT ACTIVITY. ENTRAINMENT OF ACTIVITY RHYTHM OCCURS WHEN THE ZEITGEBER IS IMPOSED. THE PHASE OF THE POST-ENTRAINMENT FREE-RUN FOLLOWS ON FROM THE PHASE OF THE ENTRAINED RHYTHM. (FROM RAJARATNAM. 1997).

1.1.4. Masking effects

Enright's criteria have been used to distinguish between effects on a rhythm that imply phase control via the pacemaker and 'masking effects'. The term 'masking' is used to describe the direct effects of a zeitgeber on an overt rhythm which do not involve phase-resetting of the underlying pacemaker (Aschoff, 1960). Masking has been observed to occur in a variety of circadian rhythms, but is especially well expressed in activity during exposure to brief pulses of light or darkness (Aschoff, 1960; Aschoff & von Goetz, 1988; Binkley & Mosher, 1985; Edlstein & Amir, 1999; Eriksson, 1978; Gander & Moore-Ede, 1983). Findings from these studies also indicate that the extent to which activity is masked depends on the circadian phase of exposure to the stimulus, as well as the particular species and individual. Other researchers have reported that a zeitgeber can exert both masking and entraining effects. For example, in a study by Albers et al. (1982), in addition to its entraining effects, light was found to elicit activity while darkness had suppressed activity in the diurnal squirrel monkey. The opposite is presumed to be true in nocturnal species. The interested reader can refer to Aschoff (1999), Rietveld et al. (1993), and Marques and Waterhouse (1994) for recent reviews on this topic.

1.1.5. Phase relationship to the zeitgeber

According to Aschoff (1965a), the phase relationship between the entrained pacemaker and its zeitgeber can be defined in terms of the phase angle difference (PAD) between reference phases in the two oscillations. For example, if activity onset in the entrained state begins 2h prior to light (L) onset, the PAD would equal +2h. On the other hand, if activity onset follows after L onset by 2h, the PAD would equal –2h. A large number of studies provide some evidence demonstrating a dependence of PAD on tau and T; PAD is more likely to be positive when tau<T, whereas negative PADs have been found to occur when tau>T (Aschoff, 1965a; Aschoff & Wever, 1962, as cited in Aschoff, 1978; Aschoff & Pohl, 1978). The results of these studies indicate that PAD becomes increasing more positive the shorter tau is and the longer T is, while the reverse is true for longer taus and shorter Ts.

1.1.6. Reentrainment after a phase shift of the zeitgeber

An attempt to extend the criteria for a zeitgeber was made by Enright to include the following guideline: a shift in the phase of circadian rhythms must occur following an abrupt shift in the zeitgeber cycle. It was argued that this process of 'reentrainment' is an important feature of circadian systems. Several days would be required to complete reentrainment and the term 'transients' was adopted to describe the cycles that intervene between the two steady states of entrainment.

The literature with regard to zeitgeber phase shifts is considerable. The method used to phase shift the LD cycle involves either advancing (phase advance) or delaying (phase delay) the onset of either the L or dark (D) phase. It is clear that subjects reentrain to a new LD cycle either by advancing or delaying activity onset (Aschoff et al., 1975; Boulos & Houpt, 1994; Ebling et al., 1992; Goodless et al., 1991; Marumoto et al., 1996; Mistlberger & Nadeau, 1992; Mrosovsky & Salmon, 1990; Puchalski et al., 1996; Ruby et al., 1998; Sudo & Miki, 1995; Takamure et al., 1991; Tsai & Sasaki, 1986; Tsujimaru et al., 1992; Yannielli et al., 1998). The results of empirical studies indicate that direction of reentrainment is a function of several factors, including the direction and magnitude of the phase shift. For example, Aschoff et al. (1975) report that inversion of a LD cycle due to 24h L was achieved by an advance shift while 24h of darkness resulted in a delay phase shift. A dependence of direction of phase shift on tau and PAD has also been demonstrated (Aschoff et al., 1975; Pohl, 1978). (It is well established that PAD depends in part on tau; the shorter tau is, the more positive will be the PAD.) Animals with short tau (and hence a positive PAD) tended to reentrain with advancing transients, while those with long tau (and hence a negative PAD) tended to reentrain with delaying transients.

The duration of reentrainment is reported to depend on several factors, including the particular species and the particular rhythm which is being measured. Aschoff et al. (1975) and Erkert (1982) used a LD cycle and observed that the duration of reentrainment is negatively correlated to zeitgeber strength in rats. Zeitgeber strength is determined by the L:D ratio which was manipulated by increasing or decreasing the level of illumination during the L or D period, and by shortening or lengthening the L phase. The duration of reentrainment was found to shorten with increasing L intensity and longer L phases. In addition, Aschoff and others (Aschoff et al., 1975; Sudo & Miki, 1995; Wever, 1966) report a dependence of reentrainment rate on the magnitude of the phase shift. This was explained in terms of the circadian system's phase response sensitivity to L (see section 1.2). For example, depending on whether the LD cycle has been shifted six or eight hours, the L-offset times will meet different points of the phase response curve and hence unequal responses will ensue.

Of particular note is the asymmetry effect in the duration of reentrainment to phase advances and phase delays. In other words, the time for reentrainment to an advance shift compared to a delay shift is different. Aschoff et al. (1975) and others (Binkley & Mosher, 1989; Boulos & Houpt, 1994; Halberg et al., 1971; Ruby et al. 1998; Sisk & Stephan, 1981; Stephan et al., 1982; Takamure et al., 1991) have described the asymmetry effect in most species examined. It was argued by Wever (1966) that the asymmetry effect is dependent on tau. Results of empirical studies are reasonably consistent with this proposition in showing that reentrainment was more rapid to phase advances than phase delays in species with tau<24h, and the opposite is found to be true when tau>24h. Others have suggested that asymmetric reentrainment is more likely causally related to the phase response characteristics of circadian systems (Aschoff et al., 1975; Pohl, 1978). This issue will be considered in more detail in section 1.2.

1.1.7. Range of entrainment

It is now well established that a zeitgeber, by definition, must entrain circadian rhythms to a range of Ts, but only within certain limits (Bruce, 1960; Enright, 1965; Klotter, 1960). The term 'range of entrainment' has been used to describe the limits within which a circadian rhythm may be entrained to a particular zeitgeber. The most commonly used method by which the range of entrainment is assessed is to alter T in steps until the upper and lower limits are reached (Wever, 1962, as cited in Aschoff, 1978; Stephan, 1983a). Difficulties however have been encountered in measuring precisely the range of entrainment. A finding by both Aschoff (Aschoff & Wever, 1976) and Wever (1972, 1975) was that tau fluctuated periodically near the limits of entrainment to a LD cycle and the range of entrainment varied for different overt rhythms. A further difficulty was described by Moore-Ede et al. (1982) who reported that the circadian system may loose its capacity to produce self-sustaining oscillations following exposure to long/short T cycles.

1.1.8. Relative coordination

The term 'relative coordination' was originally used by von Holst (1939) to describe a phenomenon which occurs when a zeitgeber is not sufficiently strong to entrain circadian rhythms but may still exert some phase control on the pacemaker. Modulations of tau typically occur such that tau will lengthen and shorten at certain phase relationships between the pacemaker and the zeitgeber. Since 1939, there have been many published reports of relative coordination, most notably those by Aschoff (1965a) and Wever (1972). Swade and Pittendrigh (1967), in describing relative coordination, indicate that stable entrainment would be expected to follow if zeitgeber strength were increased.

1.1.9. Aftereffects of entrainment

The term 'aftereffects' is adopted to describe changes in tau that occur as a consequence of the previous history of exposure to a zeitgeber. Aftereffects are long-lasting and slowly decaying changes in tau (Pittendrigh, 1960). These systematic changes reflect the lability of the circadian system, which can be distinguished from its instability or spontaneous day to day variations. Pittendrigh and Daan (1976a) have attributed circadian system lability to a number of factors, including prior exposure to constant light (LL), a change in LD cycle T, a phase shift of the LD cycle, and a change in the photoperiod duration. These researchers noted that aftereffects on tau are always in the direction of T such that tau is lengthened when tau<T and tau shortening occurs when tau>T.

1.2. Mechanism of entrainment

The following model was originally proposed last century and is now widely accepted. According to the model, steady state entrainment is achieved by a series of identical daily phase shifts. In each cycle, pacemaker tau is adjusted by an amount equal to the difference between tau and T. This is made possible because of a phase-dependent sensitivity of the pacemaker to the zeitgeber. The finding that a zeitgeber will phase advance, phase delay, or have no effect on a rhythm depending on the circadian time of exposure is consistent with this theory (Hastings & Sweeney, 1958; Pittendrigh, 1958; Pittendrigh & Bruce, 1957). The magnitude and direction of the phase shift has been demonstrated to relate to the phase of the rhythm at which the stimulus is applied. In 1960 this relationship was quantified by both DeCoursey and Pittendrigh as a phase response curve (PRC).

Several different techniques for obtaining a light-PRC have been used over the years, with the most common now involving light pulses applied at different times of the subjective day and night while the subject is free-running in constant dark (DD). The magnitude and direction of the resultant phase shift is plotted as a function of the circadian phase of the light pulse. Most studies of PRCs report the same basic shape; phase delays occur in response to light pulses applied in the late subjective day and early subjective night, while phase advances are observed in the late subjective night and early subjective day (Benloucif & Dubocovich, 1996; DeCoursey, 1960a,b, 1964; Hoban & Sulzman, 1985; Kennedy et al., 1989; Milette & Turek, 1986; Rauth-Widmann et al., 1991; Sharma & Chandrashekaran, 1997; Sharma et al., 2000). Results also show a lack of responsiveness to light during much of the subjective day. It has been argued that this indicates that daytime illumination has little effect in the entrainment process. Figure 2 illustrates the concept of a light-PRC.

Although the same general pattern is apparent in the shape of the light-PRC across species, most studies of PRC report interspecific as well as interindividual differences. DeCoursey (1960a,b) reported the light PRCs for 12 individual flying squirrels and showed some clear differences in amplitude and waveform. She suggested that group PRCs may actually obscure important differences. Daan and Pittendrigh (1976b), who derived PRCs for groups of animals rather than individual animals, found differences in the relative sizes between the phase advance and phase delay segments of the PRC between different species. For example, advances were found to be larger than delays in the golden hamster, whereas the opposite was found to be true in the house mouse. They proposed that these differences are related to differences in tau: the shorter the tau, the larger the delay/advance ratio. The relationship between tau and PRC shape has been explained in terms of the dependency of tau on the phase relationship between the morning and evening oscillators (see section 1.2.3.).

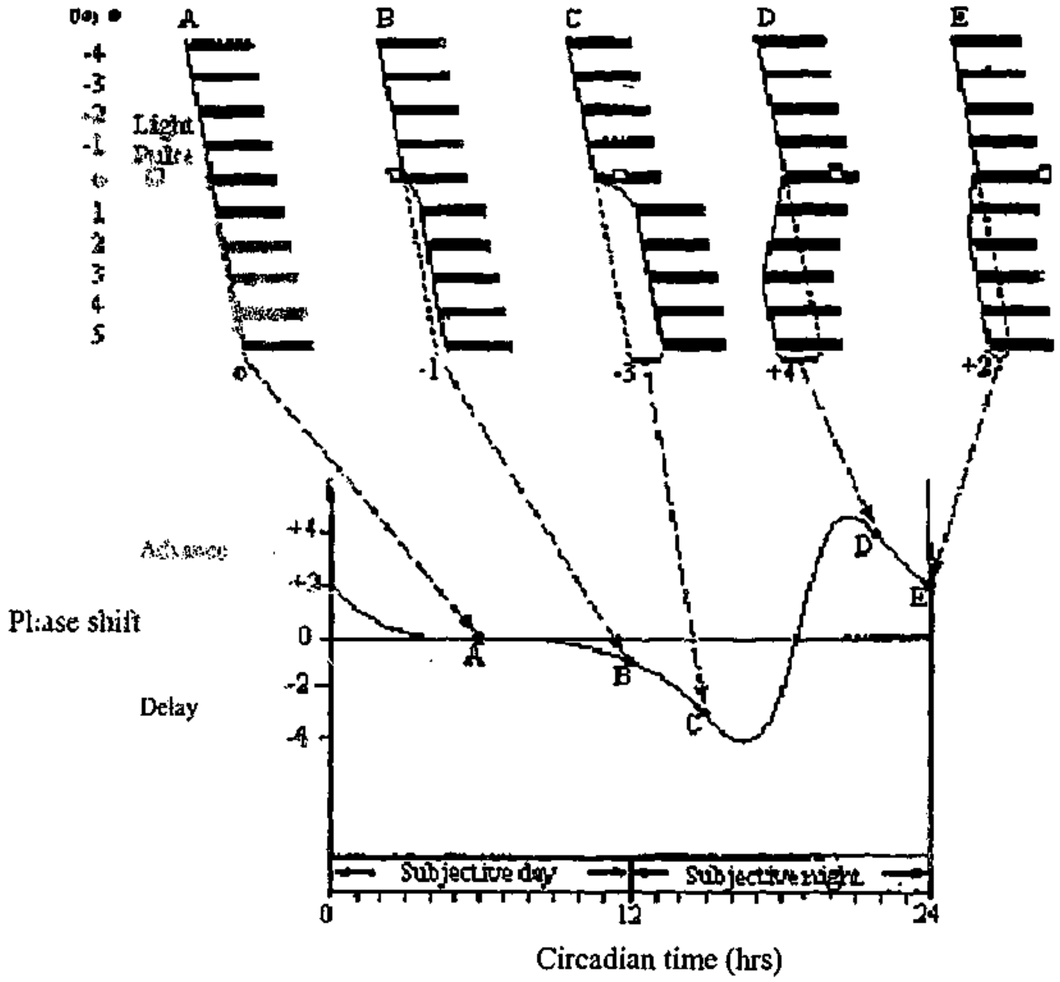


Figure 2. The construction of a PRC in a nocturnal rodent. Light pulses are applied at different times of the animals' subjective day and night on occassions A-E. The magnitude and direction of the resultant phase shift are represented on the curve below (Moore-Ede et al., 1982).

The PRC construct is generally viewed as a useful theoretical concept. For example, the PRC has been used in an attempt to predict the range of entrainment. The total range of entrainment is thought to be equal to the sum of maximal phase advances and maximal phase delays observed in the PRC (Pittendrigh, 1981). The results of studies, however, do not entirely support this hypothesis, with most researchers reporting a failure to entrain to LD zeitgebers in some animals even when the tau-T is within the range of phase shifts seen in the PRC. Pittendrigh and Daan (1976b) have however, attributed this effect to inter- and intra-individual variability in tau and PRC shape.

A number of paradigms exist for obtaining PRCs and Mrosovsky (1996) has argued that some of these other paradigms are more suitable for measuring phase shifts to zeitgebers other than light. These paradigms have been described by Aschoff (1965b) and will not be reiterated here. These authors have noted that PRC's also can be based on period differences rather than phase differences.

1.2.1. Formal aspects of entrainment

Two models based on the PRC have been developed to explain the mechanism of entrainment: the non-parametric model (Aschoff, 1960; Pittendrigh & Daan, 1976b) and the parametric model (Bruce, 1960). According to the non-parametric model, the influence of the zeitgeber on the pacemaker is based on abrupt, instantaneous phase shifts of the pacemaker which occur near the transition phases of the zeitgeber cycle. In the case of the LD cycle, the L-D (dawn) and D-L (dusk) transitions are postulated to provide key signals for entrainment; phase resetting of the pacemaker is thought to occur at one or both of these transitions. Entrainment occurs when the net phase shift is equal to the difference between tau and T. The strongest evidence in support of this model has been obtained from studies utilizing 'skeleton photoperiods' (i.e., two brief light pulses per cycle simulating dusk and dawn of a natural day) (Pohl, 1983; Rauth-Widmann et al., 1991; Schanbacher, 1988; Sharma et al., 1997; Stephan, 1983; Strubbe et al., 1986). Skeleton photoperiods are thought to entrain the circadian system with the interaction of a morning phase advance and an evening phase delay.

There are many limitations of this approach with respect to entrainment by natural LD cycles. For example, Hut et al. (1999) has shown that the PRC model is not sufficient to explain entrainment under natural condition in the European Ground Squirrel, because the model requires that the animal is exposed to at least one of the LD transitions. This is clearly not the case in the European Ground Squirrel. Thus, the parametric model has been used to explain entrainment under natural conditions. According to the parametric model, the zeitgeber acts continuously on the pacemaker and has the effect of changing the angular velocity of the pacemaker and consequently its period in a phase dependent manner. Tau response curves have now been derived for several species (Beersma et al., 1999a). In his paper titled 'Colin Pittendrigh, Jurgen Aschoff, and the Natural Entrainment of Circadian Systems', Serge Daan (2000) argues that changes in angular velocity as characterized by the tau response curve contribute to the stability of entrainment in diurnal species, and are particularly important in burrowing animals. This and other authors (Beersma et al., 1999 a, b) have suggested that the LD pacemaker(s) is most accurate when responding to light by simultaneous phase shifts and changes of velocity.

1.2. ORGANIZATION OF THE CIRCADIAN SYSTEM

1.2.1. Functional organization

This section provides a description of the most frequently employed terms used to describe the components of the circadian system. The most widely accepted functional model of the mammalian circadian system postulates the existence of multiple oscillators which are hierarchially and non-hierarchially arranged and mutually coupled (Moore-Ede et al., 1976). The term 'pacemaker' is used to describe the oscillator(s) at the top of the hierarchy which is (are) responsible for synchronizing the circadian system as a whole. The term 'oscillator' has been applied to oscillators lower down the hierarchical scale which are normally driven by the major pacemaker. The oscillators in turn drive the overt rhythms. According to this model, information is transmitted between the separate oscillatory units by neural and endocrine variables called 'mediators'.

1.2.2. Structural organization and functioning

In 1972, Stephan and Zucker and Moore and Eichler published the now classic studies providing the first clear evidence that the suprachiasmatic nuclei of the hypothalamus (SCN) contain a major circadian pacemaker. Corroboration of this view comes from a number of studies utilizing a range of techniques. The most widely used technique is based on the destruction of the SCN, with researchers reporting a loss or severe disruption of circadian rhythmicity following SCN ablation or surgical isolation (Eastman et al., 1984; Ibuka & Kawamura, 1975; Ibuka et al., 1977; Inouye & Kawamura, 1979, 1982; Moore & Eichler, 1972; Refinetti et al., 1994; Richter, 1967; Schwartz & Zimmerman, 1991; Stephan & Zucker, 1972). These findings have been supported by electrophysiological and metabolic studies, demonstrating a circadian rhythm in SCN activity (Bos & Mirmiran, 1990; Green & Gilette, 1982; Groos & Hendriks, 1982; Honma et al., 1998; Inoye & Kawamura, 1979; Mirmiran et al., 1995; Schwartz & Gainer, 1977, Schwartz et al., 1980; Shibata et al., 1982). Further support for this idea comes from the studies of Sawaki et al. (1984) and others (Anguilar-Roberlo et al., 1992, 1998; Griffioen et al., 1993; LeSauter & Silver, 1994; LeSauter et al., 1996; Li & Satinoff, 1998; Saitoh et al., 1990, 1991) who transplanted whole cell foetal grafts and dissociated cell suspensions in SCN lesioned animals and reported a restoration of circadian rhythmicity. In a similar study by Ralph et al. (1990), tau of the recipient corresponded to that of the donor. These results are consistent with those of other studies utilizing non-destructive techniques, including Fuchs and Moore (1980) and Schwartz et al. (1983) who reported phase shifts and changes in tau of the free-running rhythm following electrical stimulation of the SCN.

A pacemaker must have input and output pathways. Because this issue is outside the scope of the current thesis, the transduction pathway to the SCN is only briefly discussed. There are many recent reviews on this topic, including Inoye and Shibata (1994), LeSauter and Silver (1998), Miller et al. (1996), Morin (1994), Pickard and Rea (1997), Rietveld (1992, 1993), and van den Pol and Dudek (1993). It is now well established that the SCN respond to photic input from the retina. There is evidence supporting a critical role for the retinohypothalamic tract in relaying information from the retina to the SCN, however, other afferent pathways also have been implicated (Hendrikson et al., 1972; Moore & Lenn, 1972; Pickard & Silverman, 1981; Van Gelder, 1998). The SCN receives another input from the retina indirectly via the intergeniculate nucleus; this pathway appears to contribute to the process of photic entrainment. In addition to the direct and indirect retina-to-SCN pathways, the SCN receive afferents from several other brain regions, including a large projection from the raphe. The relevant literature with regard to the raphe suggests a role in circadian rhythm regulation.

Much less is known about the efferent pathways that mediate entrainment to LD cycles. It is known that the SCN transmit information to a number of central brain structures, including hypothalamic, thalamic, limbic, and caudal brain structures (Ibata et al., 1999; Watts, 1991; Watts et al., 1987). A particularly dense efferent projection to the subparaventricular zone of the hypothalamus has been identified; this region in turn projects to most of the same sites as the direct efferents of the

SCN. The connection with the subparaventricular zone is thought to be involved in circadian functioning.

Even less is known about the nature of the signal released by SCN cells that communicates information to other brain structures. There appear to be two output mechanisms for the SCN. In addition to classical synaptic transmission, the SCN appears to influence other neurons via endocrine or paracrine output. Evidence in support of this hypothesis comes from transplant studies. The results of these studies indicate that SCN transplants restore circadian rhythmicity in the host before any massive axonal outgrowth from the transplant has occurred (Lehman et al., 1987). Recovery is reported to occur even if the SCN is transplanted to a site some distance from the SCN, in the apparent absence of contact with any other brain regions (Anguilar-Roberlo, 1994; DeCoursey & Buggy, 1989). Furthermore, when hypothalamic knife cuts are used to isolate the SCN from synaptic contact with the rest of the central nervous system, circadian rhythmicity may be maintained (Hakim et al., 1991).

1.2.3. Evidence for a multi-oscillator system

The organization of the circadian system has not been clearly established and there is at present a lack of agreement amongst researchers as to how many structures are involved. While it is clear that the SCN is a key component, findings regarding the presence of other pacemakers are unclear. By far the most compelling evidence for the existence of multiple pacemakers comes from the study of other zeitgebers, including cycles of food availability; these empirical findings will be reviewed in chapter 2.

There now appears to be a substantial amount of evidence for the presence of multiple oscillators. The most commonly held view is that the circadian system consists of two oscillators, however, there appears to be some evidence that allows the postulation of separate, self-sustaining oscillators responsible for driving different rhythms (Aschoff, 1965b; Eastman & Rechtshaffen, 1983). This evidence will not be reviewed here. There is strong evidence to suggest that at least two distinct oscillators underlie any given rhythm, with a number of researchers noting that a single rhythm can break down into two components which initially free-run independently, but later develop a new stable phase relationship 180 degrees anti-phase to one another. 'Rhythm splitting' was first described by Pittendrigh in 1960 for the arctic ground squirrel and the Syrian hamster, and since then generally has been found to occur in response to changes to light intensity or after prolonged exposure to constant photic conditions (Pittendrigh, 1974, 1976c). Splitting has been observed in a range of circadian rhythms, including locomotor activity, feeding, drinking, body temperature, electrical brain stimulation, serum concentration of luteinizing hormone, and neural activity of the SCN (Boulos & Morin, 1985; Meijer & Rietveld, 1989; Menaker, 1959; Pickard & Turek, 1983; Pittendrigh, 1960, 1967, 1970; Rosenwasser & Adler, 1986; Swann & Turek, 1985; Turek et al., 1982). The finding in these studies of rhythm splitting, taken together with the fact that most species show two major peaks at the beginning and end of activity, is consistent with the functional model proposed by Pittendigh and Daan in 1976. According to the model, which was based on empirical data from four rodent species, the circadian system comprises of two mutually coupled oscillators which respond differentially to the effects of light. One of the oscillators controls the morning component of activity (M oscillator) and the other controls the evening component (E oscillator). The view that splitting represents a break down in mutual coupling between two oscillators is supported by mathematical demonstrations that mutually coupled oscillators have two steady states to each other; one with nearly 0 degrees difference and the other one with 180 degrees phase difference (Daan & Berde, 1978).

Over the years, the two-oscillator model has been frequently evoked by many researchers to account for empirical findings (Elliot & Tamarkin, 1994; Gorman et al., 1997; Hoffman & Illnerova, 1986; Illnervora, 1991; Oda et al., 2000; Puchalski & Lynch, 1986, 1988a,b, 1991a,b, 1994; Vilaplana et al. 1997). For example, the multi-oscillator model has been used by some of these authors to explain dissociation of free-running rhythms into several circadian components which can be entrained by a ultradian LD cycle. In 1982, Pickard and Turek proposed that the contralateral SCN act as independent oscillators. Recent studies report asymmetrical electrical activity between the SCN nuclei in vitro and there is evidence to suggest that tau of the free-running rhythm is influenced by interactions between the two SCN (Davis & Viswanathan, 1996; Zhang & Aguilar-Roberlo, 1995). Furthermore, Davis and

Viswanathan (1996) concluded a single SCN is as capable as two SCN of producing circadian rhythmicity. Despite this, the model remains a hypothetical construct.

While the above hypothesis has accrued some support over the years, many researchers now view each SCN neuron as a circadian oscillator. The fact that isolated SCN neurons kept in culture display circadian rhythms in neuronal firing is one line of evidence used to support this hypothesis (Welsh et al., 1995). In this study, circadian rhythms of widely different phases and period lengths were recorded from neurons in the same culture. Given that neuronal rhythms in SCN slice are synchronized, it was proposed that the synchronizing factor(s) for SCN autonomous cellular oscillators is usually present in cell slice but not in cell culture (Miller et al., 1996). These mechanisms will not be reviewed here, but the interested reader can refer to Miller (1993) and van den Pol (1993).

Modern molecular studies support the notion that the mammalian circadian system is multi-oscillatory. Early studies yielded remarkable results; data were collected from *Drosophila melanogaster* and single gene loci, including period (*per*) and time-less (*tim*), were found to affect pacemaker behaviour (Hall & Rosbash, 1988; Rosbash & Hall, 1989; see Hall, 1995 for review). Since the publication of these papers, the 'clock' genes in mammals have received attention; *per1*, *per2*, *per3*, and *clock* oscillate in the SCN and appear to be important for circadian clock function (Balsalobre et al., 1998; King et al., 1997; Sakamoto et al., 1998; Shearman et al., 1997; Tel et al., 1997; Zylka et al., 1998). Results of these studies indicate that all *per* genes are expressed in other brain regions, as well as peripheral tissues. Zylka and coworkers (1998) found that *per* RNA levels display prominent circadian rhythms in liver, skeletal muscle, and testis. These findings are comparable to those obtained from *Drosophila melanogaster* in which *per* is widely expressed throughout the body and in most places where *per* is expressed, its RNA oscillates. Given that light sensitive oscillators are known to exist throughout the fly body, in all areas in which *per* oscillates, it is possible that peripheral oscillators exist outside the SCN in mammalian species. This hypothesis has been supported by Yamazaki et al. (2000) who reported that peripheral tissues exhibit damped oscillation in the absence of the SCN. It was argued that the mammalian circadian system is hierarchically organized, with self-sustained oscillators in the SCN entraining damped oscillators in the periphery. This issue will be considered again in section 2.4.1.

CHAPTER 2. THE FEEDING ENTRAINABLE CIRCADIAN SYSTEM IN THE OMNIVOROUS RAT

Although the LD cycle has been studied extensively, a relatively neglected area has been the study of other zeitgebers. While the zeitgeber properties of LD cycles are well established, it is not clear whether these effects can be generalized to other zeitgebers, or whether they are specific to the LD cycle. It is possible that different zeitgebers may affect the circadian system in different ways. The extent of similarity/differences between zeitgeber effects needs to be determined before proceeding to develop elaborate theories of the circadian system which may or may not apply to other zeitgebers. Results from studies of feeding schedules have presented an unclear picture, and suggest that entrainment is less consistent and predictable than under LD cycles. In view of the variable effects of feeding schedules, it may be preferable at this stage to focus on this zeitgeber. The purpose of this chapter is to discuss the empirical data with regard to cycles of food availability. The following discussion will focus on the rat because this is by far the most thoroughly studied species. Chapter 3 will consider other species in some depth and interspecies comparisons will be made. Before proceeding, worthy of mention is the notable decline in research in this field over the last decade. The most likely explanation for this trend is that the feeding entrainable circadian system is proving to be intractable.

2.1. INTRODUCTION TO THE FEEDING ENTRAINABLE CIRCADIAN SYSTEM

When food availability is restricted to a few hours at the same time each day, activity onset in the entrained state precedes the onset of food access. In 1922 Richter coined the term 'anticipatory activity' to describe the bout of increased activity that precedes food access. Since the work of Richter there have been many published reports of anticipatory activity in a number of circadian rhythms, including wheel-running (Aschoff et al., 1983; Bolles & Lorge, 1962; Stephan, 1981, 1986b), general cage activity (Aschoff et al., 1983), food-lever pressing (Bolles & Stokes, 1965; Boulos et al, 1980; Mistlberger et al., 1996), approaches to the food bin (Aschoff et al., 1983; Davidson & Stephan, 1999; Ruis et al., 1989), and drinking (Aschoff et al., 1983; Stephan, 1981, 1986b). In addition to activity rhythms, entrainment of a number of physiological variables has been demonstrated (Challet & Pevet, 1996; Challet et al., 1997; Honma et al., 1992; Leal & Moreira, 1997; Mitome et al., 1994). The interested reader can refer to Boulos and Terman (1980) and Johnson (1992) for a review on this topic.

Since Richter coined the term 'anticipatory activity', researchers have focused on determining the nature of the mechanism that underlies anticipatory activity. The entrainment model is undoubtedly the most widely recognized and used in the empirical literature. This is not surprising since many aspects of anticipatory activity can be explained within the framework of this theory (Mistlberger, 1994). This model will be discussed extensively throughout the current thesis; empirical outcomes will be explained with reference to this model. Other researchers have postulated a specific rôle for learning processes in the generation of anticipatory activity (Mistlberger, 1994; Mistlberger & Merchant, 1995). There have been several learning hypotheses proposed over the years. A brief overview of learning theories will be presented here; the interested reader can refer to Mistlberger (1994) for a review on the topic. The major learning hypothesis of anticipatory activity includes a postulated role for a single circadian oscillator. Two assumptions are made. The clock must store in memory a representation of the circadian phase at which feeding occurs. These representations are used in computations to predict future feeding times and the predictions can be implemented by consulting the clock for current time. While this model has received some support in non-mammalian species, it has several weaknesses (Mistlberger, 1994). In a study designed to test the predictions of 'entrainment' versus 'computational' learning models of anticipatory activity in rats, Mistlberger and Marchant (1995) concluded that the latter is less compatible with empirical findings. Other learning models invoke a more traditional, associative learning mechanism. These models also are subject to serious criticism (Mistlberger, 1994; Mistlberger & Marchant, 1995). In the absence of further empirical support for learning theories, these conjectures remain purely speculative.

2.2. ADAPTATION TO CYCLES OF FOOD AVAILABILITY

The method used to assess the zeitgeber effects of feeding schedules involves providing *ad libitum* food access pre- and post- a cycle of restricted food availability. A number of investigators have indicated that body weight, food and water intake is lower during the feeding schedule relative to the pre- and post- phases (Balagura et al., 1975; Fallon, 1965; Lawrence & Mason, 1955; Moskowitz, 1958). Similar results were obtained by Reid and Finger (1954) who collected data both pre- and post- scheduled feeding and found that intake was lowest during the feeding schedule and highest after its removal. Median weight loss was cited at 31% of baseline body weight, with the greatest weight loss occurring over the first 10 to 15 days of the feeding schedule. These investigators also noted that one of eight animals failed to adapt to the feeding schedule and died. While the results are generally comparable across studies, the magnitude of the decrement in body weight, and food and water intake has differed from one study to another, but is always largest initially. These studies show reasonable consistency in reporting a period of adjustment to feeding schedules of at least 10 days.

Other changes, including changes in activity levels, have been reported to occur in response to feeding schedules. Although the results have not been entirely consistent, a general pattern is apparent. Aschoff et al. (1983) measured wheel running activity pre-, during, and post- scheduled feeding of 2h food access and found that activity increased by 150% during the feeding schedule and decreased below baseline levels afterwards. They found no effect on wheel-running activity using a feeding schedule of 4h food access. Results from this study also indicated that activity measured with devices involving stationary cage techniques remained constant. There is thus a question as to whether the data are general or a function of the type of instrument being used to measure activity. Aschoff et al. (1983) also noted that the amount of activity directed at the food and water hoppers did not change under a feeding schedule. A subsequent study by Stephan (1986a) has not supported this finding, showing lower levels of drinking (25%) and approaches to the food bin (50%) during the feeding schedule compared to the pre- and post-phases.

2.3. THE FEEDING ENTRAINABLE CIRCADIAN SYSTEM

This section provides a review of the empirical literature supporting a zeitgeber role for cycles of food availability. An analogy has been made between the putative feeding entrainable pacemaker (FEP) responsible for driving anticipatory activity and the SCN 'light entrainable pacemaker' (LEP) which mediates photic entrainment.

The terms ‘anticipatory activity’ and ‘free-running light entrainable rhythm’ have been used in the empirical literature to describe the overt rhythms representing the output of the FEP and the LEP, respectively. This terminology has been adopted in the present thesis in order that the reader be able to easily distinguish between the two rhythms. Evidence in support of this proposition is reviewed below.

2.3.1. Phase angle difference between activity onset and the onset of food access

During scheduled feeding, a unique phase relationship is established between the overt rhythms and the feeding schedule. As noted above, the onset of activity consistently precedes the onset of food access. If the interval between activity onset and the onset of food access is expressed as the PAD between an entrained pacemaker and a zeitgeber, it follows that the PAD during scheduled feeding would be positive. Thus, as noted earlier, the term ‘anticipatory activity’ is used to describe this state of increased activity preceding food access.

The PAD between activity onset and the onset of food access for different overt rhythms may vary. Generally, the PAD is more positive for wheel-running, approaches to the food bin, and lever-pressing, ranging between +2h and +4h (although PADs as long as +7h have been reported in recent study by Lax et al., 1999), and less positive for drinking (+0.5h) and general cage activity (Aschoff et al., 1983; Ruis et al., 1989; Stephan, 1981, 1986b). However, results differ across studies. For example, estimates of PAD for drinking range from +0.5h to +2h. This variability may reflect, in part, the range of criteria used to define activity onset. Many researchers do not specify the criteria used to calculate the PAD or the reason for selection of a particular method is not provided.

The PAD may vary as a function of feeding cycle T. Aschoff et al. (1983) reported data from rats housed in dim LL, and observed that PAD is positively correlated with T within the limits of 23.5h to 26h. Under these conditions, PAD ranged from less than 1h to 4h, and only positive PADs between activity onset and the onset of food access were reported. At T=27h, smaller PADs were measured than at T=26h, while at T=22h the PAD was such that activity onset coincided with the meal. Aschoff (1987) reported similar findings in an analysis of previous studies. Results from a study by Stephan (1981), however, have not been entirely consistent with this. Stephan employed as a measure of PAD the 'anticipatory ratio' (i.e., the ratio of mean activity immediately prior to food access to mean activity over an equal time span preceding this period) and found the anticipatory ratio to correlate positively with the T cycle, but observed the relationship is non-linear. The results indicated the mean anticipatory ratio was highest for T=24h, followed in order by T=27h, 23h, 29h.

The PAD to scheduled feeding may vary according to the phase relationship between the FEP and the LEP. A negative relationship has been demonstrated between PAD to the feeding schedule and the phase relationship between the two pacemakers. Stephan and Becker (1989) have presented data showing that PAD decreased as the phase relationship between the pacemakers was reduced such that activity onset coincided with or occurred just after food access when the two pacemakers crossed. It is possible that the PAD between activity onset and the onset of food access may reflect the phase relationship between the LEP and the FEP, which are mutually coupled. Evidence supporting this claim will be discussed in section 2.4.3.

2.3.2. Latency of anticipatory activity

There is some variability in the latency of anticipatory activity to scheduled feeding across studies as a result of differences in ambient lighting (i.e., LD cycle). A number of studies have presented data indicating that a two to seven day latency is typical of rats housed under a LD cycle (Mistlberger & Marchant, 1995; Rosenwasser et al., 1984; Stephan et al., 1979a). A slightly longer latency has been cited for blind rats or rats free-running in dim LL or DD, with some researchers reporting a latency of 10 days or more (Aschoff et al., 1983; Honma et al., 1983; Stephan, 1986a, b). Aschoff et al. (1983) found longer latencies near the limits of entrainment. He reported a dependence of latency on the phase relationship between food access and the LEP. These findings were later confirmed by Stephan (1986a). In 1990, Mistlberger et al. used bright LL conditions to eliminate the free-running light entrainable rhythm and reported that rats were slower to develop anticipatory activity under these conditions than other rats housed under lower LL intensities. Other studies have indicated that SCN lesioned rats develop anticipatory activity more quickly than intact rats

(Stephan, 1981, 1984; Stephan et al., 1979b). The significance of this finding will be considered in section 2.4.2.

2.3.3. Residual activity following removal of the feeding schedule

In order to differentiate between entraining effects and masking effects, it is necessary to demonstrate that the anticipatory rhythms free-run from a phase determined by the feeding schedule when the potential zeitgeber is removed. Two different methods of assessing rhythm phase have been used. The most commonly used method has involved replacing daily food access with *ad libitum* feeding. Coleman et al. (1982), however, studied rats and reported that anticipatory activity did not persist during *ad libitum* feeding but reappeared during subsequent periods of food deprivation. It was argued that this indicated that the FEP was being masked by *ad libitum* feeding in a manner analogous to masking of the LEP by bright LL. The other paradigm requires an animal be deprived of food for two or more days once the feeding schedule is removed.

When a feeding schedule is replaced with food deprivation, meal-associated rhythms have been found to persist in a number of circadian rhythms, including wheel-running activity, drinking, approaches to the food bin, and lever pressing (Bolles & Moot, 1973; Clarke & Coleman, 1986; Coleman et al., 1982; Mistlberger et al., 1996; Mistlberger & Merchant, 1995; Rosenwasser et al., 1984; Ruis et al., 1989; Stephan, 1992b; Stephan et al., 1979b). Activity can persist at the former time of feeding for 50 days or more, even when the deprivation trials are repeated after seven or more days of *ad libitum* feeding (Clarke & Coleman, 1986; Coleman et al., 1982; Rosenwasser et al., 1984; Ruis et al., 1989). These investigators found meal-associated activity to be less likely to recur and became less robust over repeated deprivations. Results from the Ruis et al. (1989) study, however, have been inconsistent in showing activity peaks did not correspond to the former mealtime in previously night-fed rats. The reason for this discrepancy is not clear.

Due to the self-limiting nature of food deprivation, estimates of free-running tau of the FEP are difficult to obtain. Investigators have used deprivation sessions, interleaved with periods of *ad libitum* feeding to track the time course of FEP. It was concluded that the FEP free-runs with a tau close to 24h (Clarke & Coleman, 1986; Coleman et al., 1982; Stephan et al., 1979b). Although Ruis et al. (1989) attempted to replicate the results obtained by these authors, results were inconsistent, and indicated a tau>24 h. This discrepancy may reflect differences in the methodology of these studies. In a study by Stephan (1981), rhythm free-runs were observed near the limits of entrainment, but tau was most likely confounded by aftereffects caused by prior exposure to different T cycles (see section 2.3.6.). Consistent with this interpretation, Mistlberger and Marchant (1995) used a range of feeding cycle Ts and found tau of the FEP approximated T during subsequent periods of food deprivation. Further studies are required to formally quantify tau of the FEP.

2.3.4. Reentrainment after a phase shift of food access

Phase shifts of food access can be achieved by advancing the time of feeding in one cycle or by delaying the time of feeding in one cycle. It is well established that a shift of food access results in a shift in anticipatory activity by the same number of hours (Stephan, 1984, 1986b, 1992a,b, 1997). The FEP may require one or more days to reentrain to the new mealtime, and the anticipatory rhythms usually reestablish the same PAD to the meal as prior to the phase shift.

Reentrainment to the new mealtime is usually achieved by delaying transients independent of the direction or the magnitude of the phase shift. In a series of studies examining the phase shifting properties of feeding schedules, Stephan (1984, 1986b, 1992a,b, 1997) generated some evidence in support of this proposition. In 1984, Stephan collected data from SCN lesioned rats exposed to delays and advances ranging from 4h to 8h, finding that reentrainment of anticipatory activity occurred by delaying transients. The exception to this was the 8h advance which was followed by both delaying and advancing transients. These findings have been confirmed in subsequent work by Stephan, however, in the 1986 study advancing transients were found to occur in response to a 4h advance in the time of food availability. This researcher also noted that 12h shifts, as well as some 10h advances resulted in rapid reentrainment without visible transients, with most 10h shifts resulting in delaying transients. In a more recent study, advancing transients were again observed following an 8h phase advance (Davidson & Stephan, 1999).

The rate of reentrainment does not appear to vary as a function of the magnitude or direction of the phase shift. The rate of reentrainment in intact rats has differed from one study to another, with estimates usually ranging from two to 11 days. In his 1986(b) study, Stephan reported that rats reentrained over six to 10 days to a 4h advance, over two to five days to a 4h delay, over four to five days to an 8h phase advance/delay, and over two to 11 days to a 6h phase advance/delay. Stephan did show however, that the magnitude and direction of the phase shift could affect the qualitative manner in which entrainment is achieved. In his 1984 study, the duration of anticipatory activity was increased and rats remained active until food became available after a phase delay of 4h to 6h, so that transients occurred only in activity onset. A similar increase in the duration of anticipatory activity was found to occur after an 8h delay, however, activity ceased prior to the new mealtime and transients occurred in both activity onset and activity offset.

2.3.5. Range of entrainment to feeding schedules

Results from a number of studies have indicated that intact rats housed under a LD cycle entrain to a limited range of feeding schedule Ts. Bolles and deLorge (1962) collected data from 18 rats housed in a diurnal environment and exposed to feeding schedules with Ts=19h, 29h, and 24h, and reported anticipatory wheel-running at 24h intervals only. A similar approach was adopted by Mistlberger and Marchant (1995) who reported that rats entrained to a 24h LD cycle also entrained to adiurnal feeding schedules, but only within the range of 24h to 26h. Using food deprivations, it was determined that meal-associated activity occurred in animals previously exposed to T=22h, but not to T cycles less than 22h, suggesting that 22h approaches the lower limits of entrainability. These results are similar to those obtained by Bolles and Stokes (1965). In a study of 28 rats, born, reared, and tested under a 19h or 29h LD cycle and later exposed to a feeding schedule with corresponding T, circadian rhythms were found to respond only to changes in illumination and not to the feeding schedule.

Intact rats anticipate a limited range of feeding schedule Ts under LL conditions. Data from rats kept in dim LL and exposed to various feeding schedules indicated that anticipatory activity occurred to T cycles ranging from 23.5h to 27h (Aschoff et al., 1983). By contrast, the free-running light entrainable rhythm was not entrained by the feeding cycle; this issue will be discussed in section 2.4.2. These results are consistent with those obtained in a previous study by Boulos et al. (1980) who observed entrainment to feeding schedules with T=23h and T=25h under LL, but not under T cycles of 18h and 30h.

Rats with SCN lesions show a similarly limited range of entrainment. Results from a number of studies (Stephan, 1979a, b; Boulos et al., 1980) have indicated that SCN lesioned rats anticipate feeding cycles with T=23h, 24h, and 25h, but not to 18h and 30h cycles. In the first systematic study of the limits to entrainment in SCN lesioned rats, Stephan (1981) compiled data from 30 rats and obtained results consistent with his original findings. Findings indicated that rats could anticipate feeding schedule Ts of 22h to 29h. He reported anticipatory ratios to be higher under long T cycles (T>27h) compared to short cycles, and concluded that anticipatory activity to most long cycles is quantitatively and qualitatively better. Findings regarding entrainment to T cycles of 31h and 33h appeared to be less clear, with a more diffuse activity onset and activity often stopped before the onset of food access. PAD was found to be highly variable, with some rats showing a substantial increase in the duration of activity about every five days.

2.3.6. Aftereffects on tau of the feeding entrainable pacemaker

There may be aftereffects of entrainment to cycles of food availability. A number of researchers have indicated that tau of the FEP may approximate T on removal of a feeding cycle. For example, Mistlberger and Marchant (1995) reported that the onset of meal-associated activity advanced when T was equal to 24h and delayed with T>24h. In a comprehensive study of aftereffects, Stephan (1981) reported that gradual changes in feeding cycle T extended the limits of entrainment. The approach adopted by Stephan was to change T sequentially from 24h to 27h, 29h and 31h. The upper limit of entrainment was found to be 31h, however, no rats showed anticipatory activity to a 31h cycle when T was changed abruptly from 24h to 31h. This inconsistency was attributed to aftereffects caused by prior exposure to feeding cycles.

2.3.7. Phase response properties of the feeding entrainable pacemaker

The method used to obtain a PRC for cycles of food availability involves depriving the animals of food for three days, and reinstating *ad libitum* food access at different times of the subjective day and subjective night while the animal is free-running either in LL or DD. The magnitude and direction of the phase shift of the free-running light entrainable rhythm caused by the deprivation can be plotted as a function of the circadian phase of food reinstatement. Using this technique, Coleman and his colleagues (1989, 1991) reported rats to experience major responses to food deprivation/reinstatement throughout the subjective day and night, with phase delays of up to 7h occurring in the late subjective night and small phase advances occurring in the early subjective night. While these results are interesting, several issues need to be considered. The main problem is that these authors used a small number of data points per circadian phase. A further question of interest, based on the finding in these studies of a lack of effect in DD, is what aspect of the experimental situation produced the phase shifts. One possible explanation for the differential effects of DD and LL is that LL may be acting on the LEP to produce these effects.

Stephan (1984) indicated that a PRC to food availability characterized by a large delay section and a small advance section would be consistent with the asymmetry of entrainment with respect to 24h. If the maximum phase advance is larger than the maximum phase delay, then it would follow that the lower limit of entrainment to food availability would be closer to 24h than the upper entrainment limit. In 1992, Stephan employed a different method, using total food deprivation probes to study the phase resetting properties of the FEP. Data were collected from rats exposed to phase shifts ranging from 4h to 10h, then fed at the new mealtime for one to four days. Subsequent food deprivation probes revealed that one to two food pulses delayed the onset of anticipatory activity the day after the food pulse, but were not sufficient to reset the phase of the FEP. Three to four food pulses were required. That transients had approached the new mealtime prior to deprivation in these animals supports the view that resetting occurs when the FEP is entrained to the new mealtime. As Stephan has pointed out, these responses are widely discrepant from those of the LEP (see section 1.2). The reason for these differences is unclear, but may reflect in part differences in methodology. Stephan acknowledges that the method used in this study is not equivalent to a standard PRC.

2.3.8. Theoretical and methodological issues

While the above findings provide support for a zeitgeber role of cycles of food availability, several issues still need to be addressed. One of the main issues in this area is that the status of the FEP prior to the feeding schedule is unknown. This issue is an important one because it has implications for the manner in which the feeding entrainable circadian system is conceptualized. Another issue relates to the PAD between activity onset and the onset of the meal. The PAD (always positive) reported under cycles of food availability is not consistent with the range of positive and negative PADs found to occur under LD cycles. Mistlberger and Marchant (1995) argued that the method by which PAD is assessed may be problematic, since a negative PAD would be expected to occur when T<24h, but may be masked by postprandial somnolence. An approach adopted by these researchers was to use food deprivations to assess the phase of activity onset, however, little or no activity was found to persist at the former mealtime to T cycles less than 24h.

It is possible, as Stephan (1984) has suggested, that a positive PAD reflects the adaptive significance of the ability to anticipate periodic food sources. The feeding entrainable circadian system may have evolved in response to the predictable daily occurrence of food availability for preparation in advance in all relevant bodily systems (e.g., metabolism, olfactory, gustatory, visual etc.), but this applies particularly to the behavioural acquisition of food. Thus, as Mistlberger (1994) has noted, the rat is able to anticipate the future occurrence of food availability and be prepared to interact with its environment at that time to ensure food acquisition. Some investigators have drawn an analogy between anticipatory locomotor activity observed in the laboratory and foraging behaviours; in the wild, locomotor activity may serve an anticipatory function in helping locate food in some species (Armstrong, 1980). As mentioned above, some adaptations may be related to digestive/physiological variables. These would be particularly important if food is available for a short time or in a small amount, since the animal must accelerate ingestion and/or enhance the efficiency of nutrient digestion and utilization.

A major methodological problem that needs attention is the possible lack of control over external cues that signal food access. Hungry rats are more sensitive to external cues and this may affect the nature of the data obtained. Ruis et al. (1989) report that hungry rats respond with increased wheel running and food approaches when the room is entered or passed by. One explanation for this finding is that hungry rats have a lowered threshold for novel stimuli and thus respond stronger than satiated rats. Given this, it is not possible to exclude the possibility that the FEP is affected by the response of hungry rats to external stimuli that signal food access, such as room entrance, including the sounds and odours of attendants, and noise and visual cues generated by the food access device. However, there is little direct evidence on this point in rats and further study seems warranted. Investigations of sodium-depleted rats in salt-access studies suggest that external cues tend to suppress anticipatory activity (Rosenwasser et al., 1988). However, as noted by Rosenwasser et al. (1988), such cues may be of greater concern for salt-anticipatory responding, since anticipatory activity occurs even when the onset of food availability is accompanied by some cues. More extensive data on external cues are available from other species. For example, the effect of external cues on the putative FEP in pigeons is clearly significant. In a study by Abe and Sugimoto (1987), pigeons did not show anticipatory food-key pecking when the food key was lit only during the access period. By contrast, anticipatory activity occurred when the key was lit continuously. These findings suggest that it may be necessary to exercise a greater degree of control over cues that signal food access than is commonly achieved in most studies.

2.3.9. Succeeding activity

It is beyond the scope of this thesis to examine the effects of feeding schedules on succeeding activity, since it is not clear at this stage whether succeeding activity is generated by the circadian system. A brief overview of the literature will be presented here. Some investigators have reported that, in addition to anticipatory activity, a second bout of activity called 'succeeding activity' occurs after the meal (Aschoff et al., 1983; Honma et al., 1983; Stephan, 1981). To date there has been little work investigating the possibility that succeeding activity is an expression of a circadian rhythm. The above-cited studies provide some evidence in support of this proposition, however this conclusion was challenged by Stephan in 1981 who argued that succeeding activity is a passively driven rhythm. One of the primary problems is the uncertainty as to what constitutes the onset and duration of succeeding activity. Data so far suggest that succeeding activity is most clearly expressed in wheel-running activity, and Aschoff and his coworkers observed the duration of succeeding activity to correlate negatively with the duration of anticipatory activity, resulting in a stable band of activity around the time of feeding. In 1991, Aschoff challenged Stephan's conclusion and suggested that succeeding activity may represent the trailing end of the FEP. Further research is needed to resolve this issue.

2.4. THE ORGANIZATION OF THE FEEDING ENTRAINABLE CIRCADIAN SYSTEM

2.4.1. Evidence for a feeding entrainable pacemaker outside the SCN

Data from behavioural studies provide support for a multi-oscillator model of the circadian system. A finding by many researchers is that anticipatory rhythms coexist with free-running light entrainable rhythms under a feeding schedule in LL, DD, or after optic enucleation (Aschoff et al., 1983; Cambras et al., 1993; Edmonds & Adler, 1977a; Honma et al., 1983; Lax et al., 1999; Stephan, 1986a,b; Stephan & Becker, 1989). In a recent study by Lax et al. (1999), data were collected from rats housed under a 24h LD cycle and subjected to a feeding schedule with T=23h. They found that rats tended to display two activity bouts with different taus, one associated with each zeitgeber. It was proposed that a dual FEP-LEP model could account for these findings, on the assumption that a single pacemaker cannot express two or more periodicities.

There is now a substantial amount of evidence for the existence of a FEP outside the SCN. Using the ablation method, Stephan et al. (1979a,b) and Phillips and Mikulka (1979) found that the free-running light entrainable rhythms were eliminated when the SCN was lesioned, but anticipatory activity persisted. These findings were subsequently corroborated using a diversity of behavioural and physiological variables known to express anticipatory activity (Boulos et al., 1980; Clarke & Coleman, 1986; Ruis et al., 1989; Stephan, 1981, 1983, 1989a,b, 1992a,b, 1999; Stephan & Becker, 1989; Stephan et al., 1979a,b,c). These researchers have reported findings indicating that SCN lesioned rats entrain to a limited range of feeding schedule Ts, transients occur after a phase shift of food access, and residual activity is evident on removal of a feeding schedule. Further support for the above proposition comes from Inoye (1982) who recorded multiunit SCN neuronal activity in vivo and found no significant effects of feeding schedules.

There have been many structures proposed to be the site of the FEP, however, ablation studies have not provided empirical support for these hypotheses. Structures involved in the regulation of food intake have received the most attention, including the adrenal glands (Boulos et al., 1980; Stephan et al., 1979a), the ventromedial hypothalamus (Challet et al., 1997; Honma et al., 1987b; Mistlberger & Retchschaffen, 1984), the paraventricular nucleus (Mistlberger & Rusak, 1988), the lateral hypothalamus (Mistlberger & Rusak, 1988), and caudal brainstem structures (Davidson et al., 2001). Interestingly, a recent study by Heigl and Gwinner (1999) has ruled out the pineal gland as the site of the FEP in house sparrows. Given that all of these studies adopted the same line of investigation, research now may need to focus on adopting a new approach, as well as a new way of looking at the old problem. It is conceivable that, as Mistlberger (1994) has noted, the FEP consists of more than one structure. It is possible that ablation of specific structures may not result in the loss of anticipatory activity if there is functional redundancy within the system.

Results of molecular studies provide support for the presence of a FEP in peripheral tissue. As noted in section 1.2.3, there is now compelling evidence for the existence of extra-SCN oscillators; *per* gene expression occurs widely in other brain regions and in peripheral organs such as lungs, heart and liver. Yamazaki et al. (2000) report that *per1* gene expression is advanced more quickly in the SCN compared to peripheral organs following a LD phase shift. A finding by Hara et al. (2001), Damiola et al. (2001), and Stokkan et al. (2001) was that restricted feeding entrained *per* gene expression in peripheral tissues without affecting the SCN. In the study by Hara et al. (2001), findings also indicated that a LD phase shift affected *per* gene expression in the SCN, but not in the liver of restricted-fed mice. These authors report that restricted feeding-induced oscillations of *per* gene levels in the liver did not require an intact SCN. Interestingly, results of biochemical studies also point to the involvement of the digestive system in the entrainment of the FEP (see section 2.4.4). There is some indication in the above results that the FEP may exist in peripheral tissue such as the liver. It was argued by Stokkan et al. (2001) that the FEP responds directly to the environment in a manner similar to some peripheral organs in *Drosophila* whose rhythms respond directly to environmental signals.

2.4.2. *Coupling between the feeding- and light- entrainable pacemakers*

The previous section reviewed evidence that the circadian system of the rat contains at least two circadian pacemakers: a LEP and a FEP. In the present section, evidence is presented which suggests that the two pacemakers may be weakly coupled.

Results of a number of studies have indicated that cycles of food availability entrain the free-running light entrainable rhythms in at least some rats (Cambras et al., 1993; Stephan, 1986a,b; Stephan & Becker, 1989). The percentage of animals reported to entrain differs across studies, but is usually low, with an overall percentage of less than 20%. Higher figures have been reported in a recent study by Challet et al. (1996) who used a daily calorie restricted feeding schedule. The issue of calorie content will be considered in detail in section 2.4.4. Results from studies by Stephan (1986a,b) indicated that entrained rats had a difference of less than 10 minutes between tau of the free-running light entrainable rhythms and feeding schedule T, but a number of rats failed to entrain despite small period differences. He suggested that this finding raises the possibility that successful entrainment depends in part on the difference between tau of the respective pacemakers (and possibly on the PAD between the two pacemakers) and is consistent with a weak zeitgeber effect of feeding schedules on the LEP. He argued that while the feeding schedule may be acting directly on the LEP, if this was the case SCN lesioned and intact rats would not be expected to respond to feeding schedules in the same way. Stephan proposed that a more likely explanation is that entrainment is the result of internal coupling between the LEP and FEP. The FEP entrains to its zeitgeber, and may in turn, via coupling entrain the free-running LEP. It was argued that coupling strength can be sufficiently strong to entrain the LEP under conditions that would otherwise cause dissociation.

A number of investigators have argued in favour of a coupled pacemaker model. Findings indicate that rats housed in LL or DD exhibit changes in phase and period of the free-running light entrainable rhythm, and a compression of activity time in response to feeding schedules (Aschoff et al., 1983; Cambras et al., 1993; Stephan, 1986a,b; Stephan & Becker, 1989). One possible explanation for these effects is that the coupling between the underlying pacemakers is altered. Other evidence in favor of this hypothesis comes from a study by Honma et al. (1983) who found that the phase of the free-running rhythm on removal of the feeding schedule affected subsequent persistence of anticipatory activity; persistence of anticipatory activity was longest when the feeding schedule ended within the activity time of the free-running light entrainable rhythm. Mistlberger et al. (1990a) collected data from eight rats and found persistence of anticipatory activity to be shorter in rats whose free-running light entrainable rhythms had been eliminated by long-term light exposure. Also relevant here is the finding that the PAD between the FEP and the LEP is preserved in food deprived rats housed under LL, DD, and LD, even after a phase shift of the LD cycle (Coleman et al., 1982; Rosenwasser et al., 1984; Stephan, 1986c).

The coupling strength between the two pacemakers is thought to be asymmetrical. Rosenwasser et al. (1984) collected data from rats housed in LD and exposed to a feeding schedule, followed by periods of *ad libitum* food access interleaved with food deprivations. He found that, as noted above, the PAD between meal-associated activity and the light-entrained rhythm was preserved in food-deprived rats, even after a phase shift of the LD cycle which occurred during *ad libitum* food access. These results have been corroborated in the work by Ottenweller et al. (1990). In a recent study by Mistlberger et al. (1996), attenuation of anticipatory activity was found to occur following inversion of a LD cycle. By contrast, Stephan (1986c) reported that a phase shift of food access had little effect on the free-running light entrainable rhythm. Based on the fact that the rate of reentrainment is shorter in SCN lesioned rats than in intact rats, and findings that SCN lesioned rats develop anticipatory activity more quickly than do intact rats, Stephan (1986c, 1992b) has proposed that the SCN has a retarding influence on the FEP.

2.4.3. Evidence for a multi-oscillatory feeding entrainable circadian system

The organization of the feeding entrainable circadian system has received little attention, but results so far support the existence of a multi-oscillatory system. In order to investigate this hypothesis, researchers have examined the effects of multiple meals on the circadian system. The results indicate that feeding schedules consisting of two daily meals separated by 6h, 7h, 8h, or 12h elicit anticipatory activity in a number of behaviours, including wheel-running (Bolles & Moot, 1973;

Edmonds & Adler, 1977b; Stephan, 1989a,b), lever pressing (Boulos & Logothetis, 1990; Mistlberger et al., 1996), and drinking (Stephan, 1989a,b). Estimates of PAD have produced variable results, however, positive PADs have been noted for all rhythms studied so far, with the exception of drinking (Boulos & Logothetis, 1990).

The findings from these studies suggest that entrainment to two daily meals is generally less stable and consistent compared to a single meal, with Stephan (1989a,b) reporting that anticipatory activity waxed and waned, frequently giving the appearance of transients. In general, fewer subjects show anticipatory activity to two meals compared to a single meal and anticipatory activity takes longer to develop. In the study by Edmonds and Adler (1977b), five of 12 intact rats anticipated two daily meals with same T, but only at the very end of the feeding schedule which lasted for 85 days. Stephan (1989b) reported that rats also show anticipation to meal schedules with T cycles of 23.75h and 25h, but not T=23.5h. However, anticipation of two meals with T=23.75h is even less stable and the PAD is atypical i.e., activity increase was coincident with or followed food access. It is not clear why the range of entrainment to two meals appears to be narrower than to a single meal. If a deficit in energy balance is required to engage the FEP, one possibility is that rats fed twice daily are less food deprived than those fed a single daily meal and the feeding schedule is thus a less potent zeitgeber. Previous research by Stephan and Becker (1989) however, does not support this proposition, since rats were able to anticipate feeding schedules with food access of up to 12h. Under such circumstances, there is presumably very little energy deficit.

The amount of anticipatory activity is reported to depend on the PAD between FEP and the LEP and the PAD between the two meals. Boulos and Logothetis (1990) collected data from five rats housed in LL and reported higher levels of anticipatory activity when food access coincided with the activity time of the free-running light entrainable rhythm. Subjects also showed more anticipatory activity when food access occurred in the D phase compared to the L phase. These investigators also indicated that anticipatory activity is affected by the size of the inter-meal interval. No differences were observed in either the amount or duration of anticipatory activity to each meal in SCN lesioned rats exposed to two meals separated by 12h. Under a 16:8h feeding cycle however, rats showed more anticipatory lever pressing at the end of 16h than at the end of 8h. Similar results were obtained by Stephan (1989a) who observed lower levels of anticipatory activity to the trailing meal when the interval between two meals was reduced to 5h. Anticipatory activity to the trailing meal had been reduced in both amount and duration, and was less consistent in some cases, appearing for a few days only. A possible explanation for this finding is provided by the deprivation hypothesis described above: a state of energy deficit is required for the FEP to become operational. Such a state is unlikely to occur immediately following a meal.

A small number of researchers have examined the effect of food deprivation on anticipatory activity following exposure to multiple meals. In the studies by Boulos and Logothetis (1990) and Bolles and Moot (1973), rats were subjected to two daily meals, then food deprived before and after a period of *ad libitum* food access. A finding by both of these investigators was that residual activity persisted for up to five days in food deprived rats, even when the deprivation was repeated, but was not apparent during *ad libitum* food access. Results of the study by Stephan (1989a) are not entirely consistent with these findings, showing that residual activity persisted for seven or more days under *ad libitum* conditions. Furthermore, unlike in the Boulos and Logothetis study, Stephan found activity onset to commence near the previous onset of anticipatory activity and continue for 6h to 8h during food deprivation. White and Timberlake (1994) also report peaks near the former time of feeding during food deprivation.

The most convincing evidence for the existence of multiple pacemakers comes from studies utilizing two feeding schedules with different Ts (Edmonds & Adler, 1977b; Stephan, 1983, 1989b). Stephan (1989b) exposed rats to two meals with T cycles of 25h and 26h and found that a third of the animals maintained relatively sustained anticipatory activity to both meals over a prolonged period. Only one rat displayed anticipation on most days. Interestingly, Stephan also found that a cessation or reduction of anticipation to one meal was often followed by its appearance or increase to the other meal. Such shifts of anticipatory activity from one meal to the other were most common at inter-meal intervals of 0 to 5h and 11h to 16h. At the short interval, anticipatory activity was most pronounced to the leading meal and diminished to the trailing meal. Stephan suggested that these shifts reflect the coupling interactions between the two pacemakers: the pacemakers were forced out of entrainment at a critical PAD when the internal coupling force exceeded the force of the entraining agent. These results were consistent with those of Edmonds and Adler (1977b) in showing that rats could anticipate two feeding schedules with different Ts.

Stephan (1986a) was able to establish a role in eliciting anticipatory activity for a maximum of two feeding entrainable oscillators. Stephan exposed SCN lesioned rats to three daily meals separated by 6-6-12h and 8-8-8h. He reported that the animals could anticipate one or two meals, but no rats displayed consistent anticipation to all three meals. This study has not been replicated and the findings therefore remain to be confirmed. It is possible, as Stephan has noted, that in a system consisting of two oscillators, each oscillator may be used to track one meal in the above studies. In single meal studies, succeeding activity may reflect the output of the second feeding entrainable oscillator. Both of these hypotheses are purely speculative.

2.4.4. The nature of the zeitgeber/transduction

A question remains as to what aspect of the experimental situation acts as the zeitgeber signal. The literature concerning the transduction pathway is reviewed below.

There is some evidence supporting a role for overt activity, or some other critical variable such as arousal or motivation, in mediating the effects of 'non-photic' agents (see Mrosovsky 1995 for a comprehensive review), but the link to feeding schedules is at best tenuous. Several features set apart feeding schedules and other 'non-photic' agents. The most notable of these is the fact that the PAD between activity onset and the onset of food access is always positive, whereas negative PADs are generally reported to occur under 'non-photic' zeitgeber (Reebs & Mrosovsky, 1989). Another effect specific to feeding schedules is the persistence of a free-running rhythm in the entrained state. 'Non-photic' zeitgebers such as access to a running wheel or cage changing have the effect of entraining the free-running light entrainable rhythm (Mrosovsky, 1989, 1995, 1996). While the SCN and the intergeniculate leaflet have been implicated in the 'non-photic' transduction pathway

(Meyer et al., 1993; Mistlberger, 1992), ablation of these structures has little or no effect on meal anticipatory activity (Challet et al., 1996; Stephan et al., 1979a,b). Mrosovsky (1995) has argued that these lines of evidence are inconsistent with hypotheses suggesting a common underlying mechanism for feeding schedules and 'non-photic' zeitgebers at any level.

Food is essential to the survival of all mammals, however, other agents are also important. Coleman et al. (1998) argued that any agent which is motivationally significant may entrain the circadian system. For example, cycles of water availability have been shown to entrain circadian rhythms (Bolles, 1968; Bolles & Duncan, 1969; Dhume & Gogate, 1982). It is not clear, however, whether this effect occurs indirectly through changes in feeding patterns. It is well established that rats eat when they drink and that the amount of food intake is positively correlated with water intake (Fitzsimons & Le Magnen, 1969; Johnson & Johnson, 1990, 1991; Kissileff, 1969; Mistlberger & Rechtschaffen, 1985; Siegel & Stuckey, 1947). Another example is salt. In 1985, Rosenwasser and colleagues reported that salt-deprived rats do not anticipate a daily opportunity to ingest salt. In a subsequent study, anticipatory activity to a salt-access schedule was found to occur in some behaviours (Rosenwasser et al., 1988). It was noted by these authors that anticipatory activity to salt-access schedules differs from that seen under feeding schedules in terms of its magnitude, its dependence on stimuli related to the LD cycle, and the range of behaviours that exhibit anticipatory activity. Thus, a question remains as to whether the anticipatory activity reported in this study is mediated by the FEP.

A number of researchers have proposed that the entraining signal is specific to ingestive events. Findings regarding the role of pre-ingestive, pre-gastric or gastric stimuli are varied, but there appears to be a substantial amount of evidence for a lack of involvement (Apelgren et al., 1985; Comperatore & Stephan, 1990; Davidson & Stephan, 1998; Moreira & Krieger, 1982; Stephan, 1986c). There is however some evidence that food nutritive (i.e., calorie) content is critical for the expression of anticipatory activity. Mistlberger and Rusak (1987) collected data from rats given *ad libitum* food access and exposed to a palatable, nutrient rich meal for 2h daily. They found that the majority of rats anticipated the feeding schedule, but not when meal size was limited to 4g. Rats did not entrain to a palatable but non-nutritive meal. It was argued in this study that anticipatory activity is dependent on both meal size and calorie content. Subsequent studies have not entirely supported this interpretation. Mistlberger et al. (1990b) observed anticipatory activity to a feeding schedule consisting of two single macro-nutrient meals in rats with *ad libitum* food access, but only when the feeding schedule accounted for at least 30% of daily caloric intake. Stephan (1997) estimated that approximately 22kcal is required to affect the FEP in the rat. Increasing meal size by adding non-nutritive bulk had little or no effect on anticipatory activity. These findings suggest that calorie content rather than gastric distention contribute to entrainment by feeding schedules.

Other recent studies provide further support for a critical role for calorie content in food entrainment. In the 1996 study, Challet and coworkers fed rats a daily calorie restricted diet and found the free-running light entrainable rhythm to be entrained by the feeding schedule. It was argued that calorie restricted feeding schedules lead to a stronger coupling between the FEP and LEP than had been previously described by Stephan (1986a, b). In a subsequent study by these authors (Challet et al., 1997), a hypocaloric diet was found to have a greater influence on LD entrained rhythms (i.e., phase shifting LD entrained rhythms) than a non-calorie restricted feeding schedule. The phase shifting effects of hypocaloric restricted feedings on LD entrained rhythms were eliminated following lesion of the intergeniculate leaflet, suggesting that the intergeniculate leaflet plays a role in conveying information between the LEP and the FEP (Challet et al., 1996). While replication of this study is required, taken together, these findings suggest that feeding schedules associated with calorie restriction are a more powerful entraining agent than time-limited feeding schedules alone. If nutritive content provides the entraining signal however, it is not clear why calorie restriction should produce a more potent zeitgeber. A more likely explanation of these findings is that such effects occur via the arousal/activity system proposed by Mrosovsky (1988, 1995, 1996) and have nothing to do with the FEP per se. It is well established that calorie restriction results in a general increase in locomotor activity.

A recent study by Davidson and Stephan (1999a) has shown that the pituitary hormones are not required for entrainment to feeding schedules. They collected data from hypophysectomized rats and found that anticipatory activity developed within a few days of exposure to restricted feeding. These findings rule out the possibility that important metabolic hormones, including growth hormone, adreno-corticotrophic hormone and thyroid stimulating hormone are involved in the transduction pathway to and from the FEP. In a subsequent study, Davidson and Stephan (1999b) attempted to elucidate how the FEP gains access to the Central Nervous System to initiate the expression of anticipatory activity. Two assumptions were made. 1. The FEP is located in a peripheral organ (see section 2.4.1), and; 2. There is communication between the FEP and the Central Nervous System. These authors found plasma glucogen levels to be lower during anticipatory activity relative to before anticipatory activity in both intact and SCN-lesioned rats. Although the functional implications of this change still need to be established, this finding supports the possibility that humoral and metabolic signals related to digestive physiology are causally linked to the expression of anticipatory activity.

CHAPTER 3. THE FEEDING ENTRAINABLE CIRCADIAN SYSTEM IN CARNIVORES AND HERBIVORES

In 1950 Beach surveyed previous research and reported that the variety of species studied had declined over the years, with the rat in particular being widely used. Although the necessity of a comparative approach has long been recognized, this trend has continued until today and is evident in all areas of research. The significance of this approach will not be discussed here; the interested reader can refer to Beach (1950) for a review.

The majority of circadian studies have been conducted using the rat. Despite the evidence for a zeitgeber role of feeding schedules in the rat, evidence in other species is less consistent and there is still some uncertainty as to whether food availability is an effective zeitgeber in all mammalian species (see Mistlberger 1994 for review). Some of the results with the rat have been corroborated in other species however, species differences have been documented in recent comparative studies (Jilge, 1991; Kennedy et al., 1991, 1995; Mistlberger, 1993a,b). The reason for these differences is unclear, but may be related to the fact that a species is omnivorous, carnivorous, or herbivorous. The possibility that differences exist between classes of species has been examined previously by Rajaratnam (1997) who developed a diurnal animal model of the circadian system, since both photic and 'non-photic' zeitgeber appear to affect nocturnal and diurnal circadian systems in different ways. Although the omnivorous rat has been thoroughly studied, considerably fewer studies have investigated carnivorous or herbivorous species; these studies will be discussed in this chapter and comparisons will be made to omnivores. The extent of similarity/differences between species remains to be determined, with a view to understanding the zeitgeber effects of feeding schedules. Table 1 provides a summary of the some of the differences and similarities between omnivores, carnivores, and herbivores. Only one species has been chosen to represent each class of species for reasons described later. Also, only findings from studies utilizing time restricted feeding schedules have been included. Results of calorie-restriction studies have been omitted.

TABLE 1.

A SUMMARY OF THE EFFECTS OF FEEDING SCHEDULES IN OMNIVORES, CARNIVORES, AND HERBIVORES

DV	Omnivorous rat	Carnivorous kowari	Herbivorous rabbit
Anticipatory activity	306 of 315 (97%) rats exposed to a single daily meal develop anticipatory activity.	19 of 22 (86%) kowari exposed to a single daily meal develop anticipatory activity.	Rabbits exposed to a single daily meal develop anticipatory activity. Jilge et al. (1987) report 5 of 5 rabbits entrained to the feeding schedule, while Jilge (1991) did not provide sufficient detail in terms of the number of rabbits found to entrain to the feeding zeitgeber.
PAD between activity onset and onset of food access	For a single daily meal, the PAD between activity onset and the onset of food access for locomotor activity and approaches to the food bin ranges from 2h to 4h, however, PADs as large as 8h have been reported.	For a single daily meal, the PAD between activity onset and the onset of food access for locomotor activity ranged from 0.25h to 3h in the study by Kennedy et al. (1991). Much larger PADs of up to 6h have been noted in other carnivores.	For a single daily meal, the PAD between activity onset and the onset of food access for locomotor activity and approaches to the food bin ranges from 1h to 3h.
Latency to anticipatory activity	Anticipatory activity to a single daily meal usually develops within 7 days.	Anticipatory activity to a single daily meal develops within 6 days.	Anticipatory activity to a single daily meal develops within 67 days.
Free-running light entrainable rhythm	Entrainment of free-running light entrainable rhythms to a single daily meal occurred in 12 of 69 (17%) rats.	Entrainment of free-running light entrainable rhythms to a single daily meal occurred in 12 of 20 (60%) kowari.	Past studies have yielded conflicting results. Jilge and Stahle (1993) concluded that the free-running light entrainable rhythm is entrained by the feeding schedule, while Jilge (1991) report free-running rhythms persist during restricted feeding.
Food deprivations	Meal associated rhythms persist in almost all rats (108/117) when a feeding schedule is replaced with food deprivation. Meal associated rhythms are less likely to persist during subsequent food deprivations, following a period of *ad libitum* feeding.	Meal associated rhythms persist in all kowari (12/12) when a feeding schedule is replaced with food deprivation. Meal associated rhythms are less likely to persist during subsequent food deprivations, following a period of *ad libitum* feeding.	Data not available
Ad libitum food access	Meal associated rhythms have been shown to persist for up to two days during *ad libitum* food access following a single daily meal.	Meal associated rhythms have been shown to persist for up to three days during *ad libitum* food access following a single daily meal.	Meal associated rhythms have been shown to persist for up to 10 days during *ad libitum* food access following a single daily meal.
Transients	Reentrainment is achieved by delaying transients, although advancing transients have been observed following a 4h and 8h phase advance of food access.	Data not available	Delaying transients occurred in response to a phase delay of food access in two rabbits. There is no evidence of advancing transients.
T cycles	Anticipatory activity develops to feeding schedules with Ts ranging between 22h and 29h.	Data not available	Data not available
Multiple meals	Anticipatory activity develops to two but not three daily meals.	Data not available	Data not available

3.1. ADAPTATIONS OF OMNIVORES, CARNIVORES, AND HERBIVORES

Species are classified as omnivorous, carnivorous, and herbivorous on the basis of their feeding patterns which vary in terms of selectivity of food intake (and hence the characteristics of the digestive system), feeding frequency, and rate of ingestion. These adaptations are detailed below because they may have implications for the circadian system.

A number of studies have examined feeding patterns in carnivores, omnivores and herbivores, and found feeding pattern to be related to food availability (Cloudsey-Thompson, 1970; Fabry, 1969; Godfrey, 1955; Hediger, 1964; Kavanau & Ramos, 1975; Kersten et al., 1980; O'Reilly et al., 1986; Raptor Group, 1982; Rijinsdorp, 1981; Spiteri, 1982; Spiteri et al., 1982). Some of these investigators have noted cyclicity in the availability and vulnerability of prey across the day and have linked the daily variation in the feeding time of carnivores to these fluctuations. Studies with herbivores and omnivores are not consistent with these findings and report an absence of cyclicity in food availability for these animals, with most studies showing food to be more or less continuously available in the environment (Fabry, 1969; Chapman et al., 1982, cited in Cheek, 1987; Hirsch, 1973; Mykytowycz & Rowley, 1958).

Differential feeding strategies apply to carnivores, omnivores, and herbivores. It is clear that carnivores require a diet of animal origin, containing a high proportion of fat, with a high calorific value. Empirical studies show consistency in reporting that carnivores will eat one brief, but large, meal each day, in which they ingest a large number of calories in a few minutes (Collier et al., 1972; Estes, 1967a,b). Findings regarding omnivores appear to be more variable, with the estimated number of daily meals varying within a relatively large range, but is usually cited as being 'high' (Armstrong, 1980; Fabry, 1969; LeMagnen, 1967; Richter, 1927). For example, Fabry (1969) described the rat as a 'typical nibbler', ingesting food more or less continuously in small amounts, whereas others (LeMagnen & Tallon, 1963, 1967, cited in LeMagnen, 1967; Richter, 1927) have reported that rats eat six to 10 times a day in discrete (brief) meals separated by long intervals. The disparate results across omnivores may be due in part to differential feeding strategies depending on species, strain and/or individual. Herbivores are known to require large amounts of low calorie, high cellulose diet and to eat many meals of long duration throughout the day and night (Ashby, 1972; Bost et al., 1968; Cheeke, 1987; Hirsch, 1973; McClymount, 1967).

Carnivores, omnivores, and herbivores vary in terms of the characteristics of their digestive tracts and the length of the digestive process (Cheeke, 1987). It is clear that animal and/or plant matter is digested by enzymes in omnivores and carnviores, but there is little or no degradation of cellulose. Herbivores, on the other hand, have a microbial population which provide enzymes to digest cellulose. The herbivore has developed enlarged chambers in the digestive tract where conditions suitable for microbial growth and fermentation can be maintained. The rate of passage of food is slow compared with carnivores and omnivores, with studies suggesting that the digestive process can last up to 30h in some herbivores, resulting in a nearly constant input of nutrients in the stomach (Collier et al., 1972; Watson, 1954).

The above adaptations are important because, as mentioned earlier, they may have implications for circadian timing. Differences associated with feeding patterns for instance may have resulted in tighter coupling between the FEP and LEP, and/or an increased sensitivity of the feeding entrainable circadian system to food availability in carnivores compared to omnivores and herbivores. Evidence in support of this proposition is reviewed in this chapter. In view of the fact that carnivores, omnivores and herbivores are markedly different in terms of both nutritional and digestive characteristics, disparate results may be expected, particularly if anticipatory activity is mediated by variables such as food calorific content (see section 2.4.4). For example, the entraining signal may be diffuse in the herbivore due to slow digestive processes (and hence slow nutrient release) at least compared to a carnivore. Herbivores may thus be less likely than carnivores to exhibit anticipatory activity, or anticipatory activity may be less consistent and stable, and/or may be greatly reduced in amount or duration, and/or take longer to develop in the herbivore. Evidence in support of this proposition is also considered in the following chapters. While the circadian system in omnivores appears to be multi-oscillatory, evidence for the existence of a FEP is less consistent in the herbivore. While the ability to prepare in advance for a meal in all relevant bodily systems is clearly significant to the carnivore, the adaptive advantages in the case of the herbivore which eats more or less continuously are less clear. It is important to note that much of the above discussion is speculative.

3.2. CARNIVORES

The purpose of this section is to discuss literature with regard to feeding schedules in the kowari. There are two reasons for this decision. First, other carnivorous species have received little attention and in some cases, investigators have failed to provide adequate information, making it difficult to compare research outcomes. Second, data from kowari appears to be representative of other carnivores, since most studies of carnivores have yielded comparable results. Although this is generally true, species differences do exist. Cross-species inconsistencies in empirical findings are noted below, and other species are cited when limited data are available from kowari.

3.2.1. The feeding entrainable circadian system in carnivores

The circadian effects of feeding schedules have been examined in a small number of carnivorous species, including mink and weasel (Zeilinski, 1986), quoll (Kennedy et al., 1990), dunnart (Coleman et al., 1989), and bandicoot (Kennedy et al., 1995). The most extensive studies have involved the kowari (Kennedy et al., 1991; O'Reilly et al., 1986). Anticipatory activity has been documented in the wheel-running rhythms of kowari and is reported to develop over a period of two to six days (Kennedy et al., 1991; O'Reilly et al., 1986). PAD between activity onset and the onset of food access is positive, with estimates ranging from 15 minutes to 3h. This variability may reflect, in part, individual differences in the sensitivity of the circadian system to the zeitgeber. Inter-species differences in PAD have also been noted. The PAD was found to be 3h to 4h in quoll (Kennedy et al., 1990) and up to 6h in bandicoot (Kennedy et al., 1995). It is often difficult to compare studies because a number of methodological factors may vary, including ambient lighting levels and the method used to calculate PAD. For example, in bandicoots the PAD can be such that activity onset coincides with the meal in LL, whereas positive PADs as large as 6h were measured in DD (Kennedy et al., 1995).

There has been little research investigating the effects of food deprivation on the circadian system in carnivores. In a study by Kennedy et al. (1991), data were collected from 10 kowari subjected to a feeding schedule of 2h food access, then food deprived before and after 27 days of *ad libitum* food access. They found that residual meal-associated activity persisted during deprivation, even when the deprivation trial was repeated, but this effect was not observed in all animals. Three animals were reported to show an increase in tau of the free-running light entrainable rhythm on removal of the feeding schedule, an effect likely to be due to aftereffects of the feeding schedule. Results of other studies by Kennedy however, have failed to confirm these findings. In studies with quoll and bandicoot, Kennedy et al. (1990, 1995) reported that residual meal-associated activity did not persist during food deprivation following a period of *ad libitum* food access. This finding is difficult to explain, but suggests that *ad libitum* feeding results in a rapid uncoupling of the overt rhythms from the FEP assumed to drive them in these species. It is desirable to replicate Kennedy's 1991 study using quoll and bandicoot as subjects.

The phase resetting properties of food deprivation/reinstatement have not been formally investigated in kowari, but the dunnart has been studied. A study by Coleman et al. (1989) reported data from seven animals and found that dunnarts experience major phase shifts to food deprivation/reinstatement throughout the subjective day and night. The magnitude of the phase shifts was not consistently linked to the circadian phase of food reinstatement. These results are generally consistent with those obtained in the rat in showing an effect on the free-running light entrainable rhythm (Coleman & Francis, 1991), but the size of the phase shifts differs markedly. The finding in this study of larger phase shifts relative to those observed in the rat is consistent with a stronger zeitgeber effect of feeding schedules in this species. Further comparative studies are required before this conclusion can be drawn, and it would be interesting to examine the effect of food deprivation/reinstatement in the kowari.

The above findings point to a zeitgeber role for feeding schedules in carnivores however, several criteria for entrainment remain to be addressed. One of the primary problems is that no studies have explored the range of entrainment to feeding schedules in carnivores. A further problem is that no studies have investigated the phase resetting properties of the feeding entrainable circadian system in carnivores. While there appear to be some clear differences between the carnivorous kowari and the omnivorous rat, the relevant literature is limited to two published studies. More research is thus required.

3.2.2. The organization of the feeding entrainable circadian system in carnivores

Although the SCN have been implicated as the site of a major circadian pacemaker in carnivorous species, there is some evidence for the existence of a FEP outside the SCN. Behavioural studies provide evidence in support of this proposition. A finding by both Kennedy et al. (1991) and O'Reilly et al. (1986) was that anticipatory rhythms coexisted with free-running light entrainable rhythms under DD conditions, with 20% and 100% of kowari displaying two bouts of activity respectively. In both of these studies, LD preceded DD and kowari were fed in the L phase; kowari showed two bouts of activity, one entrained to each zeitgeber. Interestingly, Kennedy found the amount of LD entrained activity to be negatively related to the level of anticipatory activity.

In examining whether cycles of food availability entrain the free-running light entrainable rhythm, the two studies referred to above have reported conflicting results. This inconsistency may reflect the different methodologies used. One method of assessing the effects of feeding schedules is to maintain animals under a feeding schedule in which food is replenished at the same time each day, but is restricted in amount rather than duration. This paradigm is typically used in species which cannot consume large amounts of food in a short period of time, such as minks and weasels (Zeilinski, 1986), but also has been used by O'Reilly et al. (1986) in kowari. The results of these studies indicate the presence of a free-running light entrainable rhythm, in addition to anticipatory activity. These findings however, have not been supported in a subsequent study by Kennedy et al. (1991). In the Kennedy study (as noted above), kowari were exposed to a time-limited feeding schedule of 2h food access, before and after a period of *ad libitum* food access. They reported that the free-running light entrainable rhythms entrained in a higher proportion of animals than observed previously, with 12 of 20 kowari becoming entrained. Kennedy also found no effect of free-running tau and feeding schedule T on successful entrainment, with some subjects entraining even when tau–T=0.35h. These findings suggest that the methodology can have considerable effects on the research outcomes.

Even when cycles of food availability are not sufficiently strong to entrain the LEP, changes in tau and phase typically occur. O'Reilly et al. (1986) observed modulations in tau indicative of relative coordination in four of six kowari, with the difference between free-running rhythm tau and feeding schedule T becoming smaller when activity onset coincided with the onset of food access. Under these circumstances activity is thought to be beating between the LEP and the FEP (Kennedy et al., 1990). Kennedy et al. (1991) found that LD-entrained rhythms advanced toward a meal given in the middle of the L period and he argued that this finding is consistent with the above explanation. In an earlier study of the quoll, Kennedy observed the frequency of beats to be positively correlated to the difference between tau of the LEP and FEP.

These data are consistent with those obtained in the omnivorous rat in providing support for a multi-pacemaker model of the circadian system; a LEP controlling activity associated with light and a FEP being responsible for driving anticipatory activity. Further study is, however, required to demonstrate that the FEP is a non-SCN neural structure(s). As in the rat, the LEP and the FEP appear to be coupled, however, coupling strength appears to be stronger in carnivores than omnivores, since the free-running light entrainable rhythm is more likely to be entrained by time-limited feeding schedule in carnivores.

3.3. HERBIVORES

To date there has been little work investigating the effects of feeding schedules in the herbivore and most of these studies have used the hamster. The methodology used in hamster studies, however, is varied and hence the meaning and validity of research findings is difficult to evaluate. The purpose of this section is to discuss literature with regard to feeding schedules in the hamster. Inconsistencies in empirical findings across studies are noted and are discussed with reference to methodology. Very little research is available on other herbivorous species, and hence, the hamster provides a good starting point from which to evaluate models of the herbivore feeding entrainable circadian system.

3.3.1. Adaptation to feeding schedules in herbivores

The traditional paradigm used to study the FEP cannot be applied to hamsters. In a study by Kutscher (1969), hamsters were exposed to a feeding schedule of 2h food access, following a period of *ad libitum* feeding. He found significant phase differences in food intake, with intakes dropping to 10% to 15% of *ad libitum* levels. These results have been supported in a subsequent study by Zucker and Silverman (cited in Silverman & Zucker, 1976). Similar results were reported in a study of 10 hamsters subjected to a feeding schedule of 12h food access. Food intake and body weight were found to decrease compared to *ad libitum* baseline and six hamsters died (Silverman & Zucker, 1976). These researchers proposed that a likely explanation for these findings was that the hamster has evolved in the presence of a constant supply of food as a result of food hoarding and/or hibernation and is incapable of compensating for food deprivation.

One of the primary problems in the study of the FEP of hamsters is that there is no widely accepted methodology. Several different methods of assessing the effects of feeding schedules have been used, with one method involving a cycle of water availability in which water access is restricted to a few hours per day (Mistlberger, 1993b). It was argued that this is an effective means of creating a voluntary feeding schedule, because hamsters tend to eat when they drink. There is, however, the question as to whether food and/or water serve as the zeitgeber. Alternative methods have utilized an amount-limited feeding schedule (Abe & Rusak, 1992) or a combination of these two techniques (Mistlberger, 1993a). Finally, the earliest report involved housing hamsters in a home cage, and opening the home cage for 30 minutes each day to allow access to an open field from which hamsters could hoard food (Rusak et al., 1988).

One of the most significant issues with regard to the above paradigms is experimenter entry into the laboratory at the same time each day. This is an important issue because other cues associated with the delivery of food and/or water may be operating. In a study of squirrel monkeys, Boulos et al. (1989) argued that social cues in the form of human contact each day for delivery and removal of food provided additional cues that entrained animals to a feeding schedule in earlier work by Sulzman et al. (1977a,b). Studies of social cues have employed hamsters as subjects primarily, with many researchers indicating a strong zeitgeber effect (Mrosovsky, 1988, 1996; Mrosovsky et al., 1989). While there is no evidence to suggest this finding is specific to certain species, social cues may be of greater concern in some species (e.g., hamsters and squirrel monkeys) compared to others (e.g., rat). While it is clear that feeding schedules do exert a zeitgeber effect in the absence of social cues in many species, it is possible that social cues, if present, may provide additional cues resulting in a stronger than normal zeitgeber influence. It is therefore argued that the methodology used to study the hamster may not have been adequate. Food delivery in the absence of the experimenter is desirable.

3.3.2. The feeding entrainable circadian system in herbivores

Entrainment of wheel-running rhythms has been observed in hamsters housed under LL, DD, and LD. Estimates of PAD range widely, with Rusak et al. (1988) reporting PAD to be either positive or negative; however, estimates of PAD were not provided in this report. PAD was cited as 0 ± 3h in DD and +12 ± 14h in LL by Mistlberger (1993a). In a subsequent study by this investigator, data were collected from SCN lesioned hamsters exposed to daily cycle of water availability. The results were consistent with his earlier study in showing a wide variation in PAD, with estimates ranging between +2 ± 8h. A similar study by Mistlberger in 1992 yielded positive PAD's ranging between 6h and 10h.

The work by Mistlberger has provided evidence in support of a zeitgeber role of cycles of water availability. In the 1993b study, data were collected from six SCN lesioned hamsters exposed to T cycles of 18h and 30h. This investigator reported that hamsters did not entrain to T cycles of 30h, but under the 18h cycle, tau=T in four of five subjects. A positive PAD, however, of 12h to 14h between activity onset and the onset of drinking was found. Mistlberger noted that large PADs in this range are not consistent with oscillatory theory and argued that cycles of water availability exert a masking effect on the activity rhythms. The finding of a lack of persistence of anticipatory rhythms during subsequent water deprivation is consistent with this interpretation of the results. By contrast, residual rhythms persisted during water deprivation in hamsters exposed to a feeding schedule with T=24h. This finding corroborates earlier work by Mistlberger (1993a). In the 1993(a) study, Mistlberger observed aftereffects to cycles of water availability, with tau beginning to resemble T on removal of the schedule.

In short, although these findings from hamsters appear to corroborate previous findings with other species, some discrepancies have been reported. The primary difference in the results of different researchers appears to be with regard to the PAD of entrainment. As reported previously, activity onset in the entrained state always precedes the onset of food access in the rat (e.g., Aschoff et al., 1983). Since negative PADs have been found to occur under 'non-photic' zeitgebers (see section 2.4.4), it is conceivable that, as with the 'non-photic' zeitgebers, entrainment in the above-cited studies occurred via the arousal/activity system proposed by Mrosovsky (1988, 1995, 1996) and hence may have nothing to do with feeding *per se*. More studies are required before any conclusions can be reached, however, the range of PADs from positive to negative is inconsistent with the hypothesis that entrainment is mediated by the FEP in these studies.

3.4. Summary and conclusions

While feeding schedules have been examined in relation to the circadian system in a herbivorous species, it is not clear whether the results are confounded by the methods used. Few researchers have applied the traditional methodology to the study of the hamster, but these studies have concluded that the hamster is poorly adapted to time-limited feeding schedules. This is not a surprising finding, since feeding in response to deprivation in the herbivore is an emergency response only that is greatly different from the feeding conditions experienced by herbivores in their natural environment. Alternative paradigms have been adopted to study the hamster, and while the results of these studies are interesting, their validity needs to be considered. The paradigms are varied and have not been widely used in other species; thus usage of these methods inhibits comparison to previous research with other species because discrepancies between findings may be the result of differences in methodology. The difficulties this creates are clearly of considerable importance to the study of the circadian system. In order to overcome this limitation, the circadian system will be examined utilizing time limited feeding schedules in the present thesis.

CHAPTER 4. THE HERBIVOROUS RABBIT

The limited data from herbivorous mammals emphasizes the need for the present research. The focus of this thesis will be the European rabbit (*Oryctolagus cuniculus*), primarily with the aim of developing a model of the herbivore feeding entrainable circadian system. It is important to study the herbivore in order that basic similarities and differences between classes of species are not overlooked, and to develop a framework for understanding the feeding entrainable circadian system. If systematic differences exist between classes of species, the search for greater understanding based on a combination of these classes may not be possible. (The FEP may not be similar across mammalian species in the same way that the LEP is.) As stated previously, the majority of research has been conducted using the omnivore, and it is not clear whether the results can be generalized, or whether they are specific to this class of species. Preliminary analysis of previous findings has yielded some inconsistencies between omnivores and carnivores, however, it is not clear whether these differences relate to the fact that the species is omnivorous or carnivorous. It is thus preferable at this stage to focus on the herbivore, since a comprehensive model of the herbivore feeding entrainable circadian system has not been established. Although the hamster has received much attention, few studies have used rabbits as subjects: these studies will be reviewed in this chapter. Results from these studies are inconclusive, and the absence of systematic study creates difficulties in determining whether a feeding schedule is an effective zeitgeber in this species. Use of the rabbit is beneficial since it would provide an opportunity to investigate the FEP using the traditional paradigm, thus avoiding the potential confounding effects associated with other methods.

4.1. INTRODUCTION TO THE EUROPEAN RABBIT

The European rabbit, with one species (*Oryctolagus cuniculus*) from which all laboratory rabbits have been derived, is cited as being indigenous in Europe and North Africa. *Oryctolagus cuniculus* taxonomically comes under Class: Mammalia; Order: Lagomorph; Family; Leporidae; Genus: Lepus; Species: *Oryctolagus cuniculus*. Lagomorph was originally considered a suborder of the Rodentia, but this view of close affinities was abandoned after studies showed many differences between fossil and modern members of the two groups.

In the European rabbit, the length of the head and body ranges from 125mm to 750mm, and the tail ranges from rudimentary to short and well-furred. Body weight ranges from 1.25kg to 7kg, and life span is approximately 12 years. The European rabbit has five manal digits, four pedal digits, and the soles of the feet are covered with hair. In the male the testes are in the scrotum in front of the penis, while females have two to five pairs of mammae.

The European rabbit is reported to inhabit a wide range of habitats, from treeless and barren situations to forests, open grasslands, and deserts (Rogers, 1981; Stodart, 1965a,b). Field studies have shown that European rabbits shelter in multientrance burrow systems in stable structured groups containing two to 20 adults (Cowan, 1987; Lloyd, 1977). Southern (1948) and Mykytowycz (1958) report that separate dominance hierarchies are maintained amongst males and females. Group formation is, however, not obligatory as some females did not share burrow systems. The European rabbit is known to be dependent upon cover to avoid predation, and its range size was found to be closely related to the geometry of available cover (Cowan and Bell, 1986).

The European rabbit is herbivorous and exhibits unique digestive characteristics. As early as 1882, Morot, while studying the digestive process of the European rabbit, reported excretion of both hard and soft fecal pellets. The hard feces were discarded, whereas the soft feces were expelled through the colon and consumed directly from the anus by the rabbit. It was not until these findings were confirmed by Madsen in 1939 that scientists became interested in the phenomenon of 'cecotrophy'. Cecotrophs are reported to be high in protein, and Kulwich et al. (1953) reported an important role in vitamin economy. Watson (1954) reported that the digestive process of European rabbits lasted up to 30h, with some parts of intake recycled up to four times. As discussed in section 3.1, this may effect the expression of anticipatory activity such that it is less likely to develop and/or is reduced in duration and/or takes longer to develop than in omnivorous and carnivorous species.

European rabbits do not hibernate and typically suffer famine over winter and/or summer when forage is depleted. Stodart and Myers (1966) collected data on breeding success, health and survival, with respect to seasonal changes in the quality and quantity of vegetation, and found a strong association between these variables. Similar results were obtained by Myers and Poole (1961) and others (Poole, 1960; Wallage-Drees, 1983) who reported that food shortage reduced breeding success, body weight, growth rate, and survival in European rabbits. Many of these findings parallel those of Mykytowycz (1958, 1961) who conducted similar research, finding also a tendency towards hyperphagia and obesity following seasonal food shortages. These findings suggest that the rabbit may be a good candidate for study of the feeding entrainable circadian system.

4.2. THE CIRCADIAN SYSTEM OF THE EUROPEAN RABBIT

4.2.1. Entrainment to LD cycles

The LD cycle is a potent zeitgeber for the European rabbit. Studies of wild rabbits report a nocturnal pattern of activity in general activity and food intake (Kolb, 1986; Myers & Poole, 1961; Southern, 1940; Stodart & Myers, 1964). Southern (1940) found that wild rabbits become active in the 'late afternoon' and emerge from their burrows to feed. Another bout of increased activity was identified in the late night and early morning. Reports regarding other activity types, however, are not consistent with these results. Myers and Poole (1961) found drinking to be crepuscular, with two distinct bouts occurring around dawn and dusk. Grooming, exploration, and aggression are also reported to occur during twilight. In addition to activity rhythms, rhythmic changes have been documented in various physiological factors, including the concentration of volatile fatty acids in the alimentary tract (Henning & Hird, 1972), heart rate (Eisermann, 1988), and defecation (Myers, 1955; Southern, 1940; Watson, 1954).

There have been numerous studies investigating the effects of LD cycles in the laboratory rabbit. The laboratory rabbit is frequently cited as being active during the day and generally fails to show a clear separation between activity and rest. A commonly used method of assessing nocturnality is to express the amount of activity occurring in the D phase of the LD cycle as a percentage of total daily activity.

Estimates have been found to differ across studies, ranging between 52% and 58% for locomotor activity, 40% and 64% for water intake, and 47% and 59% for food intake (Gatterman, 1978, as cited in Jilge & Stahle, 1984; Horton et al., 1975; Jilge & Stahle, 1984). Other variables have also been subject to this analysis and suggest the possibility that percentage nocturnality for different overt rhythms may vary. These findings are beyond the scope of this thesis so will not be considered here.

Most studies show reasonable consistency in reporting a crepuscular pattern of activity in the laboratory rabbit (Jilge et al., 1986; Van Hof-van Duin, 1971; Van-Hof et al., 1963). It has been argued that activity is governed by two loosely coupled oscillators (Bobbert & Bruinvels, 1986). Van-Hof et al. (1963) report the LD peak to be smaller than the DL peak and that the size of the peaks is dependent in part on the rate of illumination change. The fact that higher activity peaks have been observed under artificial LD cycles with abrupt transitions compared to natural conditions is used as evidence to support this hypothesis. While most researchers do not specify the PAD between activity onset and the onset of the LD and/or DL transition, Jilge et al. (1986) reports that activity peaks 1h to 3h prior to the LD transition and 'shortly' after the DL transition.

A number of researchers have examined LD cycles in relation to biochemical and physiological variables in the laboratory rabbit. The results suggest that synchronization occurs in a wide range of variables, including hematological parameters (Fox & Laird, 1970), plasma N-acetylserotonin (YoungLai et al., 1986), histamine metabolism (Nowak et al., 1988), insulin (Lesault et al., 1991), arterial pressure and heart period (Eijzenbach et al., 1986), plasma melatonin (YoungLai et al., 1986), melatonin in the aqueous humor (Yu et al., 1990), aqueous protein concentration (Takahashi et al., 1995), dopamine in the retina (Novak & Zurawska, 1988), noradrenaline in the brainstem (Gingras et al., 1996), intraocular pressure (Takahashi et al., 1995), visual evoked potentials (Bobbert et al., 1978a,b), sleep-wakefulness (Pivik et al., 1986), and cecotroph production (Jilge, 1976, 1979, 1980).

4.2.2. Adaptation to feeding schedules

Research in this area is limited to one published paper by Jilge et al. (1987). This researcher collected data from five European rabbits (Himalayan breed) maintained under *ad libitum* conditions and later exposed to a feeding schedules of 4h food access, and found that the feeding schedule had a negative impact on the number of meals and time spent feeding. These dependent variables were reduced compared to the *ad libitum* baseline by approximately 12 to 13 meals and 16 to 18 minutes respectively. By contrast, the number of drinking episodes and their duration remained largely unchanged. While these findings may be valid, care must be taken not to generalize from these limited data because of the small sample. To the author's knowledge, no published data are available on the effects of feeding schedules on the amount of food and water intake, and body weight in the rabbit.

4.2.3. The feeding entrainable circadian system

Anticipatory activity in the rabbit has been documented in a number of circadian rhythms, including locomotor activity, visits to the food box, drinking, and hard feces and urine excretion. Results regarding the latency of anticipatory rhythms have been inconsistent. Jilge et al. (1987) studied rabbits maintained in LL and found that anticipatory activity appeared within 20 to 35 days. Subsequently this author reported 10 to 65 days is required in rabbits housed under LL conditions and exposed to a single daily meal (Jilge, 1991). Studies have also produced variable results with regard to estimates of PAD between activity onset and the onset of food access. Jilge et al. (1987) noted a positive PAD of 1.75 ± 0.5h for locomotor activity, 1.8 ± 0.5h for visits to the food box, and 3h to 6h for hard feces excretion. The PAD was negative for urine excretion (1h to 5h) and drinking (0h). By contrast, Jilge and Stahle (1993) reported PAD to be positive for drinking and negative for hard feces excretion.

The effect of feeding cycle T on circadian rhythms has been examined. Data so far suggest that rabbits housed under a LD cycle and later exposed to a feeding schedule with same or different T entrain to both zeitgebers (Jilge & Stahle, 1993). A study by Jilge (1991) is consistent with these results, showing anticipatory activity to a T cycle of food availability of 23h 50 minutes under dim LL conditions. In this study, the anticipatory component at T=24h (containing 25 ± 11% of total daily activity) was larger than T<24h (containing 15 ± 7% of total daily activity), however, the difference was not significant. While these findings suggest that the overt rhythms are not being passively driven by external cues which are difficult to control in the laboratory, it is still not clear whether feeding schedules affect the underlying pacemaker.

The phase shifting properties of the feeding entrainable circadian system have been investigated in the rabbit. Jilge et al. (1987) reported data from just two rabbits, and found that reentrainment was achieved by delaying transients over 10 to 20 days when food access was delayed by 6h. Jilge also presented data from two rabbits showing that reentrainment to a 6h phase advance was achieved immediately without intervening transients. While this phenomenon also has been reported in rodent studies, Stephan (1984, 1986b) has produced evidence of advancing transients following an advance phase shift. While the reason for such differences is not clear, it is important to note that the study by Jilge et al. (1987) is based on a very small sample and may not be representative of the rabbit. There is, therefore, still a need for studies using larger samples.

Cycles of food availability have been shown to influence tau of the free-running light entrainable rhythm in herbivores. Jilge and his coworker reported rabbits to experience a shortening of free-running tau, with tau becoming closer to T on removal of the feeding schedule. These authors concluded that this effect is likely to be due to aftereffects of the feeding schedule. Aftereffects typically last for up to 50 days, but are likely to persist longer in rabbits entrained to T<24h (57 ± 12 days). Interestingly, the free-running light entrainable rhythm is likely to continue with a shortened tau even after the fading of aftereffects.

The above findings are largely consistent with those from omnivores in pointing to a zeitgeber role for feeding schedules in the rabbit. This conclusion needs to be treated with caution however, since the relevant literature comprises three published papers by Jilge and his coworkers and these studies are limited by small samples. No other authors have attempted to replicate these findings. More research is thus required in order that the reliability of empirical findings can be established. Another issue is that some important questions have not yet been addressed, and as a result a comprehensive model of the herbivore feeding entrainable circadian system is lacking. For example, food deprivation following a feeding schedule could be used with a view to illustrating the endogenous nature of anticipatory activity in the rabbit. Other issues have been considered above and will not be reiterated here. The primary difference between omnivores and herbivores appears to be with respect to the PAD of entrainment and the latency of anticipatory activity, with smaller PADs and a longer latency being cited in the latter group.

4.2.4. The organization of the feeding entrainable circadian system

Although the SCN has been implicated as the site of a major circadian pacemaker in herbivorous species, there is some evidence for the existence of a FEP outside the SCN. Behavioural data provide support for a multi-oscillator model of the circadian system in the rabbit. A finding by Jilge et al. (1987) was that anticipatory activity coexisted with a weak free-running light entrainable rhythm, with tau ranging from 24.15h to 24.22h. Further evidence in support of this proposition comes from the study by Jilge and Stahle (1993). Using zeitgebers with different Ts, it was found that, in addition to anticipatory rhythms, activity entrained to the DL transition at a positive PAD of 30 minutes. The DL component was found to contain 20 ± 7% of total daily activity.

In their study the proposition that the two pacemakers are coupled was supported. As the DL component approached the end of food access, the two rhythms were reported to merge, and decomposition occurred as the PAD increased beyond 4 to 6h. The level of activity was reported to be higher in the L phase when food access occurred during the day (77 ± 4%) compared to the D phase when food access occurred during the night (63 ± 3%). The component of anticipatory activity was found to be significantly smaller during food access in the D phase (16 ± 4%) than in the L phase (27 ± 2%), and activity was less concentrated around the time of feeding. Further corroboration of this view comes from the 1987 study, reporting a shortening of free-running tau following a 6h advance of food access.

The time taken for anticipatory activity to develop may vary according to the PAD between the feeding schedule and the LEP (or free-running light entrainable rhythm):

the larger the positive PAD when the feeding schedule is introduced, the longer it takes for anticipatory activity to appear. Jilge (1991) analyzed data from five animals with a positive PAD of 512 ± 22 minutes and found that the anticipatory rhythms were established within 17 ± 6 days, whereas animals with a larger positive PAD of 1250 ± 34 minutes required at least 53 ± 9 days. Using forward graphical extrapolation, these authors found that the appearance of anticipatory activity coincided with the crossing of the free-running light entrainable rhythm and food access.

At this stage it is not clear whether cycles of food availability entrain the free-running light entrainable rhythm in the rabbit. Jilge et al. (1987) observed a weak free-running light entrainable rhythm containing 3% to 10% of total daily activity in rabbits housed under dim LL. The results obtained by Jilge and Stahle (1993) are inconsistent with this, showing the free-running light entrainable rhythms to be entrained. In some of the data illustrated, however, the phase from which the rhythm free-ran after the removal of the feeding schedule was consistent with its phase prior to entrainment. These investigators argued that feeding schedules have a dual action on circadian rhythms, exerting both masking and entraining effects. Thus, in addition to its entraining effects, a feeding schedule may suppress activity associated with the free-running light entrainable rhythm in the rabbit. The extent to which activity is masked by food is not known. More research is necessary before any conclusions can be reached.

The above findings are consistent with those obtained from the omnivores and carnivores and provide support for a multi-pacemaker model of the circadian system. All the work in this area however, has used intact animals and it is therefore not clear whether anticipatory activity is driven by a separate, independent pacemaker, or whether the SCN controls them. There is therefore a clear need for research with SCN lesioned rabbits. The pacemakers appear to be coupled, but the strength of coupling has not been established. The primary difference in the results across these studies appears to be with regard to whether or not the LEP is entrained by feeding schedules. Different results may reflect, in part, methodological inconsistencies, and/or interindividual differences in the strength of coupling between the LEP and the FEP. Further research is needed to resolve this issue.

4.3. RATIONALE AND AIMS

Although the omnivorous rat has been widely used in studies of the feeding entrainable circadian system, other classes of species have been largely neglected. Thus, while the zeitgeber effects of cycles of food availability have been clearly established in omnivores, the evidence in carnivores and herbivores is less consistent. Despite the lack of research in carnivores and herbivores and the difficulties this creates in comparing the effects of feeding schedules across classes of species, the findings to date suggest that differences do exist. It appears that anticipatory activity is reduced in duration and takes longer to develop in the herbivore, while the free-running light entrainable rhythm is more likely to entrain to the feeding schedule in carnivores. These findings point to the potential significance of adopting an analysis by class approach and suggest that progress in understanding the feeding entrainable circadian system may be dependent on comparative research.

There has been little research with the herbivore. While the hamster has been examined, the results are difficult to interpret given the wide range of methodologies used and the fact that the hamster does not tolerate food deprivation well. Thus, the effect of feeding schedules on the circadian system in herbivores still needs to be established and the potentially confounding effects associated with other methods should be avoided. A small number of studies have used rabbits as subjects, with findings suggesting that this species may be suitable for use as a model of the herbivore feeding entrainable circadian system. Because of its tolerance for food deprivation, the rabbit provides an opportunity to investigate the effects of feeding schedules in a herbivore using the traditional paradigm involving time limited feedings.

The aim of the present thesis was to explore the effects of time limited feeding schedules in the rabbit, with a view to developing a comprehensive model of the herbivore feeding entrainable circadian system. An experiment was conducted in order to examine the suitability of the rabbit for use as a herbivore model of the feeding entrainable circadian system. The aim of subsequent experiments was to determine whether or not periodic food availability is an effective zeitgeber in herbivorous species; the effects of feeding schedules on the activity rhythms of the herbivorous rabbit are evaluated and comparisons are made to omnivores and carnivores in chapters 7 and 8. The functional organization of the feeding entrainable circadian system in the rabbit was examined in two further experiments, and research findings were compared to those obtained from omnivores.

PART TWO. EXPERIMENTAL SERIES

CHAPTER 5. GENERAL METHODOLOGY

This chapter describes the methodology employed in the experiments reported in subsequent chapters.

5.1. ANIMALS

Subjects were adult, female European rabbits (Dutch breed) which ranged in age from 6 months to 3.5 years. The selection of Dutch rabbit in this thesis was determined by availability. Female rabbits were chosen for use because unlike males, female rabbits not intended for breeding can be kept together (Joint Working Group on Refinement, 1993). While studies of rodents have avoided studying females due to estrous cycle complications, the oestrous cycle and method of ovulation in the rabbit when compared to other species is different; ovulation is induced by physical stimulation by the male and there is no seasonal anoestrus (Joint Working Group on Refinement, 1993). Data were collected from two cohorts of rabbits over a period of 3.5 years, with an interval of approximately three months between experiments.

The majority of rabbits were obtained from the Nanowie Small Animal Production Unit in Geelong, Victoria. The remaining rabbits were bred from stock within the School of Psychology at La Trobe University in Melbourne, Victoria. Upon arrival at Monash University, rabbits were group housed in a laboratory measuring 3.0x2.8m at the Department of Psychology Animal House. They were maintained under constant conditions of light and temperature, and tap water and food, which consisted of a mixture of pasture replacement pellets (Clarke King Co, Melbourne, Victoria), lucerne chaff, and fresh fruit (apple) and vegetables (carrot) were available *ad libitum*. These conditions were reinstated between experiments. At the beginning of each experiment, rabbits were given two weeks to acclimitise to the laboratory conditions. This was considered desirable in order that the effects of group housing be reduced (Love, 1994).

In all experiments, rabbits were fed a small pasture replacement pellet containing 17% crude protein, 12% crude fibre, and 2% fat, with an energy content of 2,223 kcal/kg (Clarke King Co).

5.2. LABORATORY APPARATUS

5.2.1. Housing

Experiments were conducted in a controlled laboratory (3.8x4.6m) environment in the Department of Psychology Animal House at Monash University, Melbourne. The rabbits were housed individually in cages in order to avoid the possible confounding effects of social interactions that occur when animals are group housed. Air temperature was maintained at 21 ± 2 degrees celsius. A wavetek VCG/Noise generator was used to generate white noise in order that extraneous disturbances be reduced. These conditions are typical of those used in circadian rhythm studies.

5.2.2. Chapters 6 and 8

The cages (52x70x78cm) were arranged in pairs and separated by an opaque perspex screen (see Figure 3). The back of the cages was made from galvanized sheeting, and the sides and the front were covered with wire mesh. Excrement passed through a perforated, polyvinyl floor into a sawdust tray. Food and water were available in separate compartments made from polyvinyl sheeting. These compartments were located externally, and could be accessed via a vertical swinging door (10x10cm) at the back of the cage so that food and water could be replenished without disturbing the animals. A plastic, removable cup was positioned in each compartment which measured 15x17x15cm and allowed no lateral movement of the cup. Lighting was provided by a single 60-watt globe located near the top of each cage. Light intensity was controlled by a dimmer switch and measured using a Tektronix J17 LumaColor lux-meter. Light intensity at cage floor level was 40 to 60 lux. Cages were cleaned weekly (or twice weekly if deemed necessary) and rabbits checked daily (including weekends) by the experimenter. The time of entry into the laboratory was varied in an attempt to eliminate, or at least reduce, the influence of social cues on circadian rhythms.

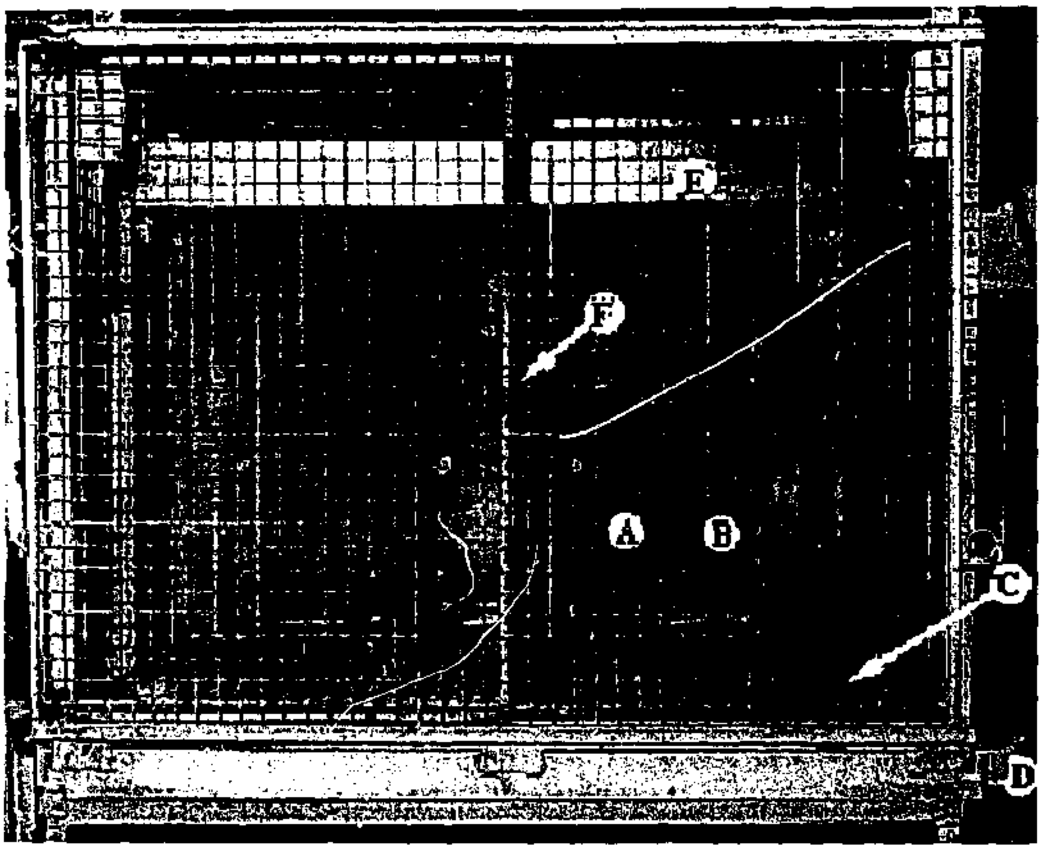


FIGURE 3. PHOTOGRAPH OF THE FRONT OF THE CAGES USED IN CHAPTERS 6 & 8. A = VERTICAL DOOR TO FOOD HOPPER. B = VERTICAL DOOR TO WATER HOPPER. C = PERFORATED FLOOR. D = SAWDUST TRAY. E = LIGHT BOX. F = OPAQUE PERSPEX SHEETING.

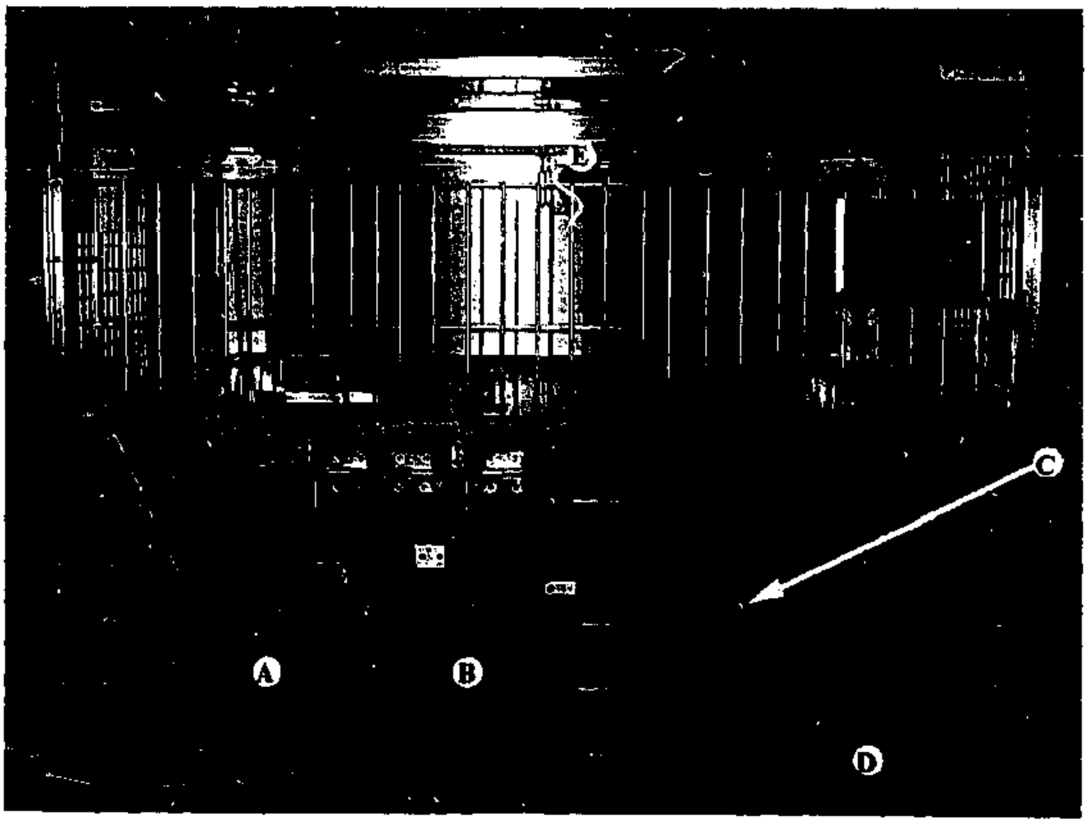


FIGURE 4. PHOTOGRAPH OF THE FRONT OF THE CAGES USED IN CHAPTERS 7 & 9. A = FOOD HOPPER. B = WATER HOPPER. C = PERFORATED FLOOR. D = SAWDUST TRAY. E = LIGHT GLOBE.

5.2.3. Chapters 7and 9

The cages (78x92x70cm) were made from plastic, and the front was stainless steel (see Figure 4). Excrement passed through a perforated floor into a sawdust tray. The same food and water apparatus as that described above was used. However, the compartments were located at the front rather than the back of the cage in order to make them more easily accessible to the experimenter. All other conditions were the same as those described above, the exceptions being that the light globe was located at the rear of the cage and staff were hired to assist with cleaning duties and the weekend monitoring of the rabbits.

5.2.4. Measuring apparatus

Feeding and drinking behaviour were measured using a microswitch fitted to the vertical door. The microswitch was activated each time the door was pushed open and a signal registered by the data logger (see section 5.3). Food and water counts were registered and stored in 15 minute bins in the data logger. The food door had a solenoid lock which was under computer control.

This system was considered suitable for use in the current study for several reasons:

- unlike the lickometer where the number of licks per unit volume is altered by contact with the apparatus, this system does not influence intake.
- this system is reliable when used continuously over an extended period of time.
- no training and pre-training of animals is involved.
- there is little spillage.
- actograms can be obtained which resemble those obtained using different techniques.
- automated techniques are frequently used in circadian research in order that social cues arising from delivery/removal of food by the experimenter are eliminated.

A passive inferred sensor (PIR) was mounted in the corner of each cage and used to measure general activity. The PIR was chosen for use because, unlike some other techniques such as the running wheel, the PIR does not influence the activity of the animal under study (Mather, 1981; Sherwin, 1998; Strong, 1957; Triechler & Hall, 1962). The PIR was used to detect radiated thermal energy from the animal. When an animal moved, a pulse output was generated by the PIR and sent to the data logger (see below). Each pulse was registered by the data logger as a single activity count. Activity counts were registered and stored in 15 minute bins in the data logger.

5.3. DATA COLLECTION

The apparatus was controlled by two data loggers located near the entrance to the laboratory. Activity was sampled and stored in 15 minute bins by the data logger. Data were downloaded each day and transferred to an IBM compatible computer for statistical and graphical analysis using the Tau program (Mini-mitter, Oregon, USA).

5.4. DATA PRESENTATION

Most of the data reported in this thesis are presented graphically in the standard actogram format used in this field of research. Actograms record the presence or absence of activity as a function of the time of day, and indicate also the amount of activity occurring in each 15 minute bin. The Tau program was used to generate actograms. Actograms have been double plotted in order to facilitate visual inspection of the data.

Visual inspection of actograms indicated that circadian rhythmicity for general activity was obscured by frequent low levels of activity. In order to enhance the clarity of the circadian rhythm for general activity, Mod1 software (Monash University, Melbourne, Australia) was used to filter low amplitude, high frequency activity. In the Mod1 program, the level of activity was indexed using a 1000 point scale. For each subject, the maximum level of activity during a sample interval was computed for each day and a score of 1000 was assigned to this value. All other data points were expressed as a proportion of the maximum value and then multiplied by 1000. The value of 1000 was arbitrary. Following scaling, the sample values ranged between 0 and 1000. The equation is shown below.

$$\frac{n}{\max} \times 1000$$

where *n* represents the sample value and max represents the daily maximum sample value.

Then the following exponential algorithm was applied.

$$\frac{1000}{e-1}\times\left(e^{\frac{n}{1000}}-1\right)$$

where n represents the scaled sample value now ranging between 0 and 1000 and e is the exponential constant. This function compresses low values and amplifies high values. Following exponential scaling, those scores less than 35% of the maximum sample value were assigned a value of 0. This procedure permitted the filtering of low noise levels without any loss of resolution. Algorithms are commonly used in this field of research in order to overcome some of the problems associated with chart recorder techniques. These problems have been outlined elsewhere and will not be discussed here (see Clarke 1983 for review).

The PIRs proved problematic since they detected activity in the laboratory. Values coinciding with laboratory entries were often not consistent with the rabbits' normal level of activity and were presumed to be errors. A pilot study was conducted in order to determine the effect of laboratory entries on the recording of general activity. An empty cage was placed in the laboratory during the first experiment and records of laboratory entries maintained. Data for both laboratory entries and PIR activity are presented in actogram format in Appendix A. The results of this study indicated a close correspondence between laboratory entries and PIR activity. Thus, these data were deleted from the actograms on the basis of inaccurate data. Bins corresponding to the time of laboratory entry were assigned a value of 0. This procedure was used in order to enhance the clarity of the circadian rhythm.

5.5. DATA ANALYSIS

The following section describes the dependent variables used in the experiments reported in this thesis.

5.5.1. Tau of the free-running light entrainable rhythm

Tau software was used to calculate tau of the free-running light entrainable rhythm. Using this software, a line of best fit was placed along activity offsets over 20 consecutive days and tau determined by the slope of the line using linear least squared regression techniques. A 20-day analysis period was used (rather than 10-days as is customary in this type of research) because activity offsets tended to be less precise in our data compared to published data. Two independent, trained raters placed the lines in order to achieve some measure of reliability. Inter-rater reliability was examined using Pearson's product-moment correlations. Estimates of tau in the present thesis were consistently highly correlated and no systematic differences between raters' estimate were found. The decision was made to use the average of the raters' estimates in statistical analyses.

A pilot test was conducted in order to examine the reliability of visual estimates, and their usefulness in terms of comparability with statistical techniques. Enright's periodogram analysis was chosen for use, since it is commonly used in this field of research. This method is not described here, but the interested reader can refer to Enright (1965). Data for approaches to the food bin were obtained over 20 days from 12 rabbits free-running in LL, with apparent single periodicities. Rabbits were housed under the conditions described in section 5.2.2. Tau software was used to derive both visual and statistical estimates. Tau software uses Enright's periodogram analysis to derive statistical estimates. Two independent, trained raters were used to derive visual estimates. Raw data are presented in Appendix A. Table 2 below shows bivariate correlations between all variables.

TABLE 2.
CORRELATIONS BETWEEN VISUAL AND STATISTICAL ESTIMATES OF TAU

	Rater 1	Rater 2
Rater 1		
Rater 2	.81**	
Enright's technique	.66*	.82**

**p*<.05
***p*<.001
n=12

As seen in Table 2, correlations amongst all variables are highly significant and no systematic differences between estimates were found. Given these findings, the decision was made to use visual estimates of tau in subsequent analyses. Similar results were obtained by Pittendrigh and Daan (1976d) who reported that visual estimates of tau are as accurate and reliable as linear regression methods. Another reason for this decision was because, as Enright (1965) has noted, statistical estimates are unreliable when multiple periodicities are present in the data. The expression of multiple periodicities in research of this type is well established and are to be expected when feeding schedules are used (Aschoff et al., 1983; Stephan, 1986a,b; Stephan & Becker, 1989).

Activity offset was used as the preferred reference phase for estimating tau of the free-running light entrainable rhythm. Enright (1966a) found activity onset to be a more precise reference phase than activity offset. Although this finding has been confirmed by many researchers, data from the rabbit in the present thesis and some other species such as the squirrel monkey has shown that offset provides the least variable estimate of tau (Rajaratnam, 1997). In the present thesis, activity onset could not be clearly determined in many cases due to poorly defined onsets. Given that the onset and offset components are thought to be controlled by separate, self-sustaining oscillators (see section 1.2.3.), it is conceivable that estimates of tau based on activity onset may differ from estimates based on offsets. In order not to systematically bias the data, activity offset was chosen as the preferred reference phase for tau estimates in the present thesis.

5.5.2. Entrainment of the free-running light entrainable rhythm by the feeding schedule

Entrainment to a feeding schedule was defined by the criteria specified by Enright (1981) which state that in order to demonstrate that stable entrainment has occurred, both period and phase control of the overt rhythm must be shown. Phase control was assumed if, on removal of the feeding schedule, the rhythm free-ran from a phase determined by the schedule and not by pre-entrainment phase. Despite the fact that activity offset was deemed to be the preferred reference phase of the free-running light entrainable rhythm, activity onsets had to be used to determine whether or not phase control of the free-running light entrainable rhythm by the feeding schedule had been achieved.

5.5.3. Anticipatory activity

As is customary in this type of research, the actograms were inspected visually in order to determine whether anticipatory activity was present. In experiment 3, actograms were plotted at a day length corresponding to the feeding schedule for easier visual assessment of anticipatory activity. The timing of activity onset is however less clear in the rabbit than in other species and hence it could not always be ascertained whether anticipatory activity was present.

Given the difficulty in interpreting actograms, alternative methods were examined. It was decided to adopt the following method: data for approaches to the food hopper (i.e., food counts) were averaged in 30 minute intervals over the final 10 consecutive days of the feeding schedule, and graphed using Microsoft Excel. A 10-day analysis period was used in order that the influence of the free-running light entrainable rhythm on anticipatory activity be reduced. Use of a 10-day analysis period was also appropriate in terms of comparison to previous research, since 10-day analysis periods are typically used in circadian rhythm research (e.g., Aschoff et al., 1983; Boulos & Logothetis, 1990; Coleman & Francis, 1991; Kennedy et al., 1991, 1995; Mistlberger et al., 1990a; Stephan, 1992b). Food counts were selected as the most appropriate measure of activity because visual inspection of the actograms indicated that anticipatory activity was more likely to occur in food counts than in general activity and drinking (i.e., water counts). The use of food counts as a DV in almost all analyses was also considered suitable due to the poor quality of many of the records for general activity. Furthermore, the results for food and water counts in the present thesis tended to be similar.

The distributions for food counts were examined visually; activity in the 3h period immediately prior to food access was compared with activity over an equal time span immediately preceding this period. A criterion of two-fold increase in activity over activity in the preceding period was used to identify anticipatory activity. A second criterion was also used; activity counts in at least one 30 minute bin must exceed 10.

Although this method has not been used previously, similar techniques have been used by Stephan (1981, 1992b) and Stephan and Becker (1989). It is appropriate that such a method be adopted to ensure a consistent approach across all experiments in the present thesis. It is notable that this criterion is somewhat arbitrary, however, it did effectively identify anticipatory activity that had been previously identified by visual inspection.

Many researchers do not indicate the method used to calculate the PAD between activity onset and the onset of food access and the rate of emergence of anticipatory activity, and there is no consistent agreement at present as to what constitutes the most appropriate method. This creates difficulties in terms of comparison of studies, since differences in results across studies may be due in part to differences in methodology. In the absence of an established criterion, the actograms were inspected visually and PAD was calculated by placing a line of best fit along activity onsets over the final 10 consecutive days of the feeding schedule. The difference between activity onset and the onset of food access was measured in *mm* and then converted to time in hours. In experiment 3, actograms were plotted at a day length corresponding to the feeding schedule for easier assessment of PAD. Two independent, trained raters were used for the reason described earlier. Generally estimates of PAD in the present thesis were highly correlated and no systematic differences between raters' estimates were found. Therefore, the decision was made to randomly select the data from one rater for use in statistical analyses.

The above method was chosen in preference to other methods because, as noted earlier, findings indicate that visual estimates of circadian parameters are as accurate and reliable as statistical methods in unimodal behaviour patterns, and superior in multimodal activity patterns (Enright, 1965; Pittendrigh & Daan, 1976d). Because of the demonstrated reliability of visual estimates and their relatively wide usage (Aschoff et al., 1983; Coleman & Francis, 1991; Kennedy et al., 1991, 1995; Mistlberger et al., 1990a; Stephan, 1981, 1984), visual estimates of the rate of emergence of anticipatory activity were also deemed appropriate for the rabbit.

5.5.4. Direction and duration of reentrainment of feeding entrainable rhythms

The direction of reentrainment was defined as the direction traveled by the transient cycles and was determined from actograms. The criterion adopted by Redman (1988) was used to calculate the duration of reentrainment. The duration of reentrainment was defined as the number of days required after the phase shift for activity onset of the feeding entrainable rhythms to regain a stable phase relation with the new feeding schedule. This method was chosen for use because, unlike other techniques, Redman's analysis takes into account changes in pre- and post- PAD.

CHAPTER 6. THE EFFECT OF FEEDING SCHEDULES ON FOOD AND WATER INTAKE, BODY WEIGHT, AND GENERAL ACTIVITY IN THE HERBIVOROUS RABBIT

6.1. RATIONALE AND AIMS

As indicated previously, the omnivorous rat is one of the most widely accepted and well-researched models of the feeding entrainable circadian system. While there is evidence for reductions in food and water intake and body weight under feeding schedules compared to *ad libitum* food access, it is clear that the rat is effective in compensating when food access is limited to a few hours per day. Relevant research is reviewed in section 2.2. Unlike the omnivorous rat, food intake per unit time is fixed in the herbivorous hamster; the inability of the hamster to tolerate feeding schedules is well-documented (see section 3.3.1). Other methods have been used to study the hamster's feeding entrainable circadian system. However, their validity remains to be determined.

Based on preliminary findings by Jilge (see section 4.2.2), it appears reasonable to expect the rabbit is suitable for use as a herbivore model, however, no systematic studies have been done. The aim of this study was to investigate the ability of the rabbit to tolerate feeding schedules, in terms of food and water intake, and body weight. Rabbits were exposed to feeding schedules of 2h, 3h, and 4h food access. These meal durations were chosen because they are commonly used in this field of research, and hence comparative data would be available from other species (e.g., Aschoff et al., 1983; Cambras et al., 1993; Honma et al., 1983; Kennedy et al., 1990, 1991; Rosewnwasser et al., 1984; Stephan, 1979b, 1981, 1984, 1986a,b). It was hypothesized that daily food and water intake, daily time spent eating and drinking, and body weight would be lowest during the feeding schedule, and highest after its removal. These effects were expected to be greatest under the 2h feeding schedule. A further purpose of the study was to explore the effects of scheduled feeding on general activity. Based on previous studies in the rat involving stationary cage techniques, no changes were expected to occur in general activity.

6.2. METHOD

6.2.1. Animals and housing

Subjects were eight female Dutch rabbits which ranged in age from 8 months to 2.5 years. Weight ranged between 3kg and 4.5kg at the start of the experiment. Rabbits were housed under the conditions described in sections 5.2.1 and 5.2.2.

6.2.2. Procedure

The experiment consisted of three phases. In the pre-phase, food and water were available *ad libitum* for 28 days. During the feeding schedule, rabbits were randomly assigned to one of three groups; group A (n=3), B (n=2), and C (n=3) fed daily for 2h, 3h, and 4h respectively. Rabbits were fed at the same time each day over 56 days. The time of initial food access was scheduled individually to the time of minimal food intake in the pre-phase. In the post-phase, food and water were available *ad libitum* for 28 days. In line with guidelines set by the Psychology Department Animal Experimentation Ethics Committee at Monash University, the post-phase was introduced immediately if body weight reduced to 80% of baseline levels during the feeding schedule.

Rabbits were checked daily and weighed on alternate days. Daily food and water intake was defined as the difference between the amount of food and water offered and the amount remaining in the cups after approximately 24h. Intakes were measured three to four times per week.

6.2.3. Design

The independent variables were phase (pre-, restricted feeding-, post-) and meal duration (group A, B, and C). Phase was a within-subjects variable and meal duration was a between-subjects variable. The dependent variables (DV) included body weight, daily food intake, daily time spent eating, daily water intake, daily time spent drinking, and daily general activity.

6.3. RESULTS

6.3.1. Data screening

Data were screened for accuracy of data entry and to identify missing values and univariate outliers. Few missing data were found; seven of eight animals completed all 56 days of the feeding schedule, with only 34 days of data included for one rabbit. For this rabbit, body weight dropped to 80% of baseline levels after 34 days of restricted feeding and the post-phase was introduced early. Most variables contained some outliers. Each case containing an outlier was examined in order to determine why the outlier occurred. Cases were examined across the three phases for consistency within and between each phase. Outliers on the variables time spent eating and time spent drinking appeared to be related to mechanical and software problems and were thus eliminated from the analysis. Outlying scores on other variables appeared to represent valid observations, since most animals with an outlying score had consistently high scores on that variable. A decision was therefore made to retain these data. These criteria have been used previously by Ross (2001).

6.3.2. Data analysis

Mean activity levels per 24h were calculated for each rabbit. This was done separately for each phase of the experiment, and data were transferred to SPSS for analysis. Data were analysed using a repeated measures analysis of variance (ANOVA). The total *n* was eight. Sample sizes were three, two, and three for groups A, B, and C respectively. In all ANOVAs, cells were weighted by their sample sizes to adjust for unequal *n*. Significant interactions were examined using a simple main effects analysis. Tukey's HSD method was used to conduct *post-hoc* tests. Summary tables for all ANOVAs and simple main effects analyses are presented in Appendix B. It is important to note that ANOVAs in the present study are low in power and that multiple such analyses were done, both aspects being statistically undesirable. However, the analyses were used to provide a descriptive aid and are not rigorous.

6.3.3. *Body weight*

Means and standard errors for body weight as a function of phase and meal duration are shown in Figure 5.

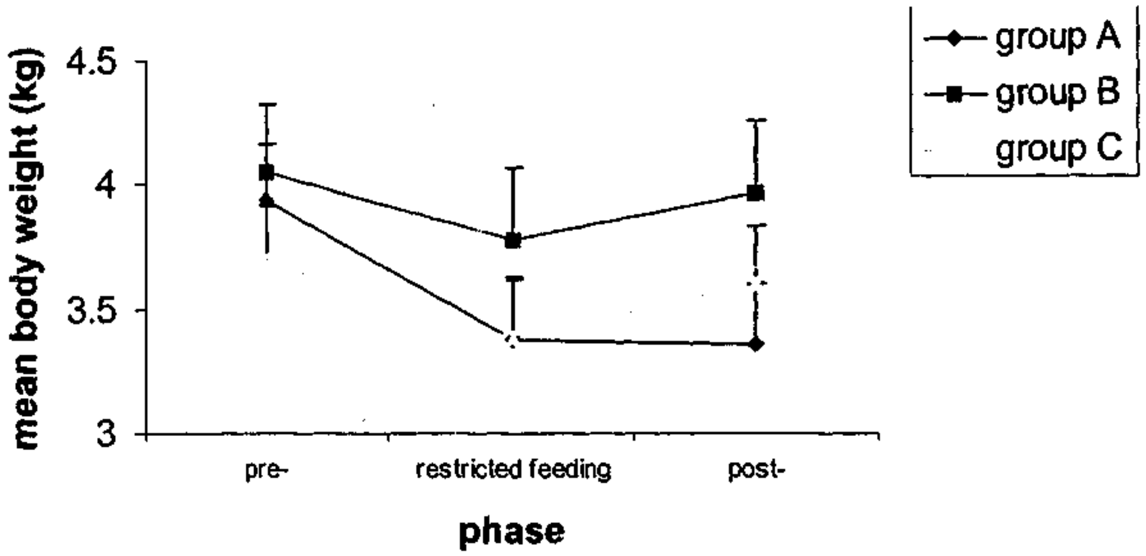


FIGURE 5. MEAN (+*SE*) BODY WEIGHT AS A FUNCTION OF PHASE AND MEAL DURATION.

Statistical analysis by two-way ANOVA indicated a significant interaction between phase and meal duration, $F(4,10)=6.77$, $p<.01$. Simple main effects analysis showed that body weight varied significantly across the experiment in group A but not in either group B or C. Body weight in group A was found to be lower during the post-phase compared to the other phases, and lower during restricted feeding than in the pre-phase. The main effect for phase was significant, $F(2, 10)=34.27$, $p<.01$. There was no significant main effect for meal duration.

6.3.4. Daily food intake

Means and standard errors for daily food intake as a function of phase and meal duration are shown in Figure 6.

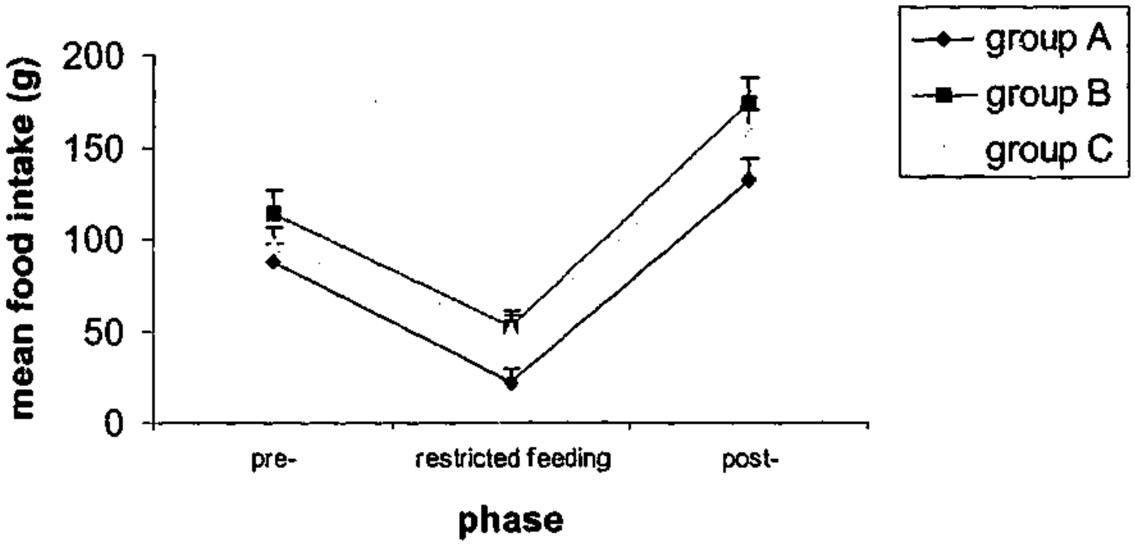


FIGURE 6. MEAN (+*SE*) DAILY FOOD INTAKE AS A FUNCTION OF PHASE AND MEAL DURATION.

Statistical analysis by two-way ANOVA indicated a significant main effect for phase, $F(2,10)=84.71$, $p<.01$. Tukey's HSD tests showed that food intake was significantly higher during the post-phase ($M=154.61$, $SE=6.97$) compared to the other phases, and higher during the pre-phase ($M=99.25$, $SE=6.39$) compared to restricted feeding ($M=41.77$, $SE=4.6$), p's$<.05$. A significant main effect for meal duration was found, $F(2,5)=7.81$, $p<.05$. Tukey's HSD tests indicated higher levels of food intake in group B ($M=113.35$, $SE=6.74$) compared to group A ($M=80.49$, $SE=5.5$), p's$<.05$. No other significant differences were found. There was no significant interaction effect.

6.3.5. Daily water intake

Means and standard errors for daily water intake as a function of phase and meal duration are shown in Figure 7.

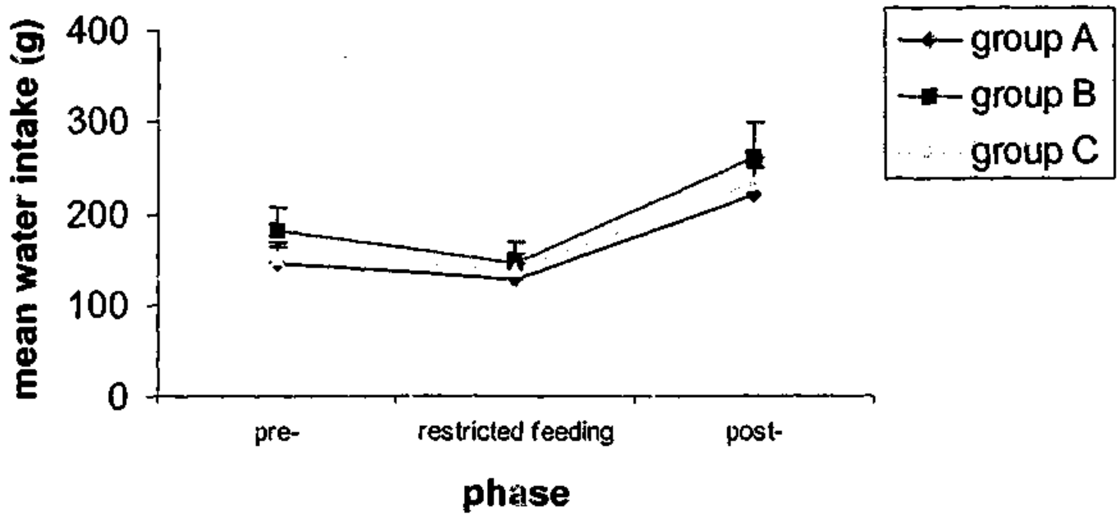


FIGURE 7. MEAN (+*SE*) DAILY WATER INTAKE AS A FUNCTION OF PHASE AND MEAL DURATION.

Statistical analysis by two-way ANOVA indicated a significant main effect for phase, *F*(2,10)=46.63, $p<.01$. Tukey's HSD tests showed that water intake was significantly higher during the post-phase (*M*=236.86, *SE*=18.32) compared to the other phases which did not differ significantly from each other (pre *M*=158.19, *SE*=12.48; restricted feeding *M*=136.08, *SE*=11.84), *p*'s<.05. The main effect for meal duration was non-significant. There was no significant interaction effect.

6.3.6. Daily time spent feeding

Means and standard errors for daily time spent feeding as a function of phase and meal duration are shown in Figure 8.

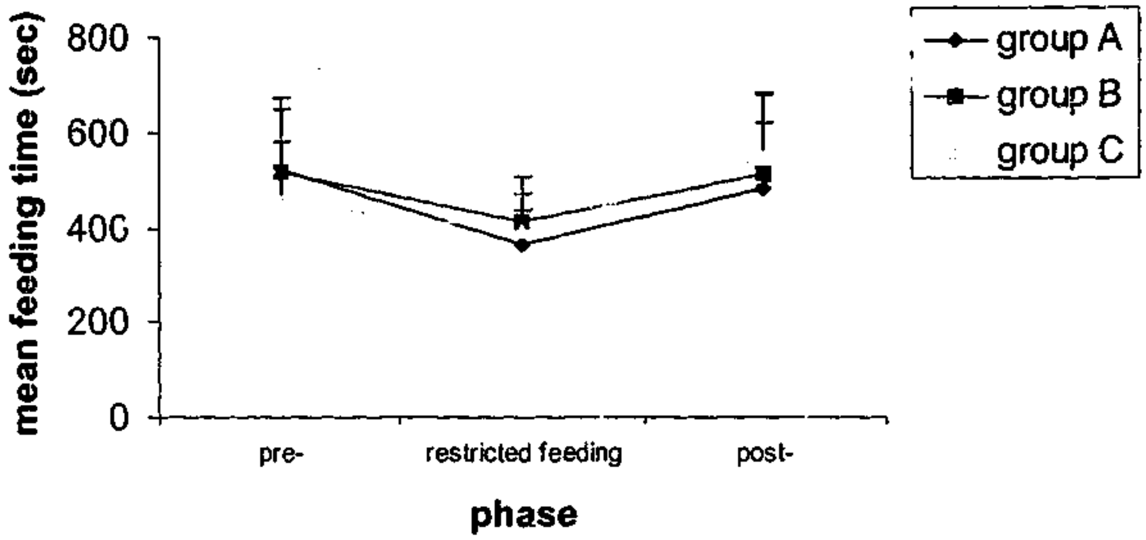


FIGURE 8. MEAN (+*SE*) DAILY TIME SPENT FEEDING AS A FUNCTION OF PHASE AND MEAL DURATION.

Statistical analysis by two-way ANOVA indicated a significant main effect for phase, *F*(2,10)=5.01, *p*<.05. Tukey's HSD tests showed that time spent feeding was significantly higher during both the pre- (*M*=497.37, *SE*=79.56) and post- (*M*=512.08, *SE*=85.4) phase compared to restricted feeding (*M*=389.6, *SE*=47.47), *p*'s<.05. No other significant differences were found. The main effect for meal duration was non-significant. There was no significant interaction effect.

6.3.7. Daily time spent drinking

Means and standard errors for daily time spent drinking as a function of phase and meal duration are shown in Figure 9.

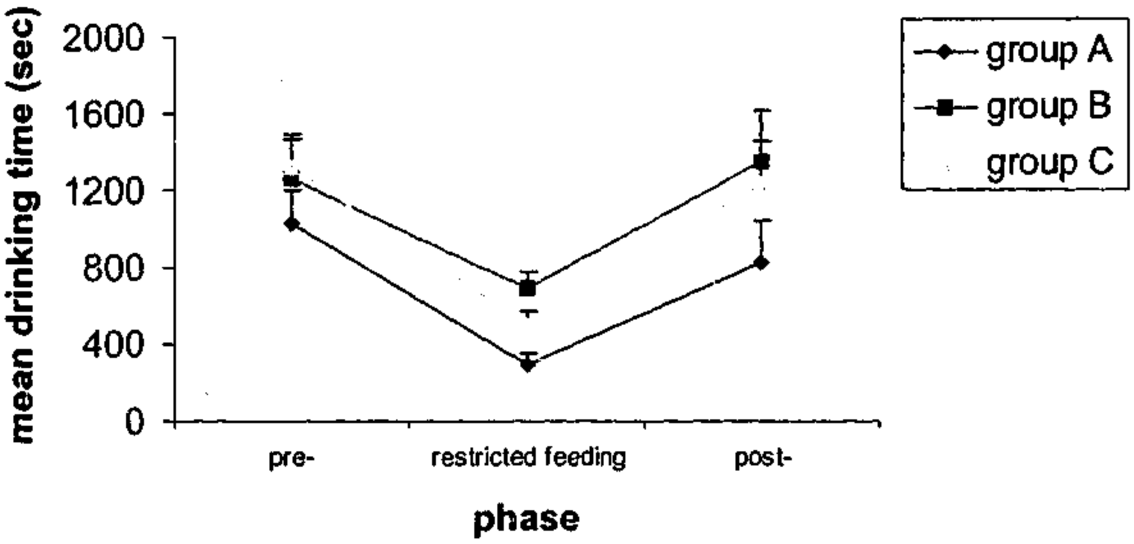


FIGURE 9. MEAN (+*SE*) DAILY TIME SPENT DRINKING AS A FUNCTION OF PHASE AND MEAL DURATION.

Statistical analysis by two-way ANOVA indicated a significant main effect for phase, *F*(2,10)=13.01, *p*<.01. Tukey's HSD tests showed that time spent drinking was significantly higher during both the pre- (*M*=1204.15, *SE*=106.11) and post- (*M*=1134.46, *SE*=136.22) phase compared to restricted feeding (*M*=494.53, *SE*=44.04), *p*'s<.05. No other significant differences were found. The main effect for meal duration was non-significant. There was no significant interaction effect.

6.3.8. General activity

Means and standard errors for general activity as a function of phase and meal duration are shown in Figure 10.

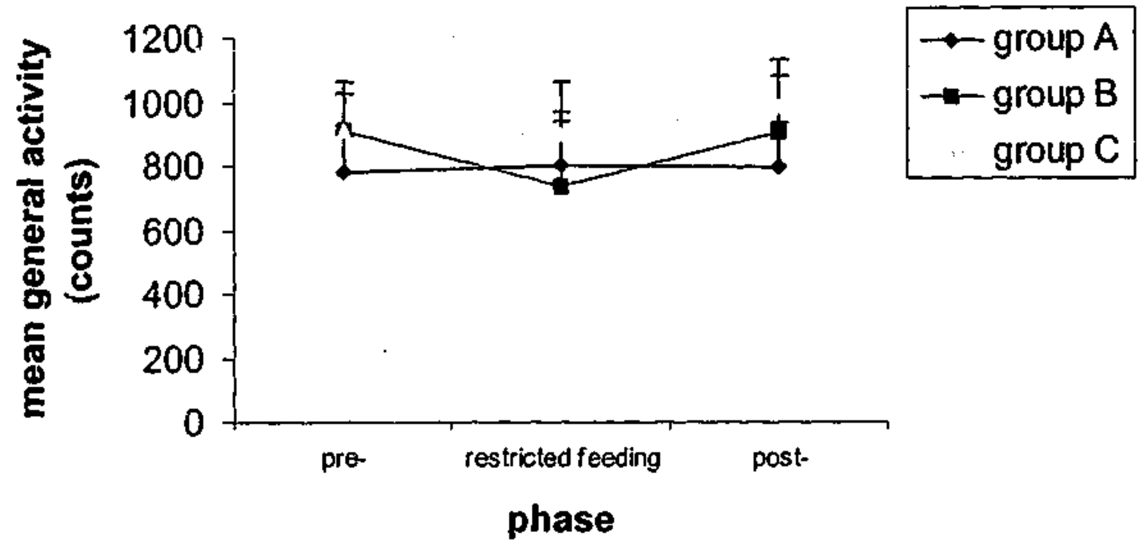


FIGURE 10. MEAN (+*SE*) GENERAL ACTIVITY AS A FUNCTION OF PHASE AND MEAL DURATION.

Statistical analysis by two-way ANOVA found no significant main effects or interaction effects.

6.4. DISCUSSION

The aim of this study was to investigate the ability of the rabbit to tolerate feeding schedules, in terms of food and water intake and body weight. The present findings suggest that the rabbit responds to feeding schedules in a manner comparable to that of the rat and hence is suitable for use as a herbivore model of the feeding entrainable circadian system. The present findings are detailed below.

6.4.1. Body weight

A significant interaction effect between phase and meal duration for body weight was found. Body weight in the 2h group showed a decrease from the pre-phase to the feeding schedule and decreased further during subsequent *ad libitum* feeding. By contrast, body weight did not change significantly across the experiment in either of the other groups. These results support the hypotheses in that the duration of food access was shown to affect the degree of weight loss and suggest that 2h meals may not be appropriate in the rabbit.

6.4.2. Food and water intake

The results of this study indicated that each DV varied across the experiment. As expected, daily food and water intake and daily time spent feeding and drinking were at their lowest during the feeding schedule and increased significantly during the post-phase. Daily food and water intake were significantly higher during the post-phase compared to the pre-phase. The latter is not a surprising finding, given that the wild rabbit exhibits extended periods of hyperphagia and polydipsia following seasonal food shortages (Mykytowycz, 1958, 1961)

The results of this study are consistent with those obtained in omnivores, showing that food and water intake were at their lowest during the feeding schedule and highest afterwards (Fallon, 1965; Lawrence & Mason, 1955; Reid & Finger, 1954). However, the extent of hyperphagia is small in the rat and lasts for only a few days (Fallon, 1965; Lawence & Mason, 1955; Reid & Finger, 1954). The conflicting data are unlikely to be attributable to the duration of exposure to the feeding schedule, since the experimental period in previous studies has rarely exceeded 30 days, whereas a longer term of 56 days was used in the present study. According to Curi et al. (1989), compensatory increases in food intake tend to last longer in meal fed rats after 4 weeks (up to 3 weeks compensatory feeding) of scheduled feeding compared to 20 weeks (up to 1 week compensatory feeding). Thus, the extent of hyperphagia would be expected to be smaller, not larger, in the present study compared to previous studies with the rat. Although the reason for this difference is unclear, differences in the nutritional status or the caloric content of foodstuff used across studies may account for the conflicting data. Support for this idea is derived from the work of Szepesi and Epstein (1976) who noted a greater tendency to overeat in calorie-restricted rats compared to *ad libitum* fed controls during subsequent *ad libitum* feeding.

The current findings are different from those of Kutscher (1969) and Silverman and Zucker (1976) who investigated the hamster. Both of these researchers reported that in the hamster food intake per unit time is fixed, and there is little compensatory overeating in response to feeding schedules; intake dropped to 10 to 15% of baseline levels during scheduled feeding and many hamsters died. This did not occur in the present study. Daily food intake was approximately 50% lower during the feeding schedule compared to baseline, suggesting that the rabbit is capable of consuming large quantities of food in short periods. The fact that food intake increased significantly in the post-phase compared to the pre-phase is also consistent with the view that the two species are different, with the rabbit (but not the hamster) showing hyperphagia and adaptability to brief periodic feedings. Also relevant here is the finding that all but one rabbit were able to maintain their body weight within 20% of baseline levels during restricted feeding. The hamster differs from the rabbit, with studies showing the hamster to be a prodigious hoarder of food, and others showing the hamster can be rendered torpid by cold exposure. It is possible, as hypothesized by Silverman and Zucker (1976), that the hamsters' 'non-compensatory' feeding style may be an effect of these adaptations. It is important to note that the present study differed from the work of Kutscher (1969) and the early work of Silverman and Zucker; data in the present study were derived using longer access periods. It is unlikely that the inability to tolerate feeding schedules found in previous studies is due to the duration of food access, since Silverman and Zucker (1976) report a failure to adjust to feeding schedules with food access periods as long as 12h in the hamster.

The finding that the amount of food intake was lower in the 2h group compared to 3h groups supports the hypotheses. The exception to this is the 4h group which fell between the other groups, but did not differ significantly from either of them. This trend was also apparent for the other DVs, but no significant differences were found. The reason for the lack of effect is not clear, but was probably due to a lack of power associated with some of the tests. This is possibly the result of the small sample size and/or greater day-to-day variability in scores within and between subjects. No previous studies have examined intake as a function of meal duration, and therefore there is no relevant literature with which to make comparisons. Replication of this study using a larger sample would be needed to investigate this issue properly.

6.4.3. General activity

In the present study, general activity did not change across the experiment. This result supports the hypotheses, and is consistent with previous research by Aschoff (1983) demonstrating no effect of feeding schedules on general activity as measured by stationary cage techniques in the rat. Aschoff et al. (1983) also noted increases in wheel-running activity; the amount of activity appeared to depend in part on the duration of food access. One explanation for this discrepancy is based on the type of instrument used to measure activity. A number of researchers have suggested that the running wheel measures a distinct behaviour as compared to other measuring devices (Mather, 1981; Sherwin, 1998; Strong, 1957; Treichler & Hall, 1962). The running wheel is reported to measure a single type of activity of a locomotor nature whereas other recording devices are sensitive to all behaviours involving movement. Running wheels are thought to introduce strong visual and auditory cues, and the wheel itself may be reinforcing by virtue of its inertia. Strong (1957) hypothesized that feeding schedules (i.e., hunger) selectively increase gross locomotor activity and decrease non-locomotor activity. An alternative explanation is that activity levels are influenced by cues generated by the running wheel. Thus, the results obtained in the present study are not surprising given that gross locomotor activity, as well as gross non-locomotor activity was measured using movement sensors, and there was no feedback mechanism operating in this apparatus.

6.4.4. Conclusion

In conclusion, this study was designed to examine the suitability of the rabbit for use as a herbivore model of the feeding entrainable circadian system. The results indicated that the rabbit is able to tolerate feeding schedules, but body weight is affected when daily food access is limited to 2h. The decision was therefore made to use 3h and 4h feeding schedules in all subsequent experiments.

CHAPTER 7. REENTRAINMENT OF CIRCADIAN RHYTHMS FOLLOWING A 5H PHASE ADVANCE AND A 5H PHASE DELAY OF THE FEEDING SCHEDULE IN THE HERBIVOROUS RABBIT

7.1. RATIONALE AND AIMS

The criteria for identifying zeitgebers were described in chapter 1 and will not be considered again here. The importance of these criteria can be appreciated in light of the fact that some environmental agents influence overt rhythms without affecting the underlying pacemaker. It is well established that the LD cycle is the most potent zeitgeber for almost all circadian rhythms, but other periodic signals are also effective as zeitgebers. Research to date supports a zeitgeber role for feeding schedules in the rat. Results of these studies indicate that: (i) anticipatory activity develops within a few days of exposure to restricted feeding; (ii) anticipatory activity resets in a series of transient cycles in response to a phase shift of food access; (iii) when food is withheld for a few days, meal-associated activity persists at the former phase of feeding, and; (iv) anticipatory activity is expressed to a limited range of feeding schedule Ts.

Most of what is known about the feeding entrainable circadian system has been derived from investigations of the omnivorous rat, with few studies using herbivores as subjects. Despite findings in the rat, evidence for a zeitgeber role of feeding schedules in herbivores is lacking and there is uncertainty as to whether periodic food availability is an effective zeitgeber in herbivorous species. More research with herbivores is therefore required. Of particular note is the absence of any data on the effects of food deprivation in herbivorous species. Furthermore, no previous studies have adequately examined the phase shifting effects of feeding schedules. A single study has been conducted by Jilge et al. (1987) using the herbivorous rabbit. However, a small sample was used and little data concerning reentrainment were presented. It was found that reentrainment was achieved by delaying transients following a phase delay of food access. No evidence of advancing transients was found. In his study, Jilge et al. (1987) documented anticipatory activity in a number of circadian rhythms and reported PADs between activity onset and the onset of food access smaller than those observed in the omnivorous rat.

The aim of the current study was to examine the phase shifting effects of feeding schedules in the herbivorous rabbit. A further aim of the study was to determine whether anticipatory activity would persist in the absence of restricted feeding. Rabbits were maintained under a 14:10h LD cycle and exposed to a feeding schedule. On day 50, the phase of food access either was delayed or advanced by 5h. Following prolonged exposure to the new mealtime, rabbits were deprived of food. Based on previous findings (see section 4.2.3), it was hypothesized that rabbits would develop anticipatory activity to the feeding schedule, but the PAD between activity onset and the onset of food access would be smaller than that of the rat. It was also hypothesized that reentrainment would be achieved by delaying transients, independent of the direction of the phase shift (see sections 2.3.4 & 4.2.3). Based on previous findings by Jilge and his colleagues, it seemed reasonable to expect that meal associated rhythms would persist at the former mealtime.

It was important to use a LD cycle in order to eliminate the possible confounding effects of the free-running light entrainable rhythm on feeding entrained rhythms. In other words, LD conditions were used to distinguish between the two rhythms; during LD entrainment any observed transients could more readily be attributed to the FEP. The author does acknowledge that reentrainment to the new mealtime may be affected by the LEP if the two pacemakers are coupled. The LD cycle was also used to assess the importance of this zeitgeber in circadian phasing in the rabbit. Although restricted feeding is the primary focus of the current thesis, the LD cycle was also of interest given its status as the dominant zeitgeber. It was expected that the LD cycle would result in a crepuscular pattern of activity. Based on previous research in the rabbit (see section 4.2.1), it was hypothesized that percentage nocturnality would be higher during *ad libitum* food access compared to restricted feeding because feeding occurred in the L phase.

7.2. METHOD

7.2.1. Animals and housing

Subjects were 12 female Dutch rabbits which ranged in age from 2.4 to 2.9 years. Weight ranged between 3.1kg and 4.4kg at the start of the experiment. Rabbits were housed under the conditions described in sections 5.2.1 and 5.2.3. Some of these rabbits had been used previously in the experiments reported in both chapters 6 and 8.

7.2.2. Procedure

Rabbits were maintained under a 14:10h LD cycle throughout the duration of the experiment. In the pre-phase, food and water were available *ad libitum* for 30 days. During restricted feeding, food access was limited to 4h per day. Rabbits were randomly assigned to two groups. Group A (*n*=6) fed in the early subjective day and group B (*n*=6) fed in the late subjective day. On day 51, food access was delayed by 5h in group A and advanced by 5h in group B. The new phase of food access was maintained for 35 days. In the post-phase, all rabbits were immediately deprived of food for three days. This was followed by 15 days *ad libitum* food access, three days food deprivation, and 20 days *ad libitum* food access. One subject in group B died during the experiment. The cause of death is unknown.

7.2.3. Design

The independent variables were phase (pre-, early subjective day, late subjective day, post-) and direction of phase shift (phase advance versus phase delay). Phase was a within-subjects variable and direction of the phase shift a between-subjects variable. The dependent variables included: percentage nocturnality for food and water counts, and general activity; PAD between activity onset and the onset of LD transition (measured as the interval between activity onset and the onset of the LD transition) for general activity; PAD between activity onset and onset of food access (measured as the interval between activity onset and the onset of food availability) for food counts, and; rate of reentrainment for food counts. Percentage nocturnality was defined as the percentage of total daily activity which occurred during the D phase of the LD cycle.

7.3. RESULTS

7.3.1. The Light Entrainable Rhythm

All rabbits showed a stable phase relationship to the LD cycle across all phases of the experiment for general activity. Actograms for general activity are presented in Figure 11. The majority of rabbits showed no or little activity associated with the LD cycle during the feeding schedule for food and water counts (see Figure 17), hence no analysis of these data are presented here. Only data for general activity will be considered in the following analysis.

In order to examine the effects of the independent variables on the timing of general activity across the day, data were summed in 30 minute bins over the final 10 consecutive days of each phase and averaged across all subjects. As seen in Figure 12, activity peaked around the LD and DL transitions across all phases of the experiment. With the exception of the early subjective day phase, there was no evidence that one peak was consistently larger than the other. In this phase, the 'lights off' peak was found to be larger than the 'lights on' peak. Individual records indicated that a general trend was apparent in that rabbits remained active throughout the D period during *ad libitum* food access. During restricted feeding, rabbits generally demonstrated a crepuscular pattern of activity, with two distinct activity bouts occurring around the LD and DL transitions separated by periods of relative inactivity.

A series of factorial ANOVAs were conducted to determine whether there were any significant main/interaction effects of phase and direction of the shift on the light entrainable rhythm. Percentage nocturnality and the PAD between activity onset and the LD transition served as the DVs. The final 10 days of each phase were used in the analysis. For subject #11, data for general activity were lost over the final 13 days of the pre-phase; days seven to 16 were used to calculate pre-phase scores for general activity in this rabbit. The total *n* was 11. Sample size for the two groups was six and five subjects for groups A and B respectively. ANOVAs were a mixed within-between model, weighting means by their sample size to adjust for unequal *n*. Tukey's HSD method was used to conduct *post-hoc* tests. Raw data and ANOVA summary tables are presented in Appendix C.

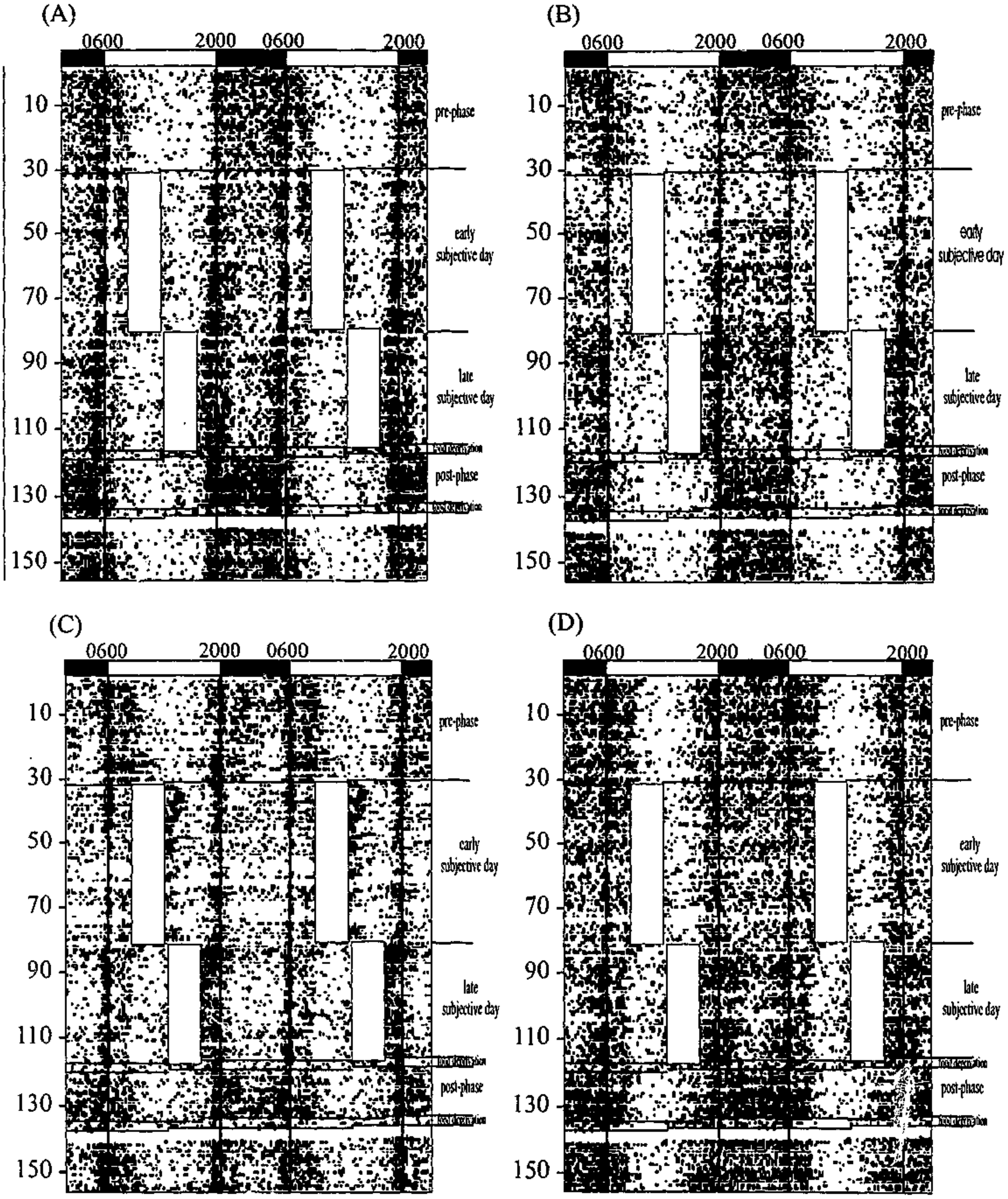


FIGURE 11. DOUBLE PLOTS OF GENERAL ACTIVITY FOR EIGHT RABBITS HOUSED UNDER A 14:10H LD CYCLE AND EXPOSED TO A FEEDING SCHEDULE. (A) SUBJECT #1; (B) SUBJECT #2; (C) SUBJECT #4; (D) SUBJECT #5; (E) SUBJECT #6; (F) SUBJECT #7; (G) SUBJECT #9; (H) SUBJECT #10. EACH LINE REPRESENTS 48 CONSECUTIVE HOURS PLOTTED IN 15 MINUTE BINS FROM LEFT TO RIGHT. EACH DAY IS PLOTTED ON THE RIGHT SIDE OF THE ACTOGRAM, AND THEN REPLOTTED AT THE BEGINNING OF THE FOLLOWING LINE. THE DARK PERIOD IS INDICATED BY HEAVY BLACK LINES. FOOD ACCESS IS DEPICTED BY HOLLOW VERTICAL LINES. EXPERIMENTAL PHASES ARE SHOWN ON THE RIGHT SIDE OF THE ACTOGRAM. DAYS OF THE EXPERIMENT ARE SHOWN ON THE LEFT SIDE OF THE ACTOGRAM; THESE CORRESPOND TO THE RIGHT SIDE OF THE ACTOGRAM. TIME OF DAY IS INDICATED AT THE TOP OF THE ACTOGRAM.

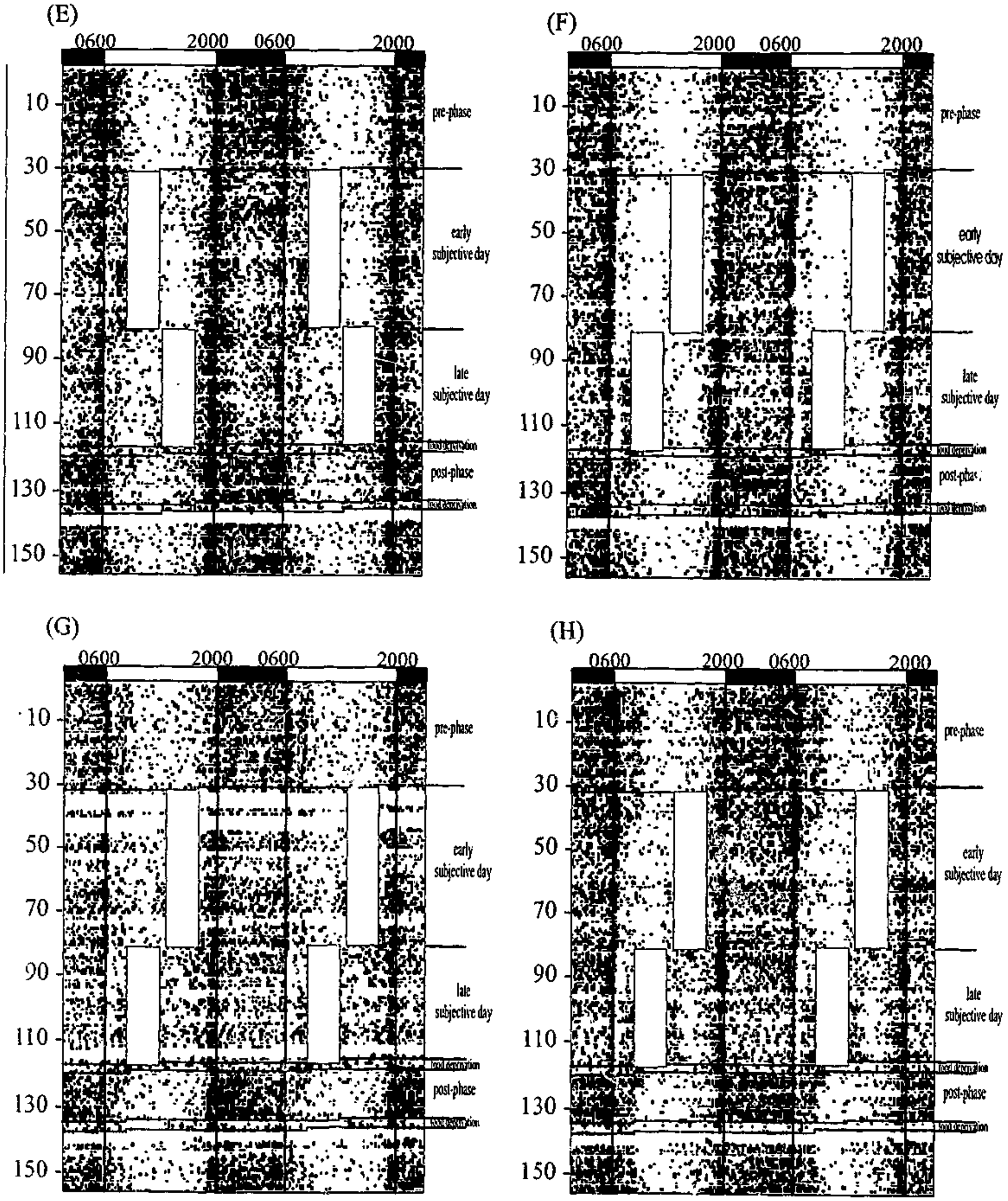


FIGURE 11 (CONT.)

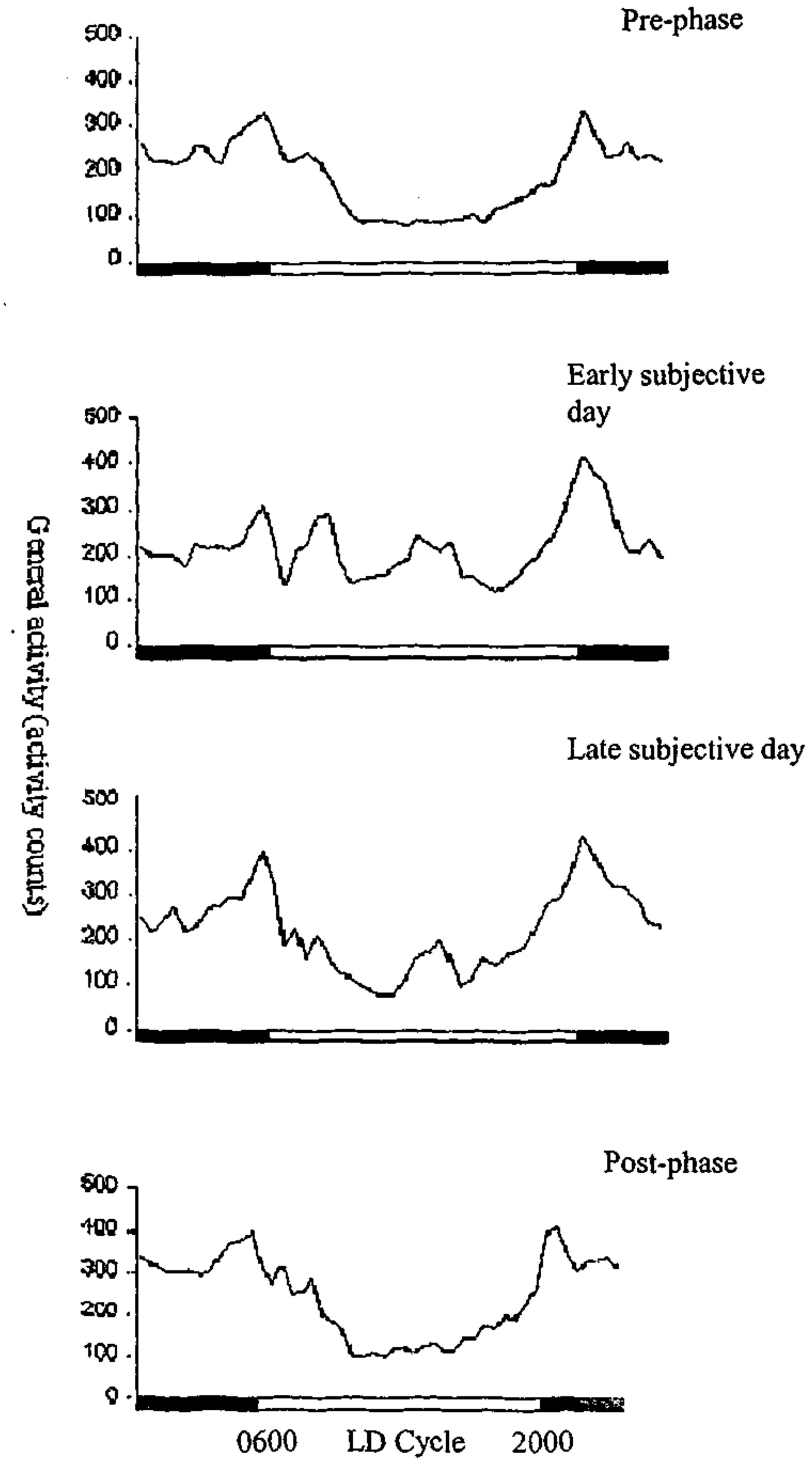


Figure 12. Mean activity counts by phase (from top to bottom: pre-, early subjective day, late subjective day, post- phase) for general activity. Data were summed in 30 minute bins over the last 10 consecutive days of each phase for each rabbit, and then averaged across all subjects. Hours of darkness are indicated by the heavy black lines at the bottom of each figure.

Means and standard errors for percentage nocturnality as a function of phase and direction of the shift for general activity are shown in Figure 13.

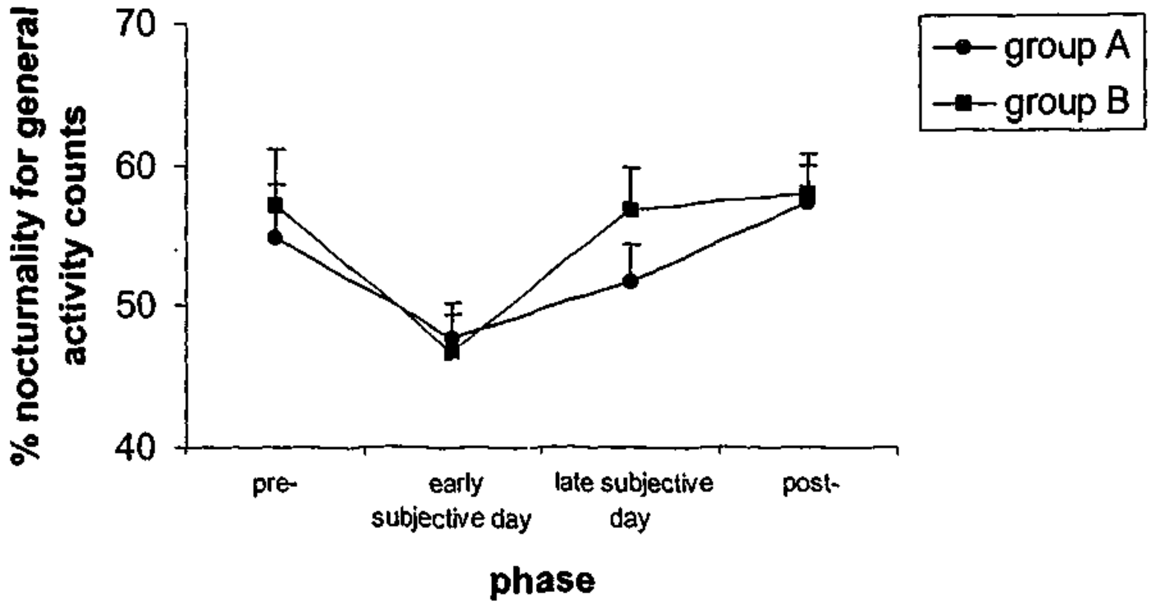


FIGURE 13. MEAN (+*SE*) PERCENTAGE NOCTURNALITY AS A FUNCTION OF PHASE AND DIRECTION OF SHIFT FOR GENERAL ACTIVITY.

Statistical analysis by two-way ANOVA indicated a significant main effect for phase, *F*(3,27)=19.28, *p*<.01. Tukey's HSD tests showed that percentage nocturnality was significantly lower during the early subjective day phase (*M*=47.31, *SE*=1.79) compared to the other phases (pre *M*=55.99, *SE*=1.71; late subjective day *M*=54.31, *SE*=1.91; post *M*=57.61, *SE*=1.96) which did not differ significantly from each other, *p's*<.05. The main effect for direction of shift was non-significant. There was no significant interaction effect.

Means and standard errors for percentage nocturnality as a function of phase and direction of the shift for food counts are shown in Figure 14.

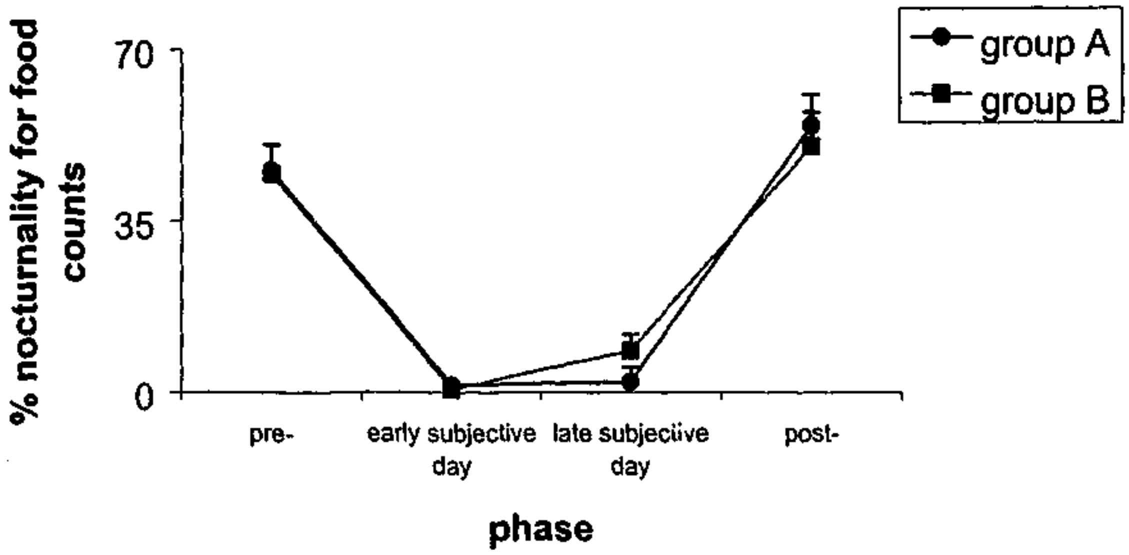


FIGURE 14. MEAN (+*SE*) PERCENTAGE NOCTURNALITY AS A FUNCTION OF PHASE AND DIRECTION OF SHIFT FOR FOOD COUNTS.

Statistical analyses by two-way ANOVA indicated a significant main effect for phase, *F*(3,27)=91.6, *p*<.01. Tukey's HSD tests showed that percentage nocturnality was significantly higher during both the pre- (*M*=45.1, *SE*=3.96) and post- (*M*=52.4, *SE*=4.61) phases compared to the early- (*M*=1.05, *SE*=0.37) and late- (*M*=5.32, *SE*=2.26) subjective day phases, *p*'s<.05. All other differences were non-significant. The main effect for direction of shift was non-significant. There was no significant interaction effect.

Means and standard errors for percentage nocturnality as a function of phase and direction of the shift for water counts are shown in Figure 15.

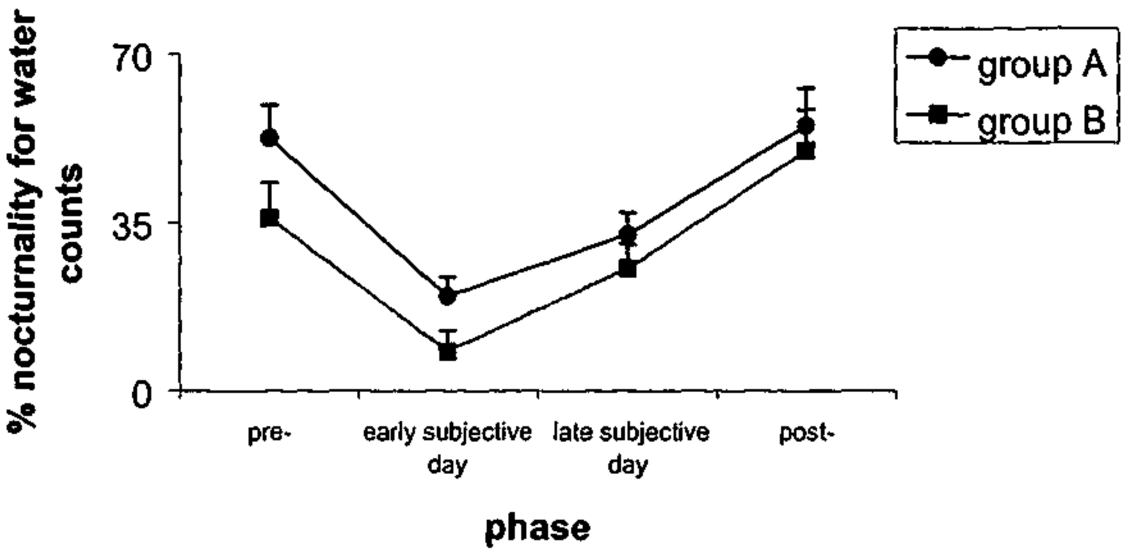


FIGURE 15. MEAN (+*SE*) PERCENTAGE NOCTURNALITY AS A FUNCTION OF PHASE AND DIRECTION OF SHIFT FOR WATER COUNTS.

Statistical analysis by two-way ANOVA indicated a significant main effect for phase, $F(3,27)=20.6$, $p<.01$. Tukey's HSD tests showed that percentage nocturnality was significantly higher during both the pre- ($M=44.22$, $SE=5.01$) and post- ($M=52.5$, $SE=5.7$) phase compared to the other phases, and significantly higher during the late- ($M=28.95$, $SE=3.42$) compared to the early- ($M=13.94$, $SE=2.93$) subjective day phase, p's<.05. All other differences were non-significant. The main effect for direction of shift was non-significant. There was no significant interaction effect.

Only data for general activity were used in the following analysis, since the majority of rabbits showed little or no feeding and drinking associated with the LD cycle during the feeding schedule. Pearson's product-moment correlation indicated that correlations between raters' estimates of PAD between activity onset and the LD transition were highly significant ($r=.73$, $p<.01$) and no systematic differences between raters' estimates were found. The decision was therefore made to randomly select the data from one rater for use in the following analysis. Mean and standard error for PAD as a function of phase and direction of the shift for general activity are shown in Figure 16.

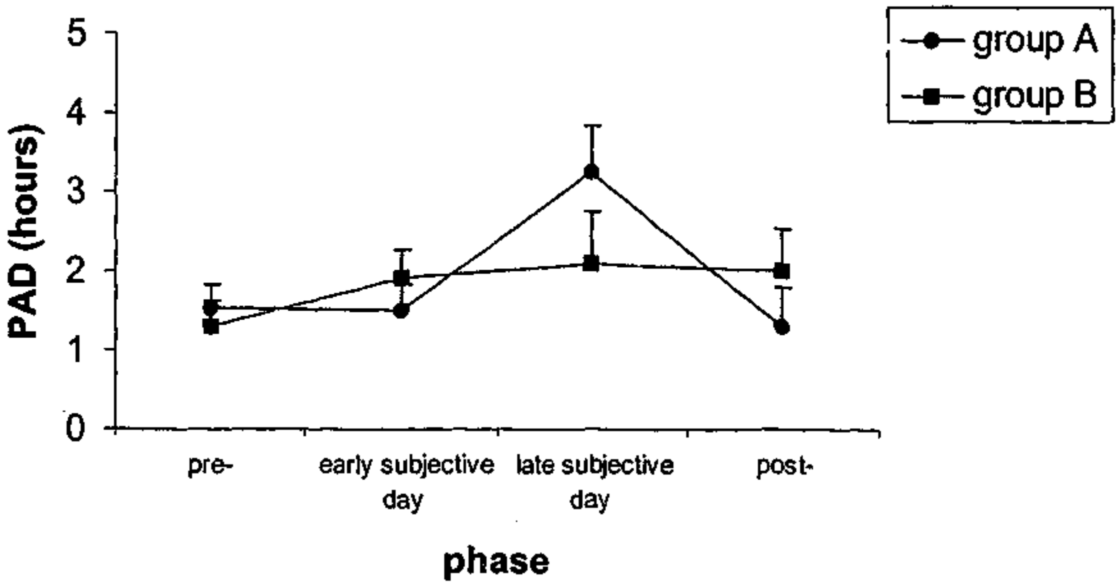


FIGURE 16. MEAN (+*SE*) PAD BETWEEN ACTIVITY ONSET AND THE ONSET OF THE LD TRANSITION AS A FUNCTION OF PHASE AND DIRECTION OF SHIFT FOR GENERAL ACTIVITY.

Statistical analysis by two-way ANOVA indicated a significant main effect for phase, $F(3,27)=3.04$, $p<.05$. Tukey's HSD tests showed that PAD was significantly higher during the late subjective day phase ($M=2.68$, $SE=0.42$) compared to the pre-phase ($M=1.42$, $SE=0.21$), $p<.05$. No other significant differences were found (early subjective day $M=1.7$, $SE=0.24$; post $M=1.65$; $SE=0.37$). The main effect for direction of shift was non-significant. There was no significant interaction effect.

7.3.2. The feeding entrainable circadian rhythm

The actograms indicated that general activity peaked prior to the feeding schedule for all rabbits fed in the early subjective day (see Figure 11). Visual inspection of the actograms however, revealed high levels of general activity during this period in the pre-phase. In order to determine whether the feeding schedule resulted in a further increase in activity during this period, activity scores obtained between 700h and 900h were summed; only data from the final 10 days were included. This was done separately for the pre- and early subjective day- phase. In almost all rabbits, activity levels were virtually unchanged between the pre- and early subjective day- phase. It was concluded that the activity increase prior to the meal in the early subjective day could not be attributed to the feeding schedule. It was thus decided not to consider these data in the following discussion.

The presence of anticipatory activity was established through visual inspection of graphical distributions of the data for food counts. The majority of the rabbits demonstrated anticipatory food count activity to at least one mealtime. Five rabbits satisfied the criteria for anticipatory activity to both mealtimes. Actograms of some of these subjects are presented in Figure 17.

A repeated measures *t*-test was performed to determine the effect of time of food access on the PAD between activity onset and the onset of food access. Only data from those rabbits showing anticipatory activity to both mealtimes were included. Two independent raters were used to estimate PAD for these rabbits. Inter-rater reliability was examined using Pearson's product-moment correlations; the inter-rater correlation was highly significant ($r=.91, p<.01$). The decision was therefore made to use data from one rater in the following analysis. Scores ranged from 1.25h to 3h, with an overall mean of 1.98h ($SD=0.56$). Mean PAD was 1.65h ($SE=0.15$) and 2.3h ($SE=0.26$) for the early- and late- subjective day phases respectively. Although a strong trend was suggested in the data, no statistically significant effects were found, $t(4)=2.08, p > .05$. Raw data are presented in Appendix C.

The actograms for food counts were examined visually in order to identify transients. Following a phase delay of food access, there was no evidence of transients either in activity onsets or activity offsets. Three of these subjects remained active until food

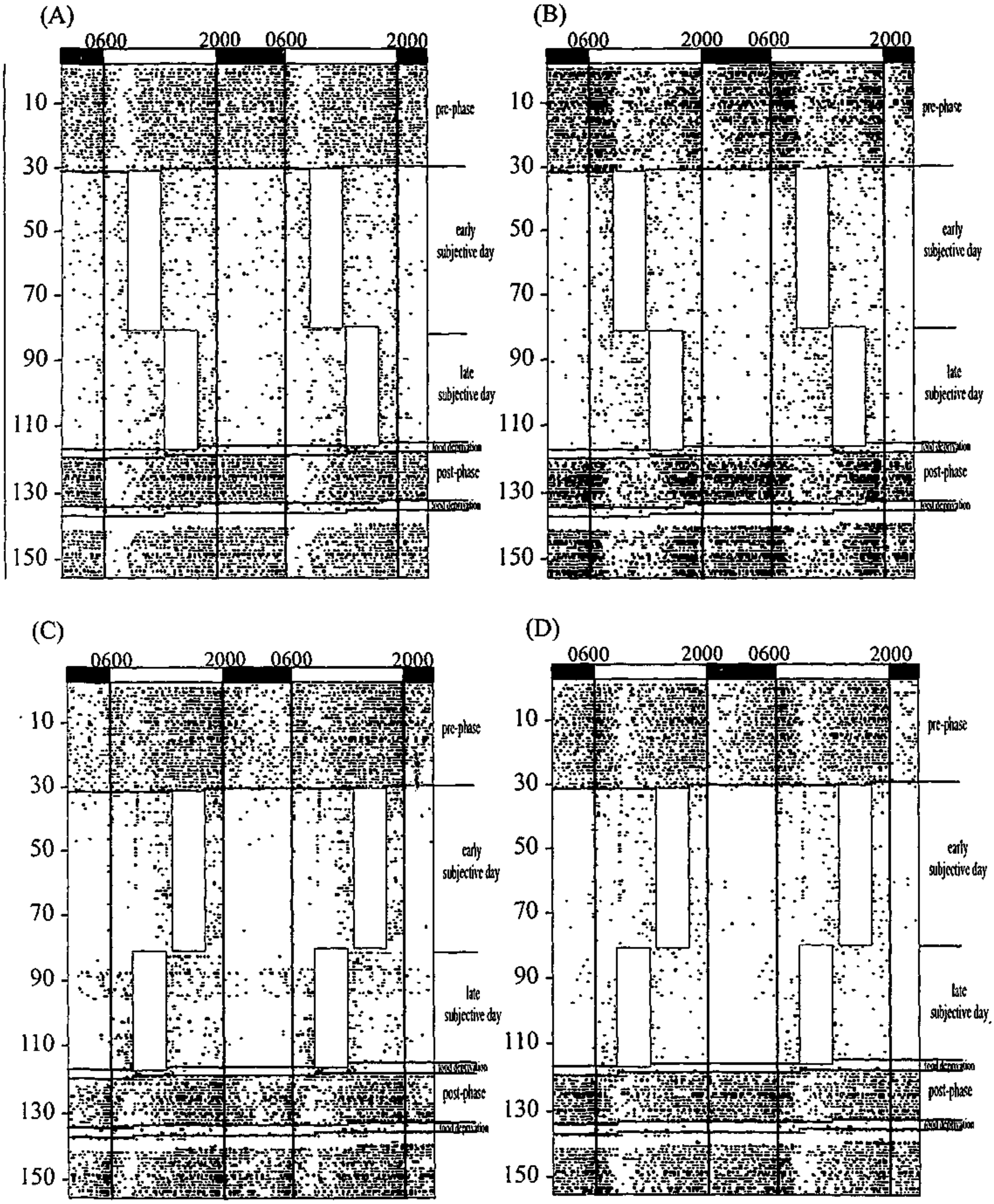


FIGURE 17. DOUBLE PLOTS OF FOOD COUNTS FOR FOUR RABBITS SHOWING ANTICIPATORY ACTIVITY IN BOTH THE EARLY SUBJECTIVE DAY AND THE LATE SUBJECTIVE DAY. (A) SUBJECT #2; (B) SUBJECT #5; (C) SUBJECT #9; (D) SUBJECT #10. CONVENTIONS AS FOR FIGURE 11. ALL RABBITS SHOW RESIDUAL ACTIVITY DURING THE FIRST DEPRIVATION SESSION.

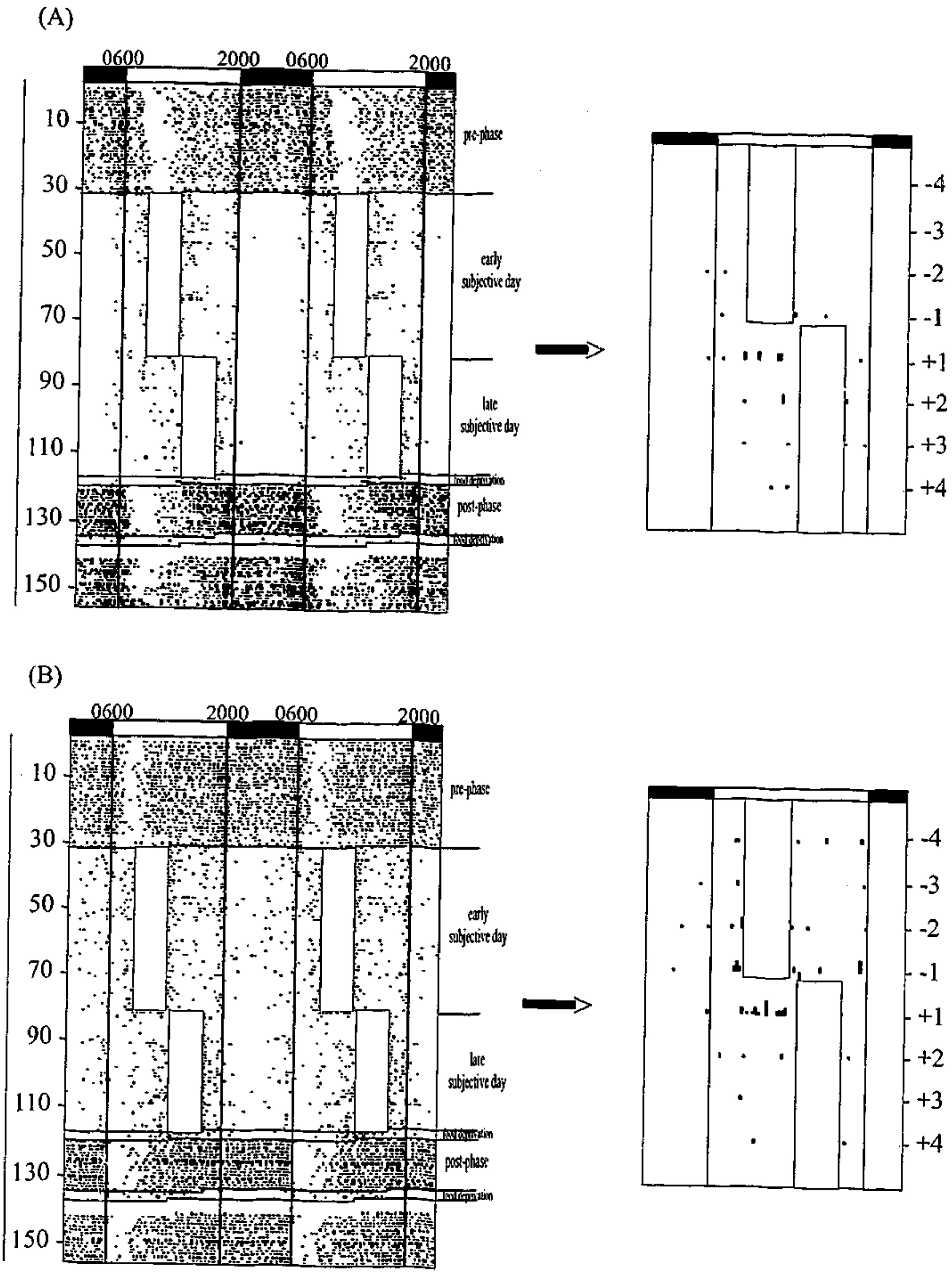


FIGURE 18. DOUBLE PLOT AND SINGLE PLOT DETAILS OF FOOD COUNTS FOR TWO RABBITS EXPOSED TO A 5H PHASE DELAY OF FOOD ACCESS. (A) SUBJECT #1; (B) SUBJECT #2. FOLLOWING THE PHASE SHIFT, THESE RABBITS REMAINED ACTIVITY UNTIL THE NEW PHASE OF FEEDING. RESIDUAL ACTIVITY DURING FOOD DEPRIVATION CAN BE SEEN IN SUBJECT #2. CONVENTIONS AS FOR FIGURE 11. DAYS BEFORE AND AFTER THE PHASE SHIFT ARE SHOWN ON THE RIGHT SIDE OF THE SINGLE PLOTS.

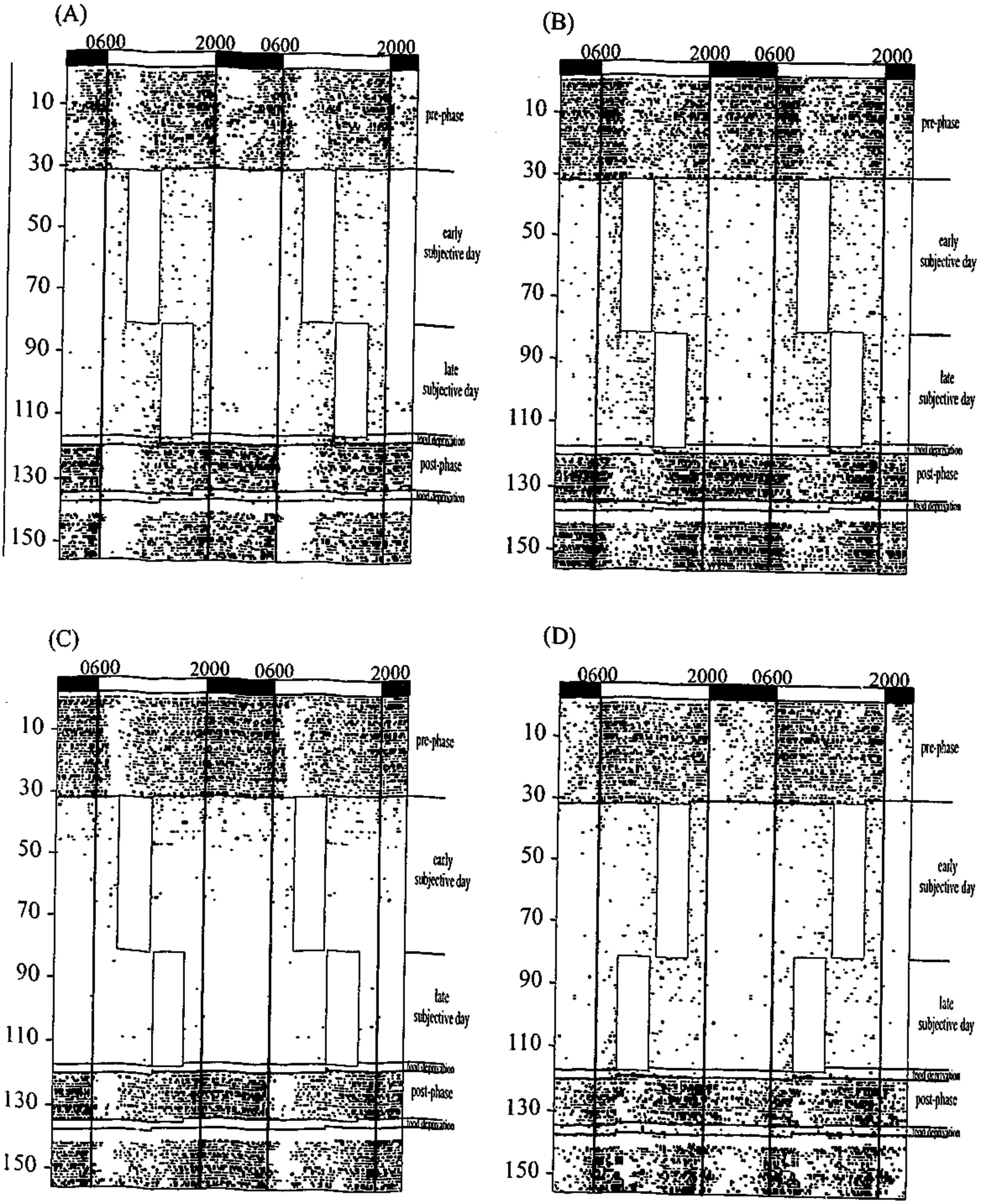


FIGURE 19. DOUBLE PLOTS OF FOOD COUNTS FOR FOUR RABBITS EXPOSED TO A 5H PHASE SHIFT OF FOOD ACCESS. (A) SUBJECT #3; (B) SUBJECT #5; (C) SUBJECT #6; (D) SUBJECT #11. CONVENTIONS AS FOR FIGURE 11. NO TRANSIENTS CAN BE SEEN. RESIDUAL ACTIVITY DURING THE FIRST DEPRIVATION SESSION CAN BY SEEN FOR SUBJECT #'s 3, 5, AND 11.

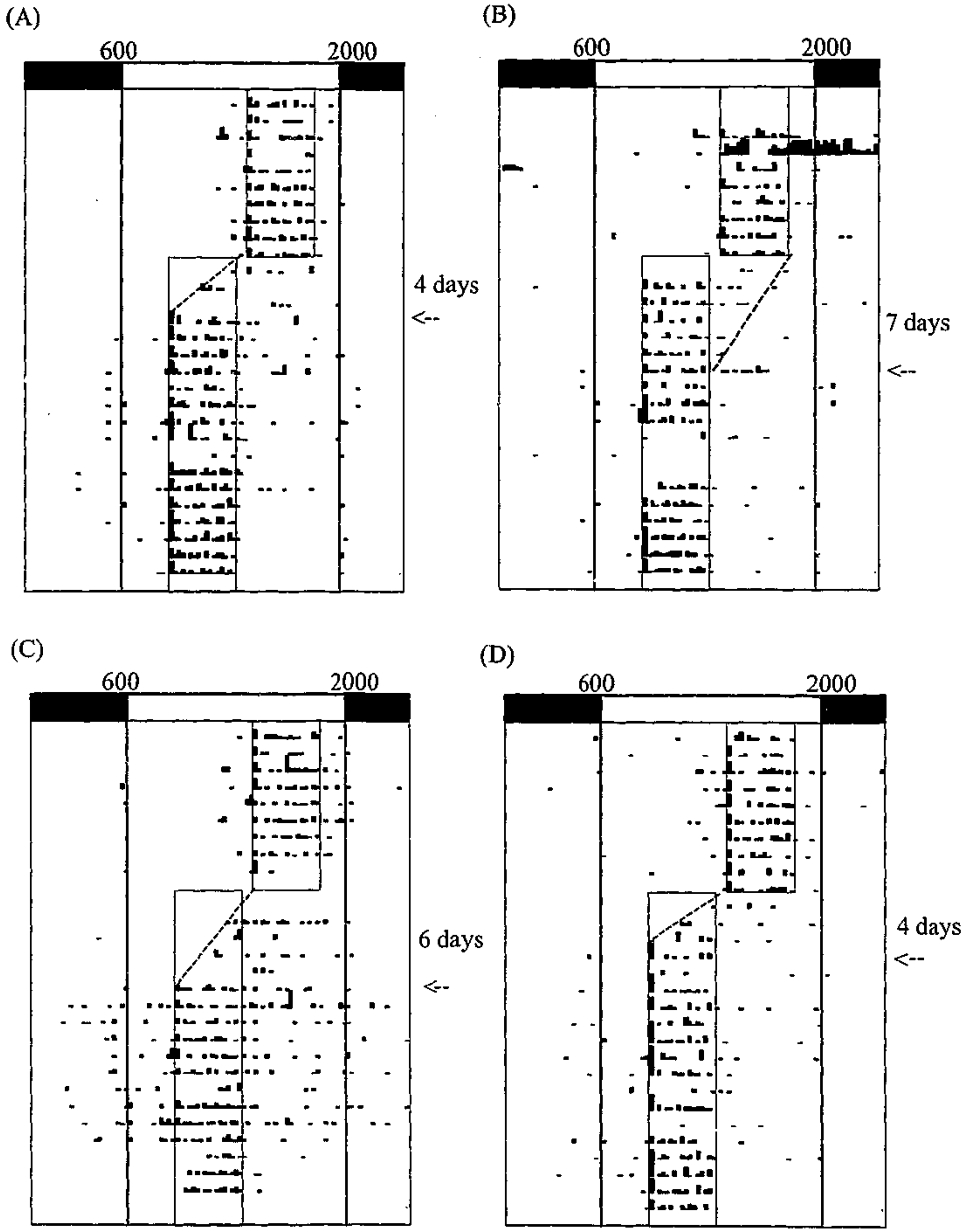


FIGURE 20. SINGLE PLOTS OF FOOD COUNTS SHOWING ADVANCING TRANSIENTS IN FOUR RABBITS. (a) SUBJECT #7; (b) SUBJECT #8; (c) SUBJECT #9; (d) SUBJECT #10. TRANSIENTS ARE INDICATED BY DOTTED LINES. THE DURATION OF REENTRAINMENT IN EACH CASE IS SHOWN ON THE RIGHT SIDE OF THE ACTOGRAMS. ARROW HEADS REPRESENT END OF REENTRAINMENT.

became available at the new mealtime. Representative actograms of these rabbits are presented in Figures 18 and 19.

Advancing transients in food counts were observed following a phase advance of food access in four of five rabbits. The actograms of these subjects are presented in Figure 20. These rabbits reentrained over a period of four to seven days. Small advances in activity onset were evident in the actograms of three of these rabbits. Advancing transients in activity offset were less clear and occurred in one rabbit only. For this rabbit, activity onset appeared at the new phase position almost immediately without intervening transients. A similar pattern of activity was found in the remaining rabbit in that activity appeared at the new mealtime without intervening transients.

Visual inspection of the actograms for food counts indicated that nine of 11 rabbits displayed residual activity at the former phase of feeding during the first food deprivation session. Residual activity did not persist during the second deprivation session following a period of *ad libitum* food access.

7.4. DISCUSSION

7.4.1. The light entrainable rhythm

In the first part of this analysis, it was intended to obtain some general chronobiological data for the rabbit. The aim was to examine the effects of LD cycles on the light entrainable rhythms in this species. It was also aimed to determine whether the temporal distribution of activity under LD conditions could be altered by a feeding schedule.

The results of this study are consistent with previous research demonstrating stable entrainment to LD cycles in the rabbit and suggest that a 14:10h LD cycle has a potent effect on the activity rhythms of this species. As expected, across all phases, rabbits showed a bimodal distribution of general activity over the night, with peaks occurring at lights on and lights off. In general, these results support previous research and suggest that the rabbit is crepuscular (Jilge & Stahle, 1984; Van-Hof et al., 1963; Van Hof-Van Duin, 1971). The present findings are also consistent with past findings in that the LD peak was larger than the DL peak when food access was limited to a few hours in the early subjective day, but not in the other phases. It should be noted that previous results have been inconsistent, with some investigators reporting a larger LD peak, while others have noted a larger DL peak, and others no difference at all (Jilge & Stahle, 1984; Van-Hof et al., 1963; Van Hof-Van Duin, 1971). The reason for the conflicting data is unclear, however, methodological differences may account for some of the variability in findings (see below). While some of these investigators argue that masking is operating in the data, others disagree and suggest that the evidence lends support to the two oscillator model originally proposed by Pittendrigh and Daan (1976b), and later applied by Bobbert and Bruinvels (1986) to the rabbit. According to this model, the two oscillators of the LEP become entrained to the LD cycle such that one oscillator is coupled to each of the transitions.

Rabbits were more active nocturnally than diurnally under *ad libitum* conditions for general activity. Mean percentage nocturnality was found to be lower for the other DVs, with scores approximating 50%. These findings are reasonably consistent with the results of other studies in the rabbit, particularly given the methodological inconsistencies (Gatterman, as cited in Jilge & Stahle, 1984; Horton et al., 1975; Jilge, 1991; Jilge & Stahle, 1984, 1993). For example, a number of differences across studies are apparent, including measuring devices, group versus individual housing, pre-experimental adaptation time, light intensity, and sound isolation of the experimental room. The major difference between the present study and past studies included the use of a 14:10h LD cycle in the present study rather than a 12:12h LD cycle. The implications of this are unclear, but one possible effect is that there are relatively more daylight hours in which to express behaviour. Spiteri (1982) however, found that percentage nocturnality for feeding decreased by only 5% when the L phase was extended from 12h to 14h. It is interesting to note that studies with the rat have yielded higher percentages than those in the present study, with estimates usually >70% for general activity, feeding and drinking (Armstrong, 1980; Greenwood et al., 1980; 1981; Johnson & Johnson, 1990; Spiteri, 1982; Zucker, 1971). This is not unexpected given that the laboratory rabbit is crepuscular and is frequently cited as being active during the L period (Horton et al., 1975; Jilge & Stahle, 1984; Kennedy, 1994).

The present findings indicate that the laboratory rabbit is less strictly nocturnal than its wild counterpart. One possible explanation for this finding is that the laboratory rabbit is not subject to the same survival pressures as the wild rabbit and has reacted by becoming increasingly diurnal. Viewed from an ecological perspective, the above-cited activities can be described as endogenously controlled avoidance behaviour to predation at unsafe times. An alternative explanation is that extraneous factors had a direct effect on activity during the L phase. For instance, the higher levels of diurnal activity in the present study could be attributed to the presence of technicians and maintenance of rabbits only during the L phase.

Percentage nocturnality varied markedly across the experiment. Consistent with the hypotheses, it was found that percentage nocturnality in food counts was lowest during the feeding schedule and significantly higher during *ad libitum* food access. In other words, food counts showed a marked decrease from baseline to the feeding schedule, changed little across the feeding schedule, and reverted to baseline levels on removal of the feeding schedule. The fact that percentage nocturnality was lowest during scheduled feeding is presumably the result of the forcing effect of the meal. The results were similar for water counts, whereas general activity appeared to be less affected by the feeding schedules than the other DVs. The results indicated that general activity was generally stable across the experiment, with only one exception. Percentage nocturnality in general activity was lowest during food access in the early subjective day, but scheduled feeding in the late subjective day had little effect on the temporal distribution of general activity. The percentage nocturnality scores obtained in the current study were <50% for feedings occurring in the early subjective day, while scores exceeded 50% for the other phases. These findings are difficult to interpret, but suggest that while the rabbit may become less nocturnal during scheduled feeding, it does not display a complete reversal of activity.

Despite the limitations inherent in comparing across DVs, these results appear to suggest a differential effect of scheduled feeding on feeding, drinking, and general activity. The stronger influence of feeding schedules on the temporal distribution of food and water intake compared to general activity has been documented by Spiteri (1982). The fact that general activity appeared to be least affected is consistent with the view that general activity occurs more independently of the feeding rhythm than does drinking, perhaps because general activity is less tightly coupled to the rhythm of food intake (Panskepp & Krost, 1975). This is supported by findings demonstrating a close temporal relationship between food and water intake (Fitzsimons & Le Magnen, 1969; Kissileff, 1969; Siegel & Stuckey, 1947).

PAD between activity onset and the LD transition for general activity varied across stages of the experiment. PAD during scheduled feedings in the late subjective day was significantly higher compared to the pre-phase, and higher than in the other phases, although did not differ significantly from either of them. The mechanism of action is unclear, but two mechanisms have been proposed in the empirical literature. One possible mechanism for such an effect is an internal coupling between the LEP and the FEP: the feeding schedule entrains the FEP which in turn via coupling affects the LEP, modifying the PAD of entrainment (Stephan, 1986a,b,c). Alternatively, changes in PAD may be due to the direct effects of the feeding schedule on the overt rhythms. Both of these explanations fail to account for the lack of effect of scheduled feeding on PAD in the early subjective day. To the author's knowledge, there are no published data on this topic and further research is thus needed.

A potential limitation of this study was the fact that masking effects may have been operating in the data. There is some evidence that activity may be negatively masked by complete darkness in a variety of species, including the rabbit. For instance, Kennedy et al. (1994) used a 14:10h LD cycle and observed a crepuscular pattern of activity in the majority of rabbits. When released into constant conditions, however, activity onset appeared to originate from the D phase rather the LD and DL transitions in some rabbits, suggesting that masking was operating in the data. It is unclear whether the data in the present study represent entraining effects or masking effects. Future studies could observe rabbits under constant conditions following exposure to a LD cycle in order to distinguish between these effects.

7.4.2. The feeding entrainable circadian rhythm

The aim of the second part of this analysis was to examine the effects of restricted feeding schedules on the feeding entrainable rhythms in the rabbit, with a view to demonstrating that feeding schedules act as an effective zeitgeber in a herbivorous species. While previous studies have made it clear that the rabbit develops anticipatory activity in response to feeding schedules, this conclusion must be based on the phenomena of persistence in the absence of periodic input and re-entrainment through transients. To this end, the effects of 5h phase shifts of food access were examined, and comparisons between advance and delay shifts made. The response of anticipatory activity to food deprivations was also assessed.

The results of this study support the view that feeding schedules have a zeitgeber effect on activity in the rabbit. The majority of animals demonstrated anticipatory activity to at least one mealtime, with five of 11 rabbits anticipating both mealtimes. Individual estimates of PAD between activity onset and the onset of food access ranged between 1h 15 minutes and 3h, with a mean of almost 2h. These results are generally consistent with those of other researchers, although slightly higher PADs have been cited in omnivores and carnivores (Aschoff et al., 1983; Kennedy et al., 1995; Ruis et al., 1989; Stephan, 1981, 1986b; Stephan & Becker, 1989). Reasons for this discrepancy will be discussed in chapter 8. These results support the view that there exist cross-species similarities in circadian timing, but also point to cross-species differences between omnivores, carnivores, and herbivores, with smaller PADs being apparent in the latter group. It should be noted that activity onsets in the present study were much less precise than those reported in omnivores and carnivores and hence interpretations based on PAD should be made with caution. Despite this however, inter-rater reliability was high.

No significant effects of mealtime on PAD were found. It should be noted however, the lack of effect of this variable may have been caused by the small sample used in the present study, resulting in a lack of power associated with the test. The results did indicate a trend towards smaller PADs in rabbits fed in the early- compared to the late- subjective day. This finding is consistent with studies demonstrating an effect of PAD between the LEP and the FEP on anticipatory activity (Honma et al., 1983; Stephan & Becker, 1989). These studies showed that the amount of anticipatory activity was reduced as the free-running rhythm crossed food access and the persistence of anticipatory activity was positively correlated to its PAD to the free-running light entrainable rhythm when the feeding schedule was removed. The most likely explanation for these findings is that the two pacemakers that drive these rhythms are coupled, and at certain phase relations the pacemakers may interact with each other. The present findings provide only limited support this hypothesis and suggest that the PAD of entrainment to restricted feeding may change at various phase angles between the two pacemakers.

While anticipatory activity is the primary focus of this section of the discussion, a finding worthy of mention was the result that the light-entrainable rhythms appeared to be effected by the feeding schedule. For food counts, there was little or no LD-related activity during restricted feeding, with the majority of rabbits showing a single bout of activity associated with the feeding schedule. A slightly different pattern of activity occurred for drinking, with two bouts of activity being apparent (one associated with each zeitgeber), however, activity associated with the LD cycle was greatly diminished compared to baseline. As indicated in section 7.3.1, data for general activity was difficult to interpret due of the overlap between the light entrainable rhythm and the feeding entrainable rhythm, however, in all cases, the light entrainable rhythm was clearly visible during the feeding schedule. In 1986(a) Stephan proposed that both the LEP and the FEP compete for control over activity during scheduled feeding, so that activity becomes temporarily uncoupled from the LEP. This explanation is unlikely here since a crepuscular pattern of activity persisted throughout the study for general activity. In light of evidence supporting a strong temporal relationship between general activity, food counts, and water counts, a more likely explanation is that the LD-entrained rhythms remained coupled to the LEP, but LD food- and water- related activity was being masked by the feeding schedule.

In the present study the delivery and removal of food was under automated control. This is an important issue because noise cues associated with the firing of the solenoid locks may have been operating in the data. Support for this theory comes from the finding that two bouts of activity, one associated with each meal, were usually present in the data during the feeding schedule. One bout was generated by the presence of food, but a second, much smaller component of activity was most likely due to noise cues generated by the equipment in those rabbits for which no food was present at this time. It is important to note that activity generated by the presence of food was most pronounced and phase-led the noise cues, suggesting that the presence of food was having an effect over and above that which could be attributable to noise cues.

7.4.3. Phase shifts of food access

The results of the present study indicated that phase shifts of food access induce gradual reentrainment of feeding related activity in a series of transient cycles, thus giving support to the zeitgeber hypothesis. These results are consistent with those of rat studies however, transients reported in the present study were less clear and more ambiguous than those obtained in omnivores (Stephan, 1984, 1986b, 1992a, 1997). Thus the following conclusions should be treated with caution. Replication of this study is required.

The fact that reentrainment was achieved by advancing transients for food counts is inconsistent with the hypotheses. The process of reentrainment to feeding schedules is typically achieved by delaying transients in the rat, although advancing transients following a phase advance of food access have been reported by Stephan (1984, 1986b). The present results are generally consistent with those of Stephan in showing that a period of less than 10 days generally is required to complete the phase shift. The findings are contrary to those of Jilge et al. (1987) who found that the rabbit reentrained immediately without intervening transients after a 6h advance phase shift, while reentrainment to a 6h delay shift of food access was achieved by delaying transients. Although the conflicting data may be attributable to differences in methodology (e.g., size of the phase shift, ambient lighting conditions, registration devices, sound isolation of the experimental room, rabbit strain etc.), there is no evidence to suggest that such factors affect the reentrainment process. Based on the present findings of small advancing transients, it could be hypothesized that the PRC for food pulses has a small advance section; this would suggest that lower limit of entrainment does not deviate too far from 24h (see section 1.2). This issue will be addressed in chapter 8.

There was no evidence of transients in activity onset/offset for food counts following a phase delay of feeding time. This finding is inconsistent with the hypotheses and previous findings: Stephan (1984, 1992a,b) reported evidence of delaying transients in response to delayed food access. A common finding in the present study was that rabbits remained active until food become available after the delay shift. This finding is similar to the results of the 1984 study by Stephan. One possible explanation for this finding is that the termination of anticipatory activity is controlled by a passive or forced response to food availability. This has been suggested by Stephan, however, the fact that activity decreased before food was available at the displaced phase after an 8h shift illustrates that this is not the case. Another explanation for this effect is not readily apparent. However, it would be interesting to investigate the effects of larger meal phase shifts in the rabbit in order to replicate Stephan's findings.

A common finding of the present study was that food count activity appeared at the new phase position after one day without intervening transients following a delay phase shift. This finding is consistent with studies by both Jilge et al. (1987) and Stephan (1984, 1992a). It is possible to explain these results by considering a role for two oscillators in the regulation of feeding entrainable rhythms. According to this explanation, the feeding entrainable circadian system consists of two oscillators; one oscillator entrains to the new phase position, while activity is decoupled from the other oscillator at the previous phase position. Alternatively, rapid reentrainment could be the result of strong phase resetting. Both of these explanations seem unlikely however given the rapid rate of phase resetting of one day. A more likely explanation is that feeding schedules produce a strong masking effect in the rabbit.

7.4.4. Food deprivation

The results of the present study demonstrated that anticipatory activity persists in the absence of periodic input in the herbivorous rabbit. That residual meal-associated activity in food counts was found to persist during food deprivation is consistent with the hypotheses and past research (Bolles & Moot, 1973; Clarke & Coleman, 1986; Coleman et al., 1982; Mistlberger & Merchant, 1995; Rosenwasser et al., 1984; Ruis et al., 1989; Stephan, 1992b; Stephan et al., 1979b). The data agree also with the work by Clarke and Coleman (1986) who reported that meal-associated activity did not persist during *ad libitum* feeding. These and other researchers (see Mistlberger, 1994 for review) postulated that failure to persist under constant conditions could be viewed as the result of strong oscillator damping, or it could reflect uncoupling of the

FEP from the overt rhythms. The fact that meal-associated activity reappears during food deprivation after a week or more of *ad libitum* feeding in the rat would suggest that coupling between the FEP and overt rhythms is gated by the animal's motivational state. Alternatively, *ad libitum* feeding may mask the expression of anticipatory activity.

Residual meal-associated activity at the former mealtime appeared in almost all rabbits, despite the fact that some of them did not meet the criteria for anticipatory activity. This finding may suggest that the criteria used to define anticipatory activity was not liberal enough, since all rabbits demonstrating residual activity also displayed some activity prior to the feeding schedule. Alternative explanations are also available. While this effect may reflect differences between classes of species, it is also possible that this is a feature specific to the rabbit. For example, in the rabbit soft feces are expelled through the colon and consumed directly from the anus in a process known as cecotrophy (see section 4.1). The soft feces follow the same digestive process as normal feed. Some parts of intake may be recycled up to four times and depending on the type of food, the rabbit's digestive process may last from 18h to 30h, averaging 20h. Thus, the nature of the digestive system may create a situation where the gut is never empty and input of nutrients is essentially constant. This is an important issue, particularly if the expression of anticipatory activity is gated by the animal's motivational state; this may inhibit anticipatory activity in the rabbit. This is not the case during food deprivation in which an energy deficit is created. This is an important possibility and may suggest that the FEP is entrained but not expressed.

Meal-associated activity did not persist during food deprivation after 15 days of *ad libitum* feeding. These results are not consistent with those of a number of researchers who have noted residual activity to persist at the former phase of feeding for 50 days or more in food deprived rats, even if the deprivation trials are repeated after seven days or more of *ad libitum* feeding (Clarke & Coleman, 1986; Coleman et al., 1982; Rosenwasser et al., 1984; Ruis et al., 1989). The present results are difficult to interpret. The same explanations as before can be invoked to explain the present results. These theories have not been empirically tested and as Mistlberger (1994) has noted, choosing between them would require physiological analysis of the

FEP itself, if such a pacemaker can be localized. Other explanations have been proposed to explain why meal associated rhythms do not persist during deprivation, although these explanations seem less likely. For example, Mistlberger et al. (1990a) suggested a role for non-circadian factors such as weight and age. Findings regarding obese rats indicate a delayed generation and reduced amplitude of anticipatory activity (Persons et al., 1993). The rabbits in the present study, however, were not obese and appeared to be of normal size for rabbits of their age. Moreover the feeding schedule promoted weight loss. The fact that almost all rabbits displayed meal-associated activity during the first food deprivation session is inconsistent with these hypotheses.

7.4.5. Conclusion

The present findings point to a zeitgeber role for feeding schedules in the rabbit; rabbits displayed anticipatory activity in response to the feeding schedule; transients were apparent after a phase shift of food access, and; meal-associated rhythms persisted during food deprivation.

CHAPTER 8. CIRCADIAN LIMITS OF ENTRAINMENT TO FEEDING SCHEDULES IN THE HERBIVOROUS RABBIT

8.1. RATIONALE AND AIMS

Whereas a good deal of chronobiological data exist for the omnivorous rat, the rabbit belongs to a less explored class of species. The zeitgeber effects of feeding schedules are less well documented in herbivores compared to omnivores, and it is at present not clear whether periodic food availability is an effective zeitgeber in herbivorous species. The results of the previous study indicated that: (i) rabbits develop anticipatory activity in response to feeding schedules; (ii) anticipatory activity resets in a series of transient cycles after a phase shift of food access; (iii) when food is withheld for a few days, meal-associated activity persists at the former phase of feeding. While these results are consistent with a zeitgeber role for feeding schedules in the herbivorous rabbit, one of the criteria for identifying zeitgebers remains to be satisfied. As noted in the introduction (see section 1.1.7), a zeitgeber by definition should entrain circadian rhythms to a range of Ts, but only within certain limits.

Research in the omnivorous rat has demonstrated that entrainment is possible only within a limited range of feeding cycle Ts, usually ranging from 23h to 27h (e.g., Aschoff et al., 1983). These authors also found that the PAD between activity onset and the onset of food access is positively correlated with T. The results of this and other studies rule out the possibility that the effects of feeding schedules are governed by subtle 24h cues which cannot be controlled in the laboratory. Relevant research is detailed in section 2.3.5.

The range of entrainment in herbivores is unclear. While the effects of T cycles have been investigated in the hamster (Mistlberger, 1993a), the method used involved cycles of water availability in which water access was restricted to a few hours each day. The validity of this paradigm remains in question since it is not clear whether food and/or water serve as the zeitgeber. Using the rabbit as subject, Jilge and Stahle (1993) examined entrainment in relation to non-24h feeding schedules. However, this study was not designed to investigate non-circadian intervals. Rabbits were exposed to feeding cycle Ts of 23.5h and 24.1h and found to entrain. Thus, the absence of systematic study of the range of entrainment in herbivores creates difficulties in determining whether feeding schedules affect the underlying pacemaker; the lack of data highlights the importance of the present study. Past research suggests that the rabbit would provide an opportunity to investigate the range of entrainment in a herbivorous species.

The primary aim of this study was to examine the range of entrainment to cycles of food availability in the herbivorous rabbit. A secondary aim of this study was to explore the effects of restricted feeding on the free-running light entrainable rhythm. Rabbits were maintained under constant light and exposed to feeding cycle Ts of 21h, 23h, 24h, 27h, and 30h. Based on previous findings, it was hypothesized that entrainment would occur to feeding schedule Ts ranging from 23h to 27h. In view of Aschoff's (1983) findings, it was hypothesized that the PAD between activity onset and the onset of food access would increase with feeding cycle T. Based on data from omnivores, carnivores, and herbivores (see sections 2.4.2 & 3.2.2 & 4.2.4), changes in phase and tau of the free-running light entrainable rhythm were expected to occur in response to the feeding schedule. Given that some previous studies have found feeding schedules to entrain the free-running light entrainable rhythm in the rabbit, while others have not (see section 4.2.4), this aspect of the study was viewed as exploratory and specific hypotheses were not generated.

8.2. METHOD

8.2.1. Animals and housing

Subjects were 16 female Dutch rabbits which ranged in age from 1.5 to 2 years. Weight ranged between 3.4kg and 4.1kg at the start of the experiment. Rabbits were housed under the conditions described in sections 5.2.1 and 5.2.2. Some of these rabbits had been used previously in the experiment reported in chapter 6.

8.2.2. Procedure

Eight rabbits were exposed to the following sequence of feeding schedules: T=24h for 66 cycles with 3h food access per cycle; T=21h for 68 cycles with 3h food access per cycle, and; T=23h for 60 cycles with 3h food access per cycle.

Eight rabbits were exposed concurrently to the following sequence of feeding schedules: T=24h for 66 cycles with 3h food access per cycle; T=27h for 53 cycles with 4h food access per cycle, and; T=30h for 47 cycles with 4h food access per cycle. One rabbit in this group died during the experiment. The probable cause of death was kidney failure.

In order to explore the impact of restricted feeding (RF) on the light entrainable rhythm, animals were given *ad libitum* food access before and after each feeding schedule. All *ad libitum* sessions were approximately 30 days long, with one exception. *Ad libitum* 4 (see below) lasted for only 22 days.

8.2.3. Design

Phase was the independent variable with seven levels; *ad libitum* 1, 24h RF, *ad libitum* 2, 21h or 27h RF, *ad libitum* 3, 23h or 30h RF, and *ad libitum* 4. The dependent variables included: PAD between activity onset and the onset of food access; latency to anticipatory activity; anticipatory ratio, and; phase and tau of the free-running light entrainable rhythm. Activity was defined in terms of general activity, and food and water counts.

8.3. RESULTS

8.3.1. Entrainment to a 24h cycle of food availability

Mechanical failure on day 63 of restricted feeding resulted in high levels of feeding (and drinking) activity for almost all subjects which were inconsistent with the animal's normal level of activity. Thus these data were omitted from the following analyses on the basis of inaccurate data. The process described in section 5.5.3. was used to identify anticipatory activity. Visual inspection of graphical distributions indicated that many rabbits satisfied the criterion for anticipatory activity, with nine rabbits showing anticipatory activity in food counts. Representative actograms of these subjects are presented in Figure 21.

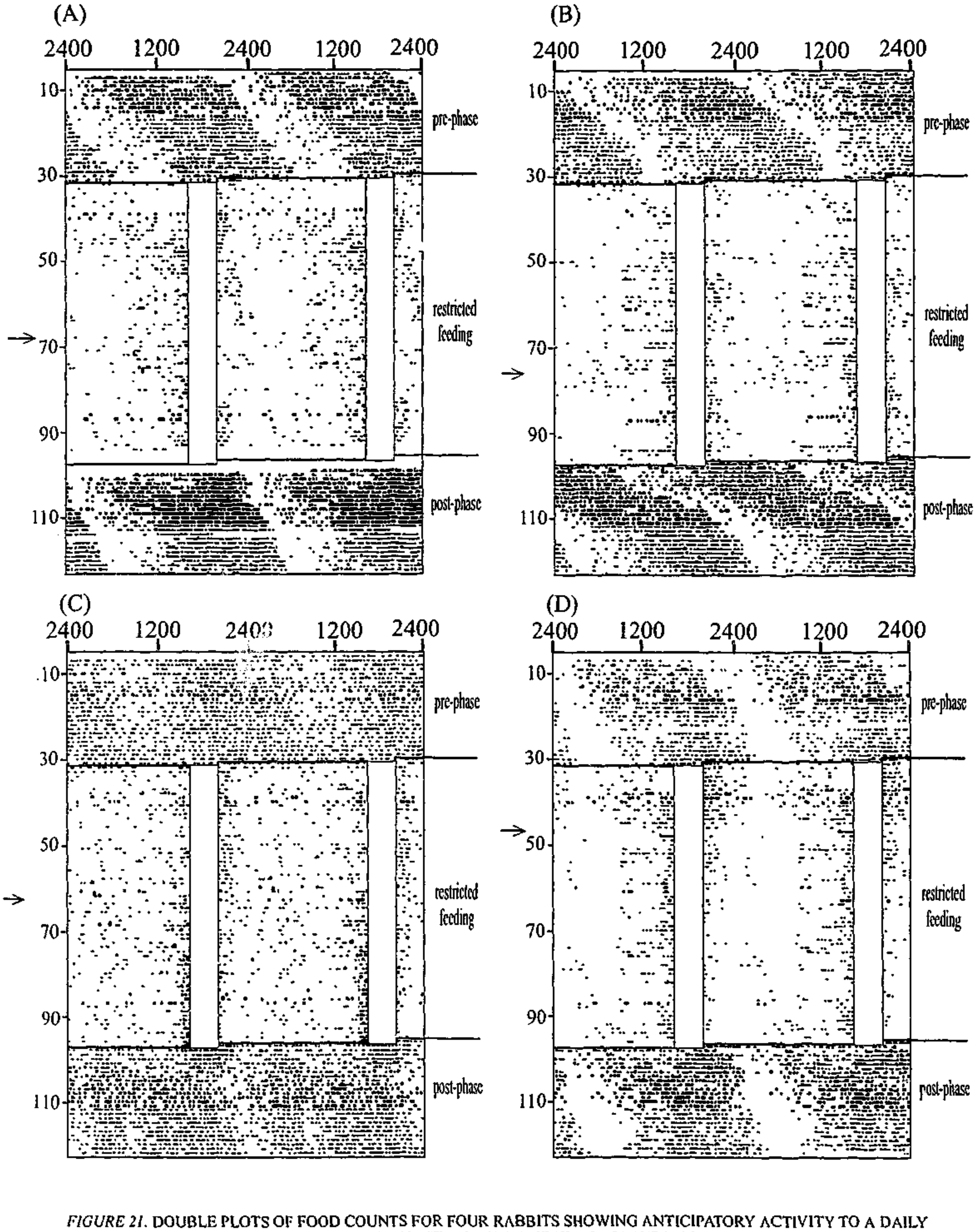


FIGURE 21. DOUBLE PLOTS OF FOOD COUNTS FOR FOUR RABBITS SHOWING ANTICIPATORY ACTIVITY TO A DAILY FEEDING SCHEDULE. (A) SUBJECT #8. (B) SUBJECT #10. (C) SUBJECT #12. (D) SUBJECT #15. CONVENTIONS AS FOR FIGURE 11. ARROW HEADS INDICATE EMERGENCE OF ANTICIPATORY ACTIVITY. HIGH LEVELS OF ACTIVITY CAN BE SEEN ON DAYS 86 (DAY 56 RF)AND 93 (DAY 63 RF) IN ALL PLOTS.

Two raters were used to estimate PAD between activity onset and the onset of food access. Inter-rater reliability was examined using Pearson's product-moment correlations. The results indicated a highly significant inter-rater correlation (r=.89, *p*<.01) and no systematic differences between raters' estimates were found. The data from one rater was therefore selected at random and is reported here. Raw data are presented in Appendix D. PAD ranged from 1h to 3h, with a mean of 1.61h (*SD*=0.61).

The effect of a daily feeding schedule on the latency to anticipatory activity was explored. Only data from those rabbits showing anticipatory activity were included. A total *n* of 9 was used. Anticipatory activity appeared after a mean of 38 days (*SD*=9.36). Individual estimates ranged between 16 and 46 days. Raw data are presented in Appendix D.

In order to maintain comparability to past research, anticipatory activity in the present study was quantified using Stephan's (1981) anticipatory ratio. The anticipatory ratio was computed by dividing the amount of activity during the 3h immediately prior to food access by the amount of activity recorded within the preceding 3h. Activity was defined in terms of food counts. The mean anticipatory ratios for all T cycles were calculated and are as follows: 0.9, 5.7, 8.9, 3.4, and 1.0 for T cycles of 21h, 23h, 24h, 27h, and 30h, respectively.

In order to ascertain whether the free-running light entrainable rhythm was influenced by aftereffects to the feeding schedule, tau for the pre- (*ad libitum* 1) and post- (*ad libitum* 2) phase was calculated for food counts. A line of best fit was placed along activity offsets over the final 20 days of each phase. This was done separately by each rater and the scores were then correlated. Inter-rater reliability was examined using Pearson's product-moment correlations; the inter-rater correlation was highly significant (r=.52, *p*<.01). Discrepancies between raters' estimates of tau were most likely attributable to the highly variable activity offsets. Data were averaged across both raters for use in subsequent analyses. Raw data are presented in Appendix D. Mean (+*SE*) estimates of free-running tau by phase are shown in Table 3.

TABLE 3.

TAU OF THE FREE-RUNNING LIGHT ENTRAINABLE RHYTHM BY PHASE FOR T=24H.

	Pre-phase	Post-phase
Mean	24.42	24.23
+*SE*	0.21	0.13

As seen in Table 3, tau changed between the pre- and post- phase. The majority of rabbits showed a shortening of tau across these phases. In these rabbits, changes in tau were in the direction of T. A lengthening of tau was observed in two rabbits. The remaining rabbit showed no changes at all across the experiment. A related samples *t*-test indicated a significantly longer tau in the pre-phase compared to the post-phase, $t(14)=3.02, p<.01$.

For eight rabbits, data were lost over the final days of the feeding schedule and the first two days of the post-phase due to equipment failure. These animals were excluded from the following analysis due to insufficient data. In order to determine whether entrainment of the free-running light entrainable rhythm had occurred, the actograms were inspected visually. Of the total seven rabbits, only one animal showed some, albeit weak evidence of entrainment to the feeding schedule. As seen in Figure 22, activity onset appeared to delay towards food access to become anticipatory activity and stable entrainment was maintained until the post-phase. Entrainment is indicated by the phase of the post-phase rhythm, as the rhythm free-ran from a phase consistent with the feeding schedule and not pre-entrainment. It should be noted that this rabbit free-run with a tau at (or at least very close to) 24h in the pre-phase. This is thus not a good demonstration of entrainment as tau-modulation by the feeding schedule is not shown.

Visual inspection of the actograms indicated that the free-running light entrainable rhythm was apparent during the feeding schedule in general activity and/or water counts for all rabbits, but was less discernable for food counts. The free-running rhythm was less discernable during the feeding schedule compared to the pre-phase, but a well- defined rhythm was reestablished during the post-phase. Representative

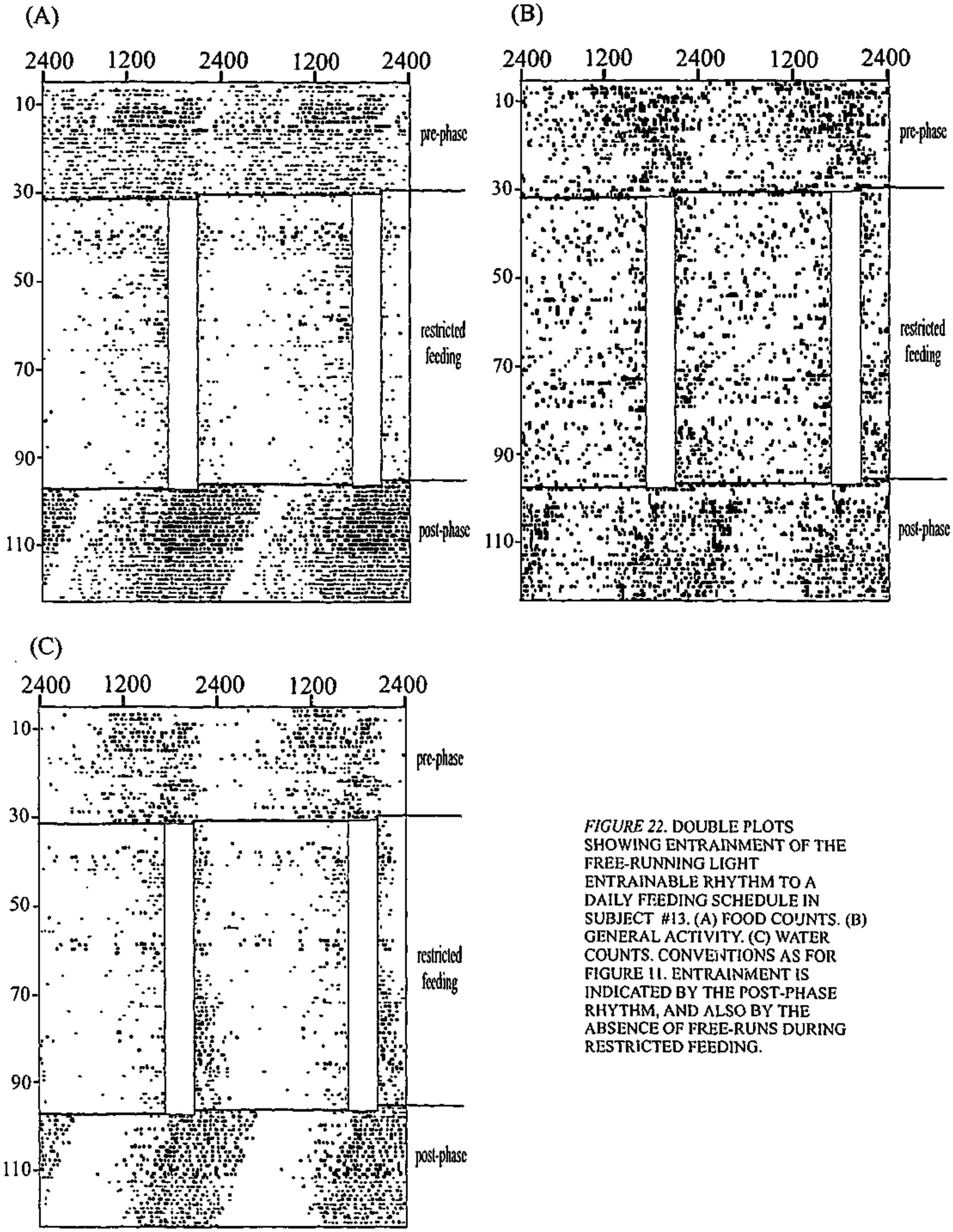


FIGURE 22. DOUBLE PLOTS SHOWING ENTRAINMENT OF THE FREE-RUNNING LIGHT ENTRAINABLE RHYTHM TO A DAILY FEEDING SCHEDULE IN SUBJECT #13. (A) FOOD COUNTS. (B) GENERAL ACTIVITY. (C) WATER COUNTS. CONVENTIONS AS FOR FIGURE 11. ENTRAINMENT IS INDICATED BY THE POST-PHASE RHYTHM, AND ALSO BY THE ABSENCE OF FREE-RUNS DURING RESTRICTED FEEDING.

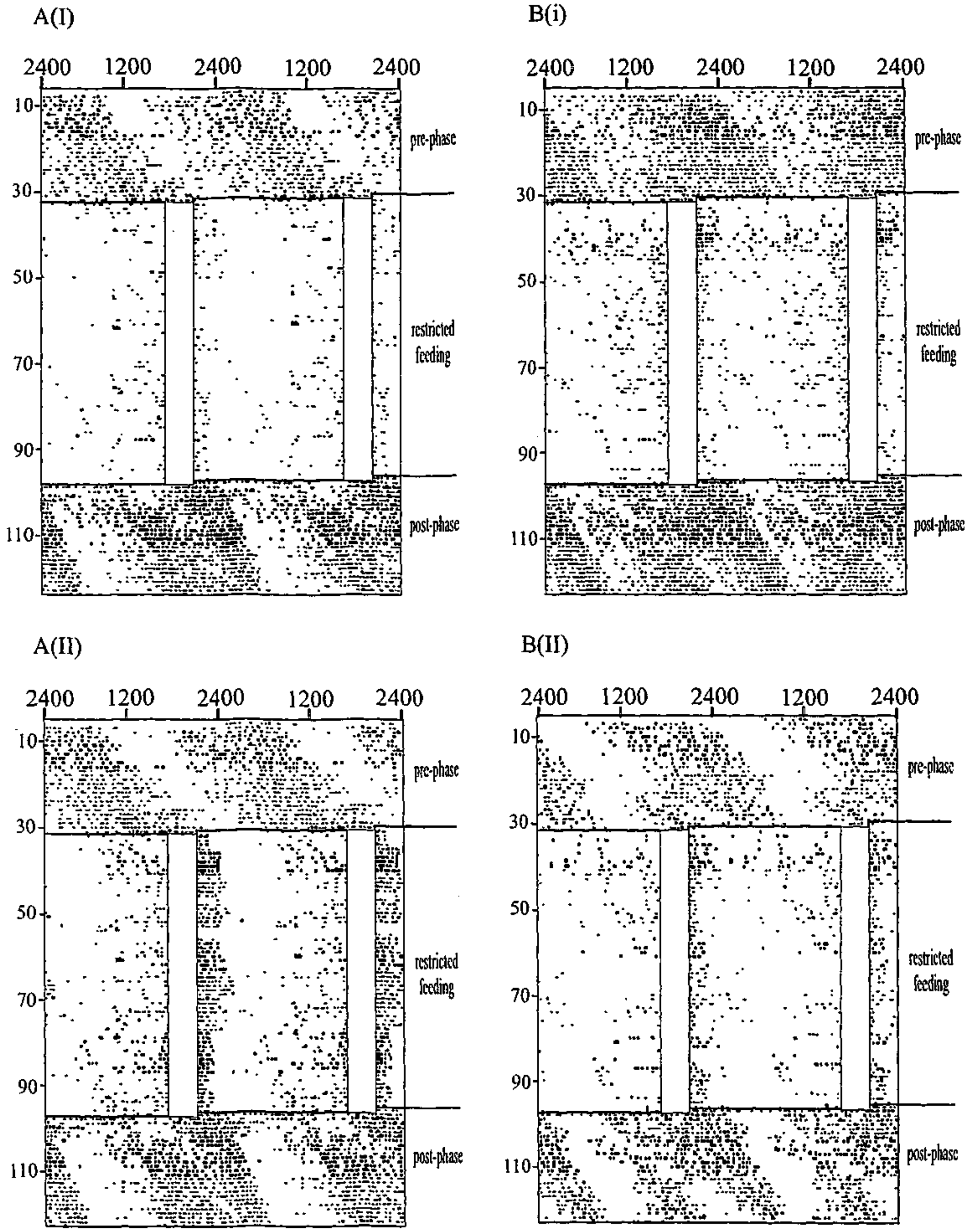


FIGURE 23. DOUBLE PLOTS OF FOOD COUNTS (I) AND WATER COUNTS (II) FROM FOUR RABBITS EXPOSED TO A DAILY FEEDING SCHEDULE. (A) SUBJECT #9. (B) SUBJECT #11. (C) SUBJECT #14. (D) SUBJECT #15. IN THE POST-PHASE, ACTIVITY CHARACTERIZED BY TWO SEPARATE COMPONENTS CAN BE SEEN.

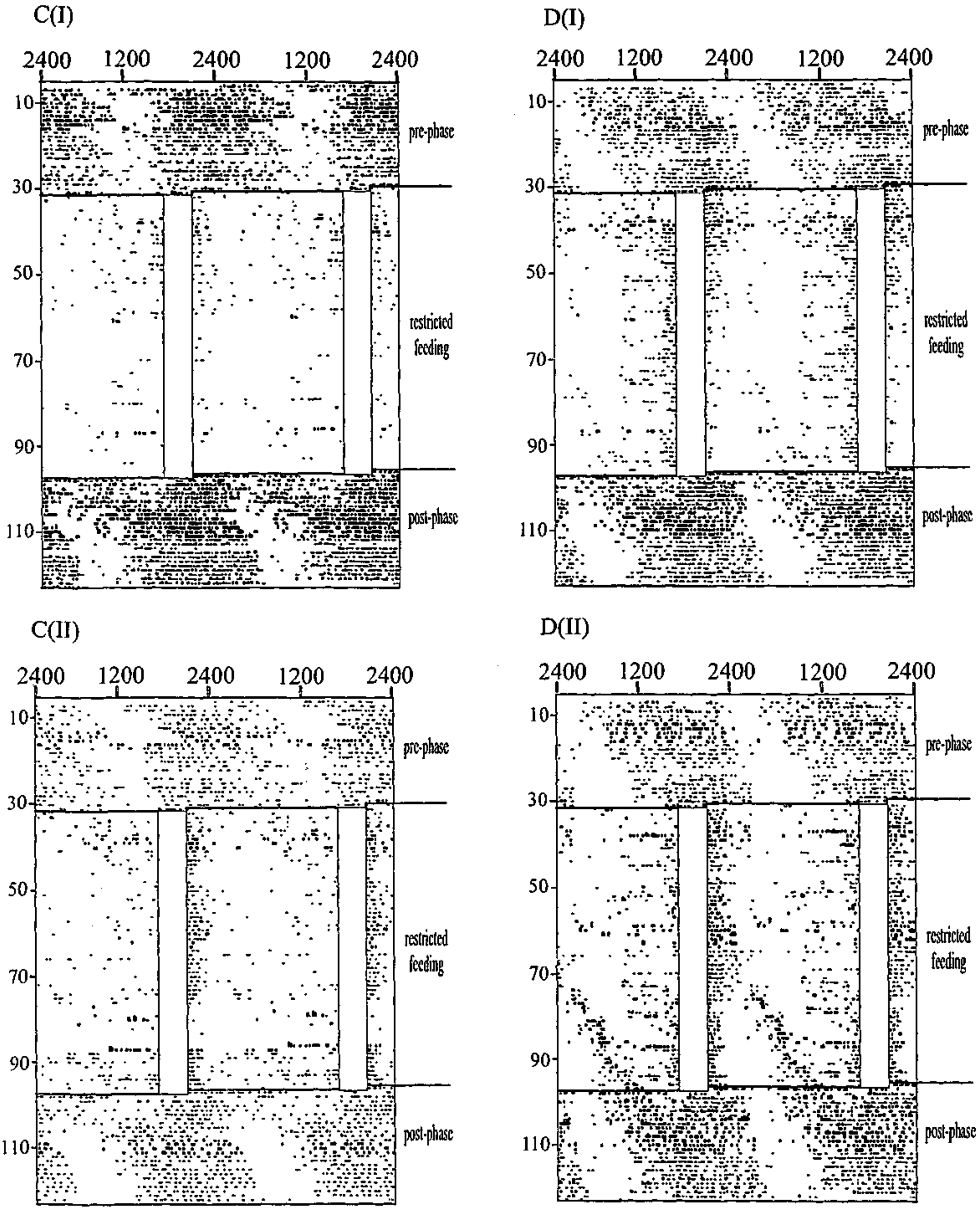


FIGURE 23 CONTINUED

actograms are presented in Figures 23, 26, and 33. In order to determine whether the phase of the free-running light entrainable rhythm had been affected by the feeding schedule, regression lines were computed through activity offsets over the last 20 days of the pre-phase and extrapolated forward to the first day of the post-phase for food counts. It was decided to use activity offsets in preference to onsets for reasons described in section 5.5.1. A change in the phase of the free-running light entrainable rhythm had occurred in all rabbits. In view of the fact that changes in tau had occurred, these data need to be treated with caution.

With the exception of two rabbits, the post-phase was characterized by two separate components; activity associated with the feeding schedule, and activity free-running from a phase largely consistent with pre-entrainment. The two components merged after approximately 10 days. Representative actograms of these subjects are presented in Figure 23.

8.3.2. Entrainment to cycles of food availability with T<24h

Visual inspection of graphical distributions, in addition to examination of the actograms, indicated that rabbits exposed to T=21h failed to meet the criteria for anticipatory activity. Representative actograms are presented in Figures 24 to 26.

Inspection of the actograms revealed that the free-running light entrainable rhythm rhythm was discernable (albeit disrupted to some degree) during the feeding schedule for water counts and general activity, but was less discernable for food counts. In order to determine whether the phase of the free-running light entrainable rhythm had been affected by the feeding schedule, regression lines were computed through activity offsets over the last 20 days of the pre-phase (*ad libitum* 2) and extrapolated forward to the first day of the post-phase (*ad libitum* 3) for food counts. As for T=24h, changes in the phase of the free-running light entrainable rhythm were common. A trend was apparent in some of the data in that the rhythm free-ran from a phase largely determined by the feeding schedule, and not by the phase of the rhythm in the pre-phase. This is illustrated in Figure 24.

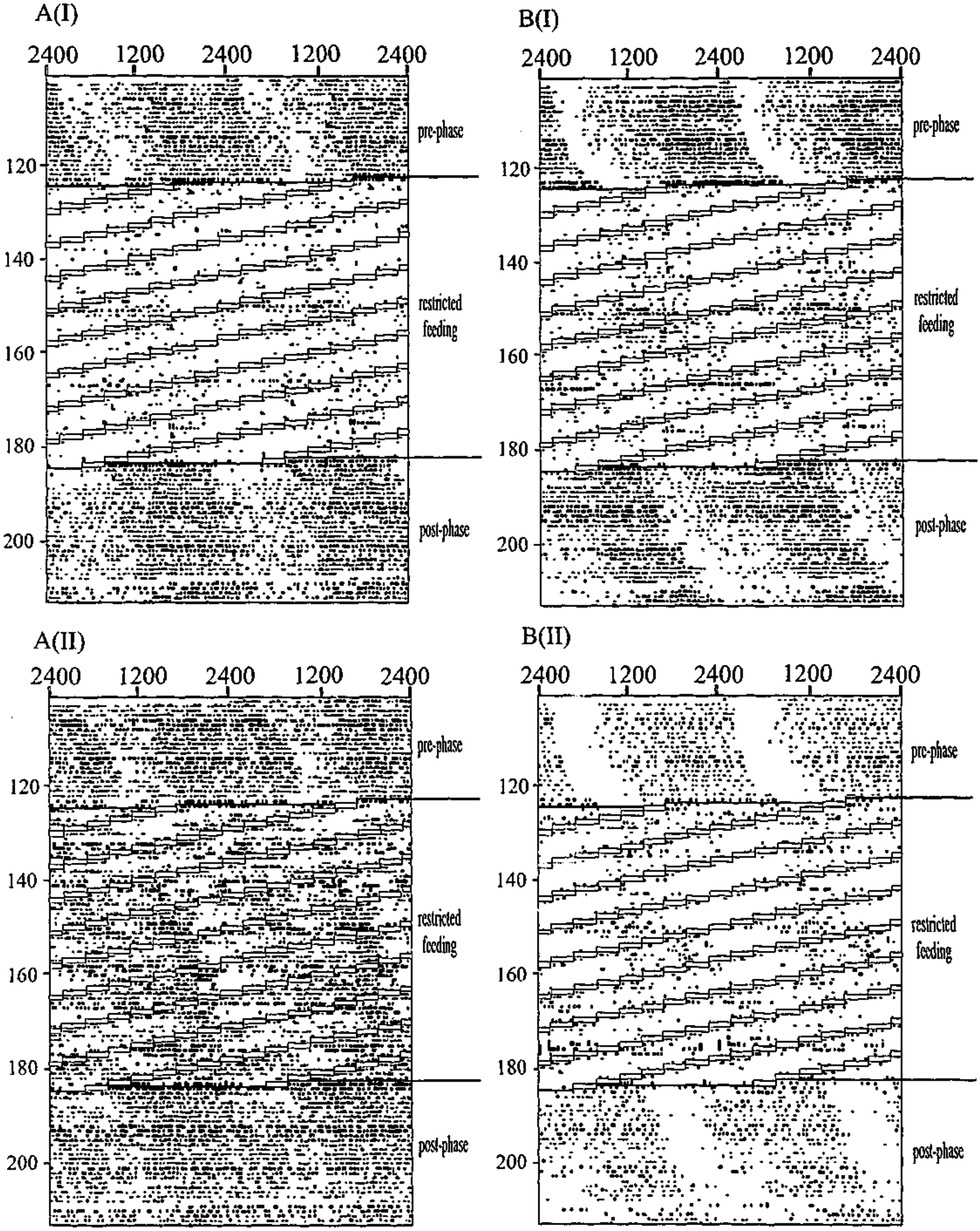


FIGURE 24. DOUBLE PLOTS OF FOOD COUNTS (I) AND WATER COUNTS (II) FROM SIX RABBITS EXPOSED TO A FEEDING SCHEDULE WITH T=21H. (A) SUBJECT #1. (B) SUBJECT #2. (C) SUBJECT #3. (D) SUBJECT #5. (E) SUBJECT #6. (F) SUBJECT #8. FOOD ACCESS IS DEPICTED BY HOLLOW BARS. DAYS OF THE EXPERIMENT ARE SHOWN ON THE LEFT SIDE OF THE ACTOGRAM; THESE CORRESPOND TO THE RIGHT SIDE OF THE ACTOGRAM. POST-PHASE FREE-RUNS ARE DETERMINED BY THE FEEDING SCHEDULE IN SUBJECT #'S 1, 3, 5, & 8.

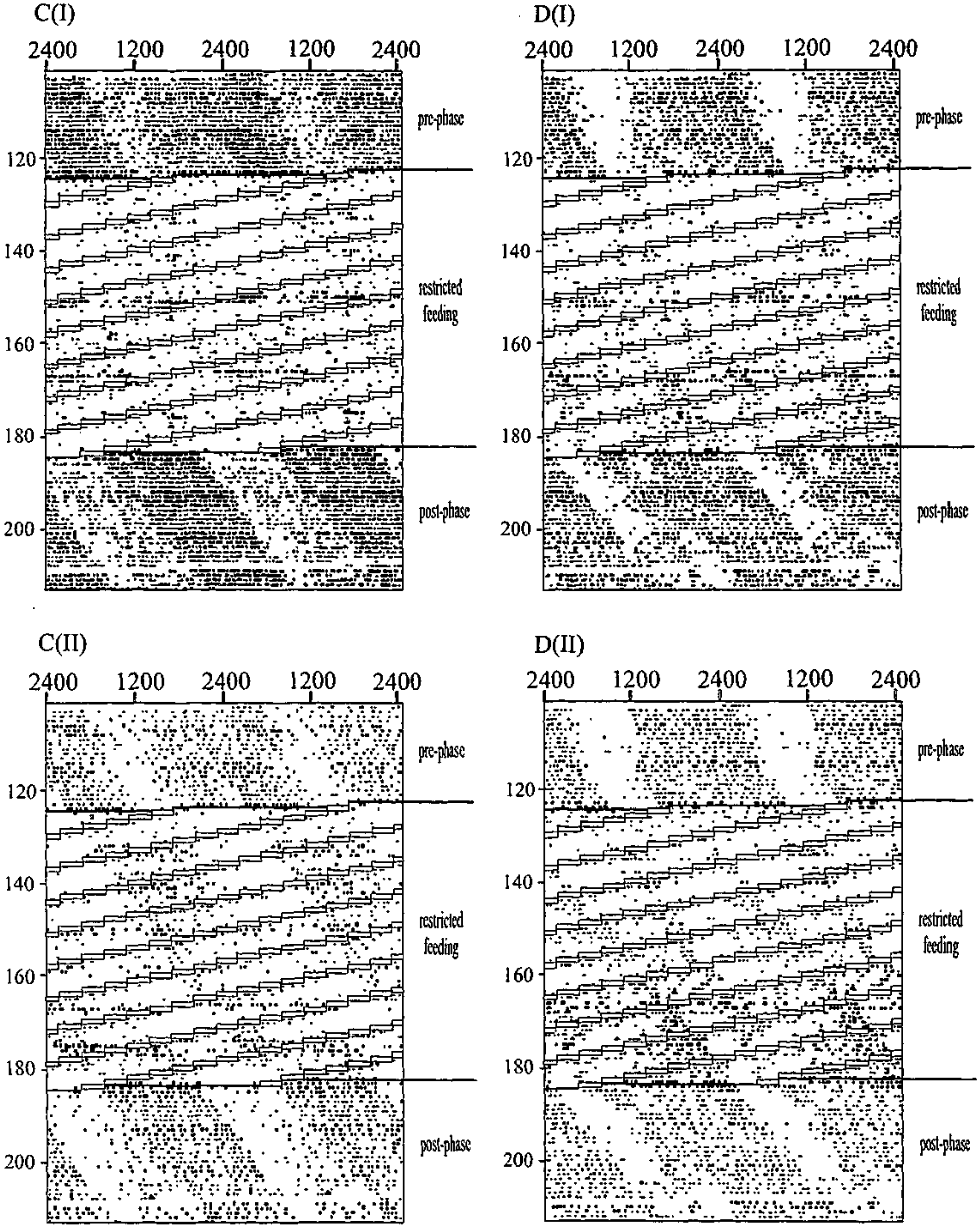


FIGURE 24 CONTINUED

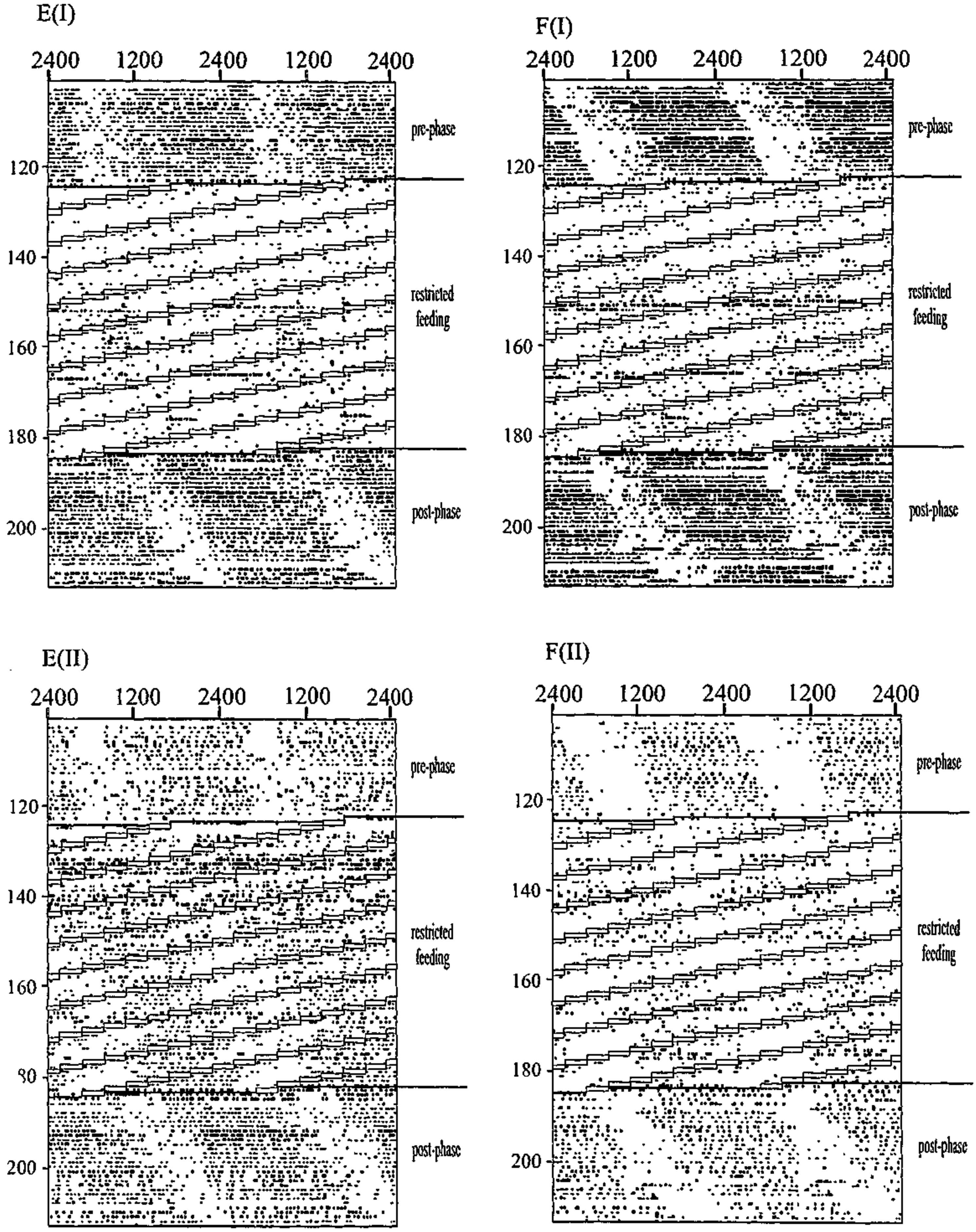


FIGURE 24 CONTINUED

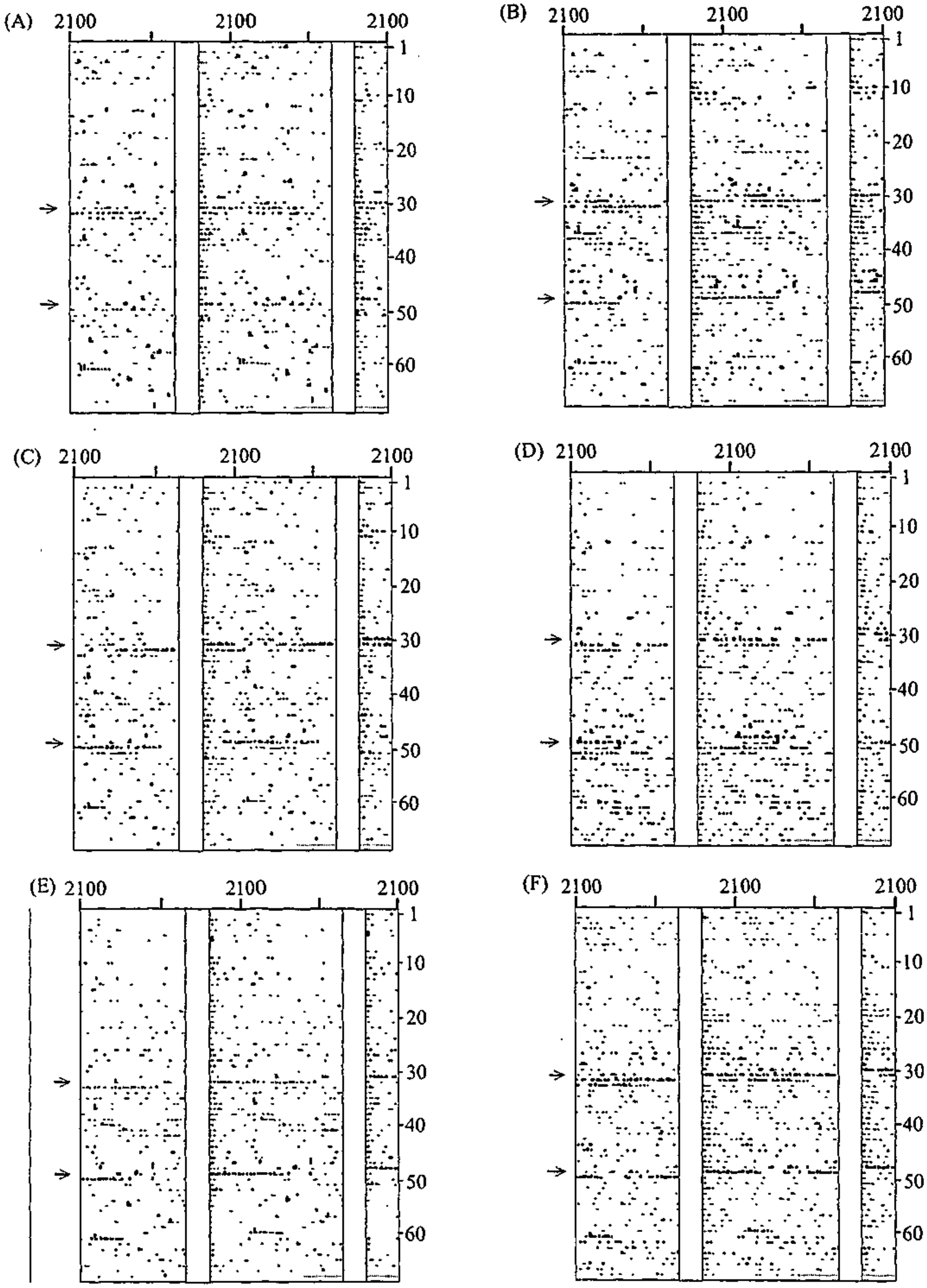


FIGURE 25. DOUBLE PLOTS OF FOOD COUNTS FROM SIX RABBITS EXPOSED TO T=21H. (A) SUBJECT #1. (B) SUBJECT #2. (C) SUBJECT #3. (D) SUBJECT #5. (E) SUBJECT #6. (F) SUBJECT #8. DATA ARE PLOTTED ON A 21H TIME SCALE. RESTRICTED FEEDING CYCLES ARE SHOWN ON THE RIGHT SIDE OF THE ACTROGRAM. FOOD ACCESS IS DEPICTED BY HOLLOW VERTICAL BARS. ARROW HEADS INDICATE DAYS OF AD LIBITUM FOOD ACCESS CAUSED BY MECHANICAL FAILURE. NO ANTICIPATORY ACTIVITY CAN BE SEEN IN THESE RABBITS.

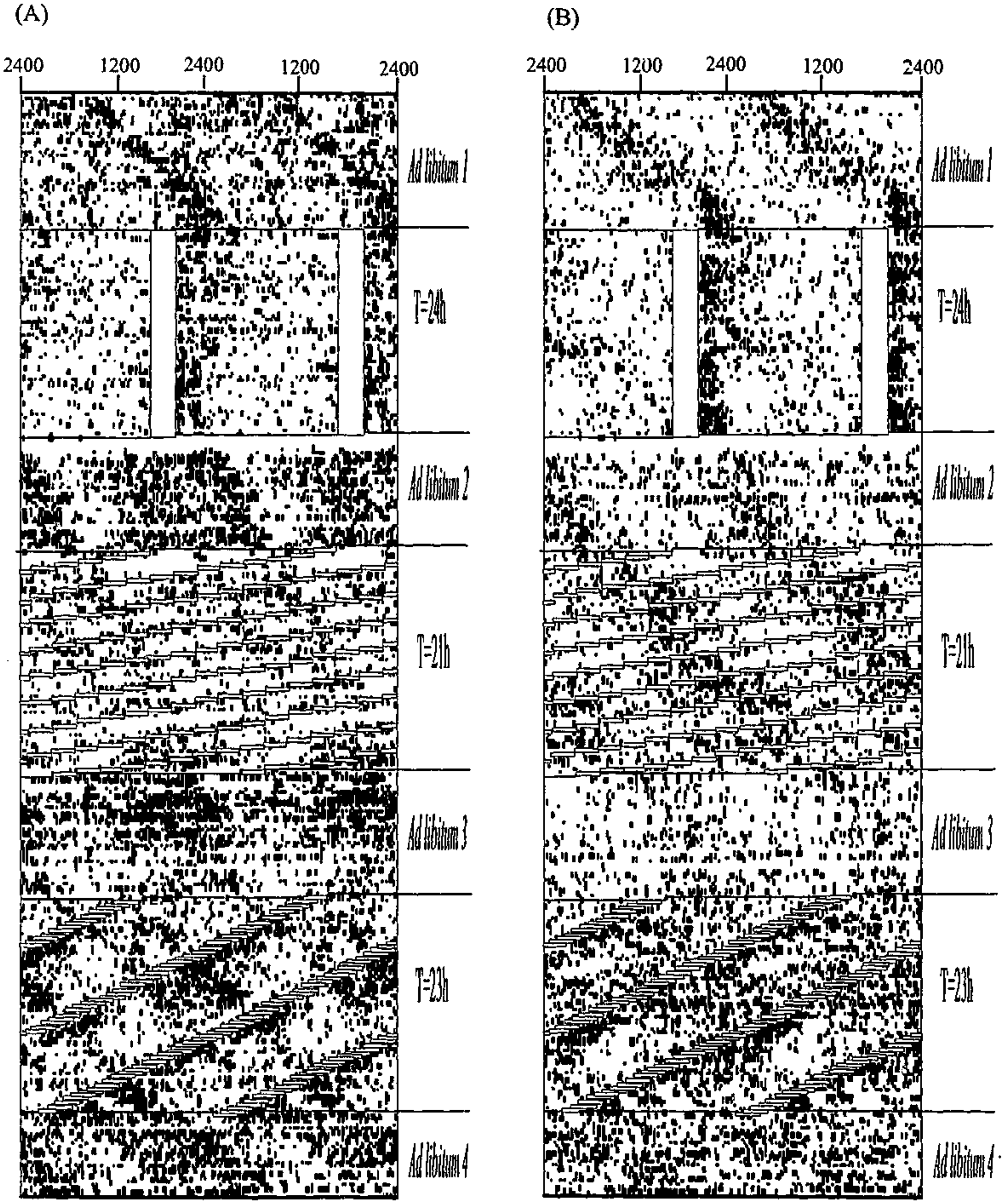


FIGURE 26. DOUBLE PLOTS OF GENERAL ACTIVITY FOR FOUR RABBITS. (A) SUBJECT #1. (B) SUBJECT #2. (C) SUBJECT #3. (D) SUBJECT #8. EXPERIMENTAL STAGES ARE SHOWN ON THE RIGHT SIDE OF THE ACTOGRAM. FOOD ACCESS IS INDICATED BY HOLLOW BARS. TIME OF DAY IS INDICATED AT THE TOP OF THE ACTOGRAM.

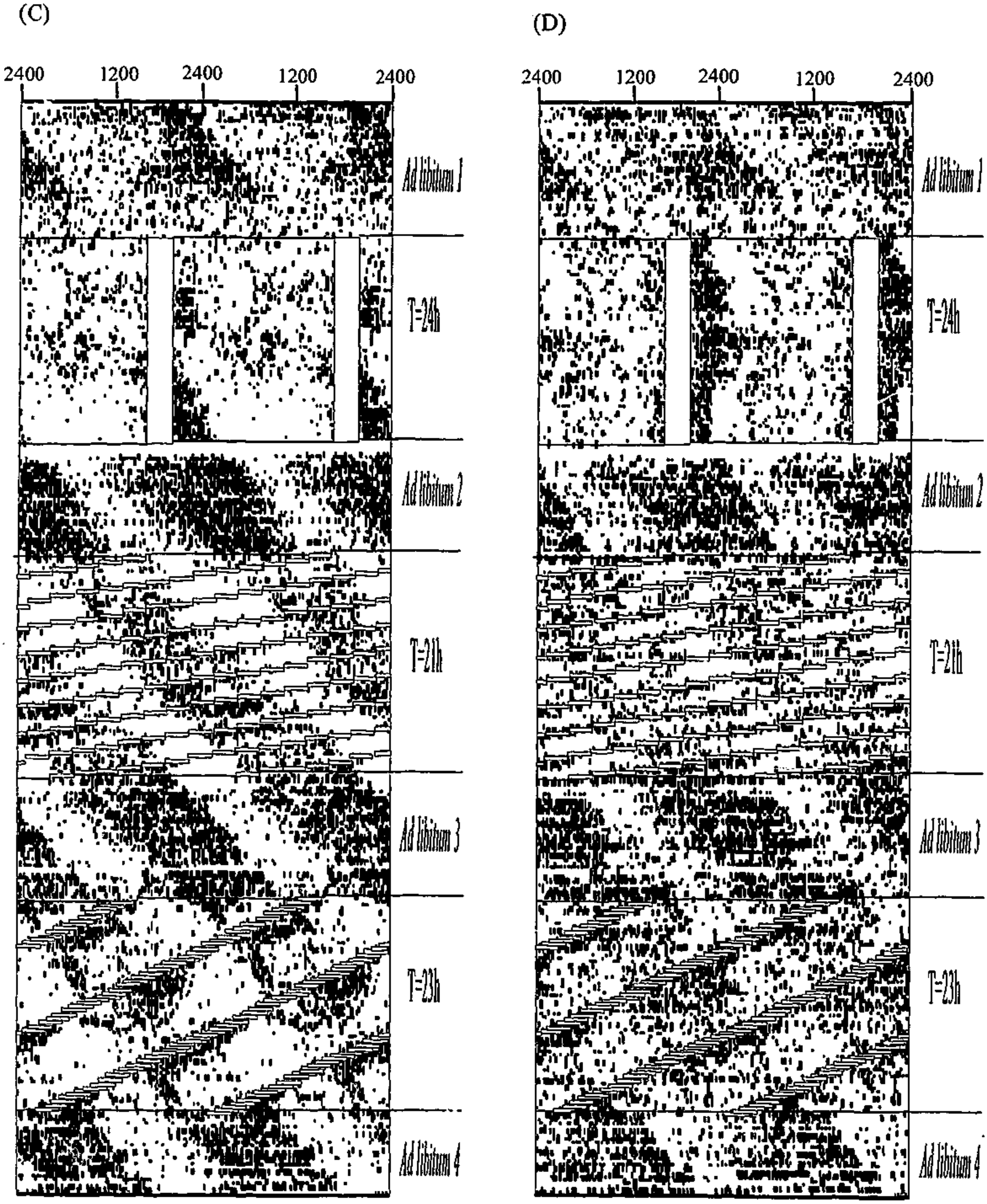


FIGURE 26 CONTINUED

Mechanical failure resulted in *ad libitum* food access and hence high levels of feeding activity during cycle 49-50 of restricted feeding with T=23h. Hence these data were omitted from the following analysis. It is important to note that such problems were common in the final stages of the experiment. For T=23h, three rabbits showed anticipatory activity. Anticipatory activity emerged within 39 cycles (*SD*=6.0). Raters' estimates of PAD were almost identical and the data from one rater is thus reported here; PADs ranged between 1.25h and 2h. Visual inspection of the actograms indicated that the free-running light entrainable rhythm did not entrain to the feeding schedule for these rabbits; post-phase (i.e., *ad libitum* 4) control of the rhythm by the feeding schedule did not occur. Actograms of these subjects are presented in Figures 27 to 29.

For the remaining rabbits, activity free-ran during the feeding schedule for general activity and/or water counts for each rabbit, but was less discernable for food counts. Activity free-runs were dampened during restricted feeding compared to the pre-phase (i.e., *ad libitum* 3) and a clearly defined light entrainable rhythm was re-established in the post-phase. This is illustrated in Figures 26 and 30. For subject #1, the activity rhythm showed two components in the post-phase: a small component of activity associated with the feeding schedule, and a larger component of free-running light entrainable activity. The same procedure as described above was used to determine whether the phase of the free-running light entrainable rhythm had been affected by the feeding schedule. Changes in the phase of the free-running light entrainable rhythm for food counts had occurred in all but one rabbit.

In order to ascertain whether tau of the free-running light entrainable rhythm was influenced by the feeding schedule, tau for *ad libitum* 2, 3, and 4 was calculated for food counts. The same procedure as used for T=24h was used here. Inter-rater reliability as determined by Pearson's product-moment correlations was highly significant ($r=.81$, $p<.01$). Data were averaged across both raters for use in the following analysis. Mean (+*SE*) estimates of free-running tau by phase are shown in Table 4.

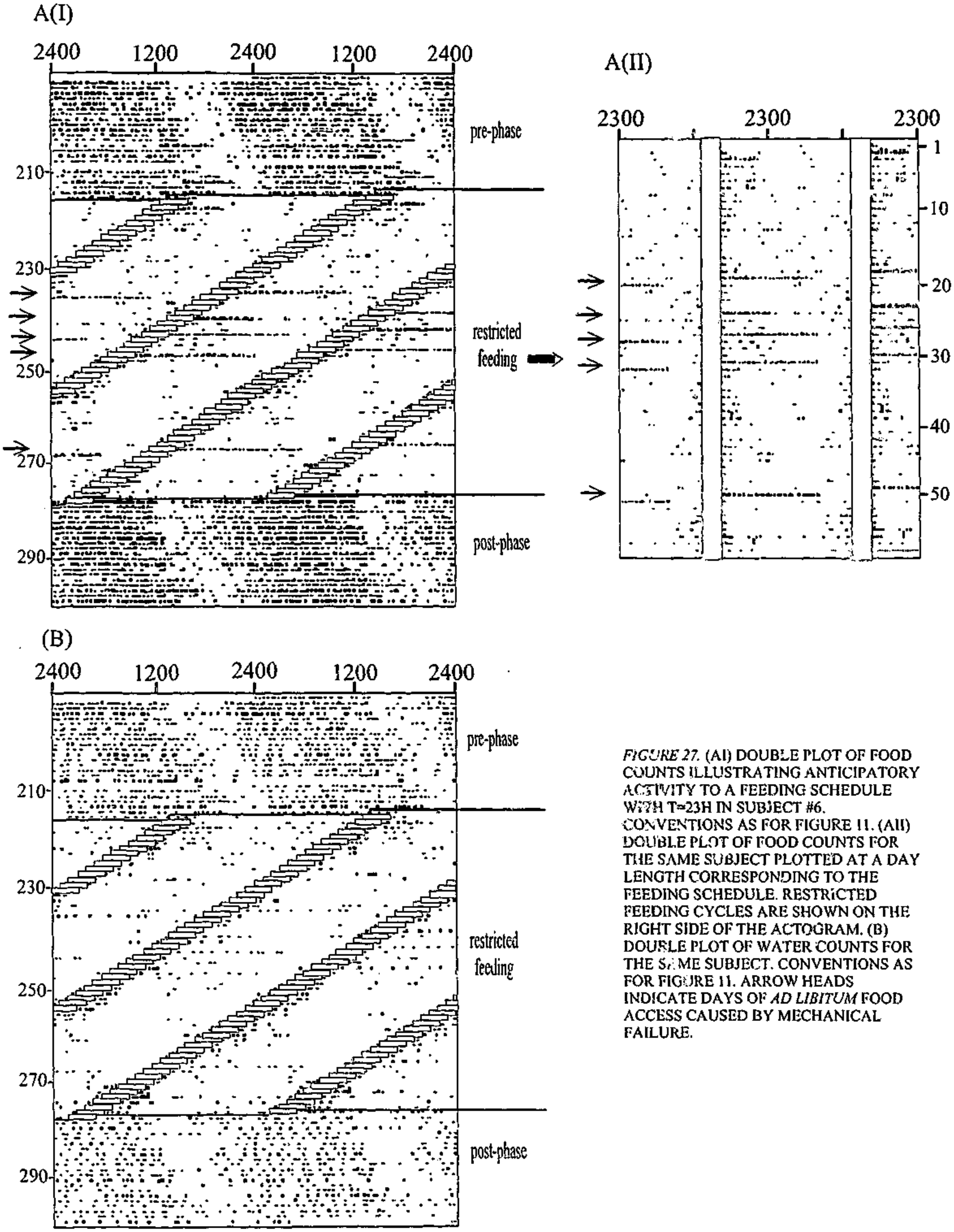


FIGURE 27. (AI) DOUBLE PLOT OF FOOD COUNTS ILLUSTRATING ANTICIPATORY ACTIVITY TO A FEEDING SCHEDULE WITH T=23H IN SUBJECT #6. CONVENTIONS AS FOR FIGURE 11. (AII) DOUBLE PLOT OF FOOD COUNTS FOR THE SAME SUBJECT PLOTTED AT A DAY LENGTH CORRESPONDING TO THE FEEDING SCHEDULE. RESTRICTED FEEDING CYCLES ARE SHOWN ON THE RIGHT SIDE OF THE ACTOGRAM. (B) DOUBLE PLOT OF WATER COUNTS FOR THE SAME SUBJECT. CONVENTIONS AS FOR FIGURE 11. ARROW HEADS INDICATE DAYS OF *AD LIBITUM* FOOD ACCESS CAUSED BY MECHANICAL FAILURE.

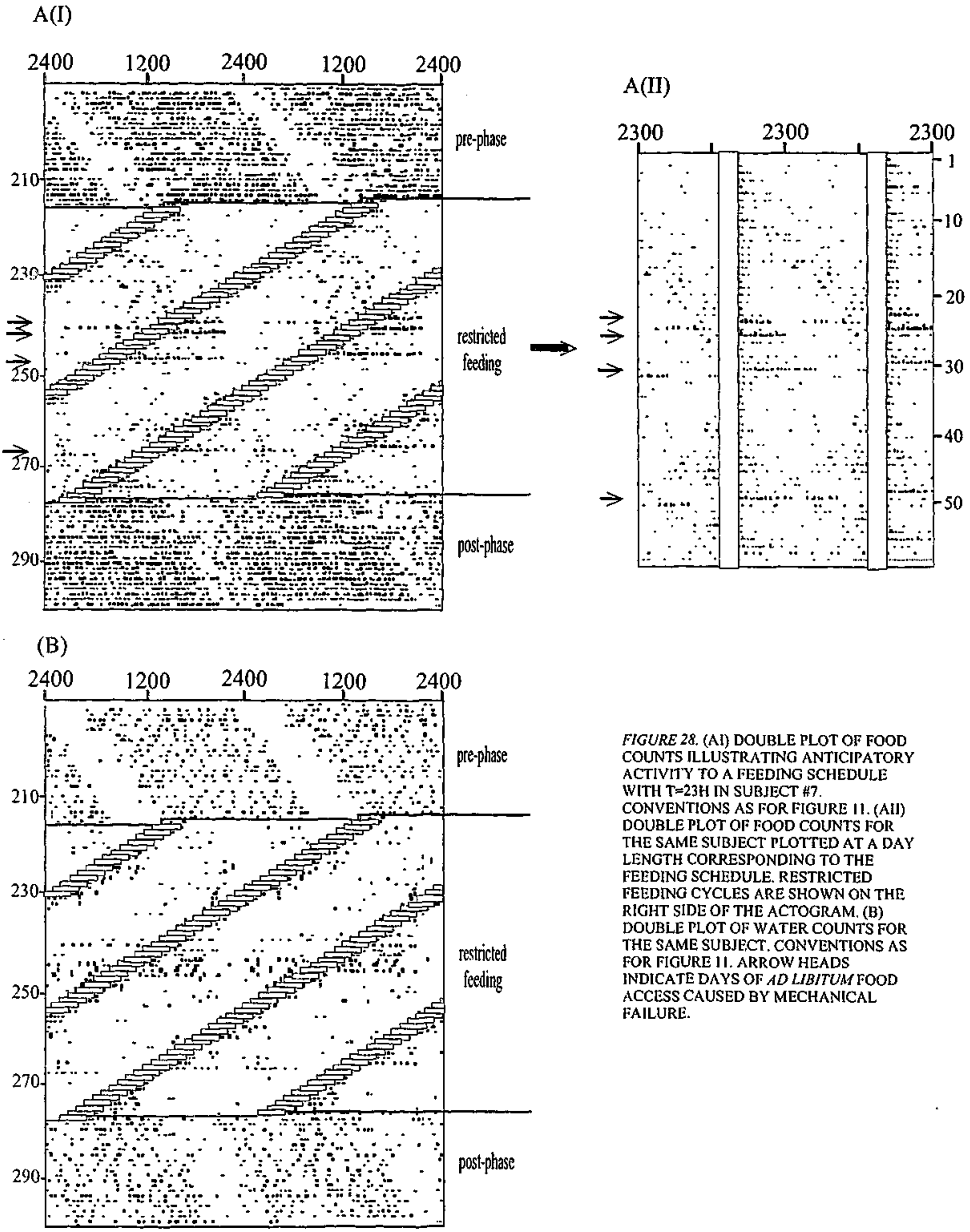
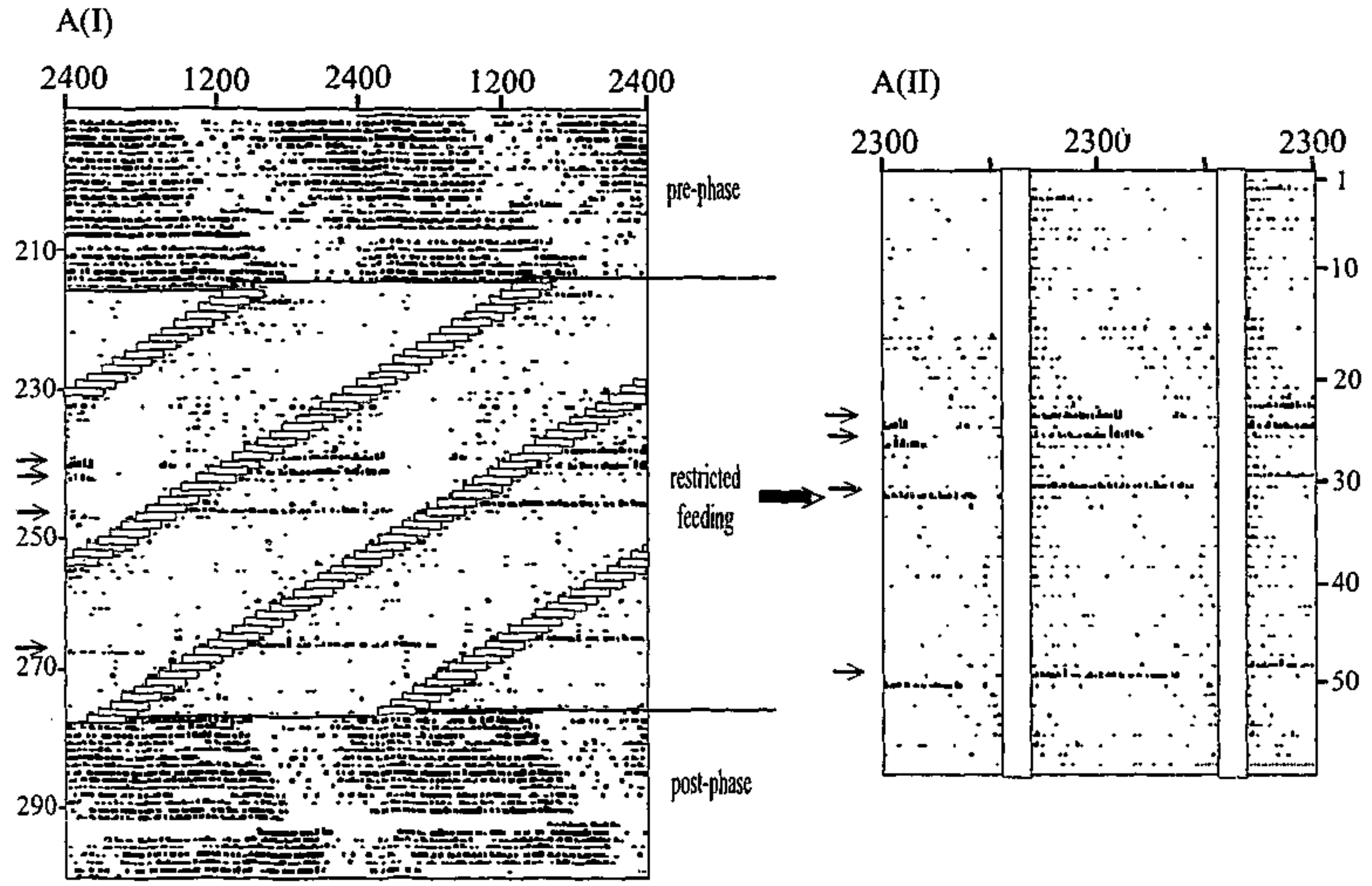


FIGURE 28. (AI) DOUBLE PLOT OF FOOD COUNTS ILLUSTRATING ANTICIPATORY ACTIVITY TO A FEEDING SCHEDULE WITH T=23H IN SUBJECT #7. CONVENTIONS AS FOR FIGURE 11. (AII) DOUBLE PLOT OF FOOD COUNTS FOR THE SAME SUBJECT PLOTTED AT A DAY LENGTH CORRESPONDING TO THE FEEDING SCHEDULE. RESTRICTED FEEDING CYCLES ARE SHOWN ON THE RIGHT SIDE OF THE ACTOGRAM. (B) DOUBLE PLOT OF WATER COUNTS FOR THE SAME SUBJECT. CONVENTIONS AS FOR FIGURE 11. ARROW HEADS INDICATE DAYS OF *AD LIBITUM* FOOD ACCESS CAUSED BY MECHANICAL FAILURE.



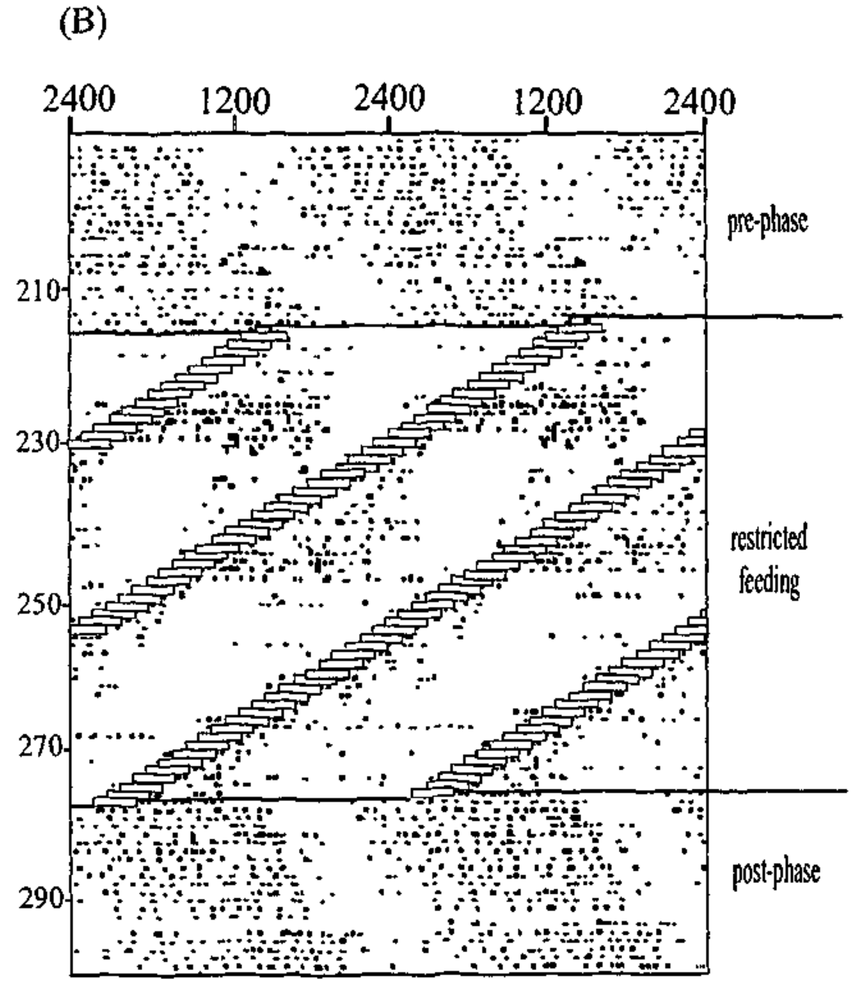


FIGURE 29. (AI) DOUBLE PLOT OF FOOD COUNTS ILLUSTRATING ANTICIPATORY ACTIVITY TO A FEEDING SCHEDULE WITH T=23H IN SUBJECT #8. CONVENTIONS AS FOR FIGURE 11. (AII) DOUBLE PLOT OF FOOD COUNTS FOR THE SAME SUBJECT PLOTTED AT A DAY LENGTH CORRESPONDING TO THE FEEDING SCHEDULE. RESTRICTED FEEDING CYCLES ARE SHOWN ON THE RIGHT SIDE OF THE ACTOGRAM. (B) DOUBLE PLOT OF WATER COUNTS FOR THE SAME SUBJECT. CONVENTIONS AS FOR FIGURE 11. ARROW HEADS INDICATE DAYS OF *AD LIBITUM* FOOD ACCESS CAUSED BY MECHANICAL FAILURE.

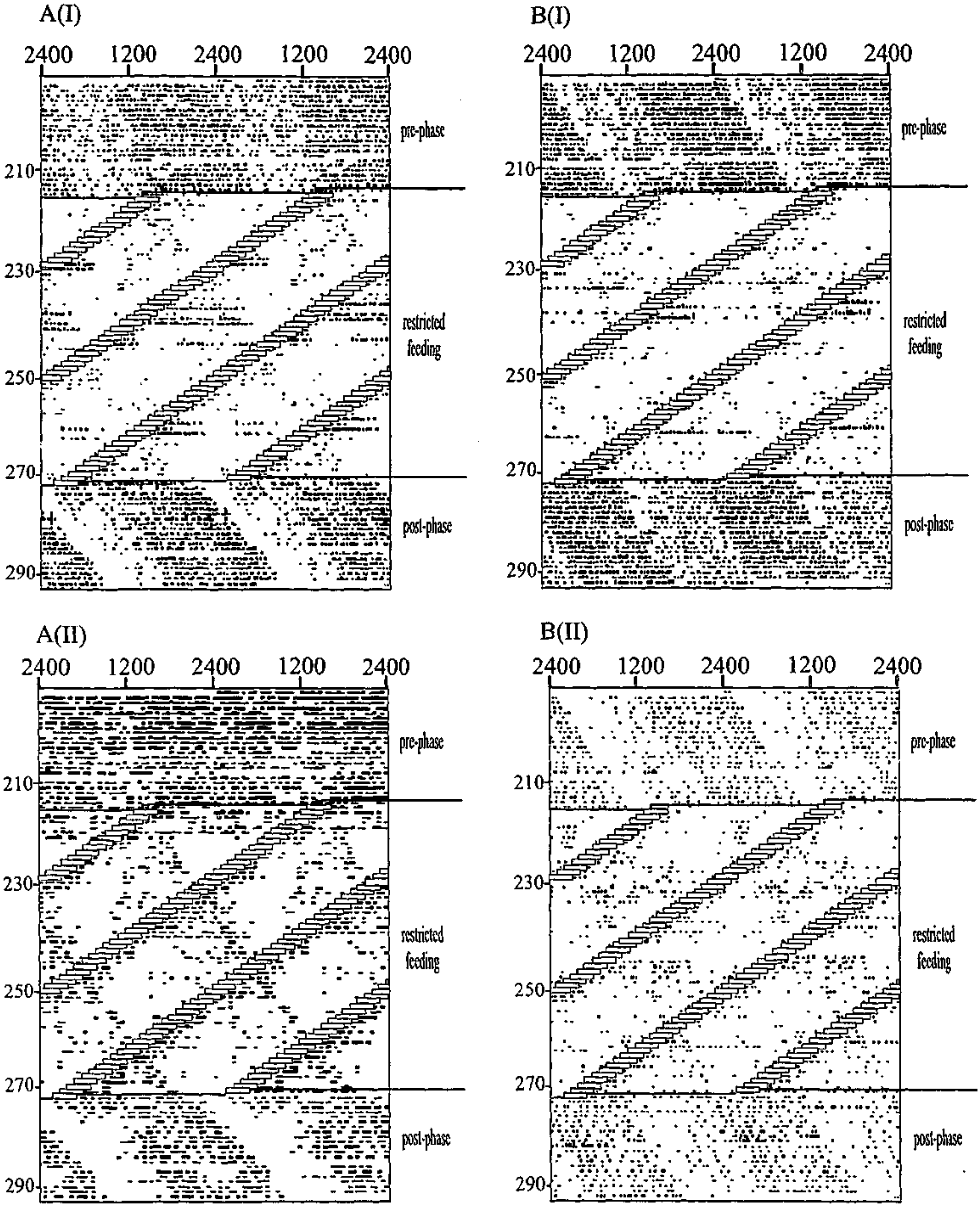


FIGURE 30. DOUBLE PLOTS OF FOOD (I) AND WATER COUNTS (II) FROM FOUR RABBITS EXPOSED TO A FEEDING SCHEDULE WITH T=23H. (A) SUBJECT #1. (B) SUBJECT #3. (C) SUBJECT #4. (D) SUBJECT #8. CONVENTIONS AS FOR FIGURE 11. FOR SUBJECT #1, ACTIVITY CONSISTED OF TWO SEPARATE COMPONENTS OVER THE FIRST FEW DAYS OF THE POST-PHASE.

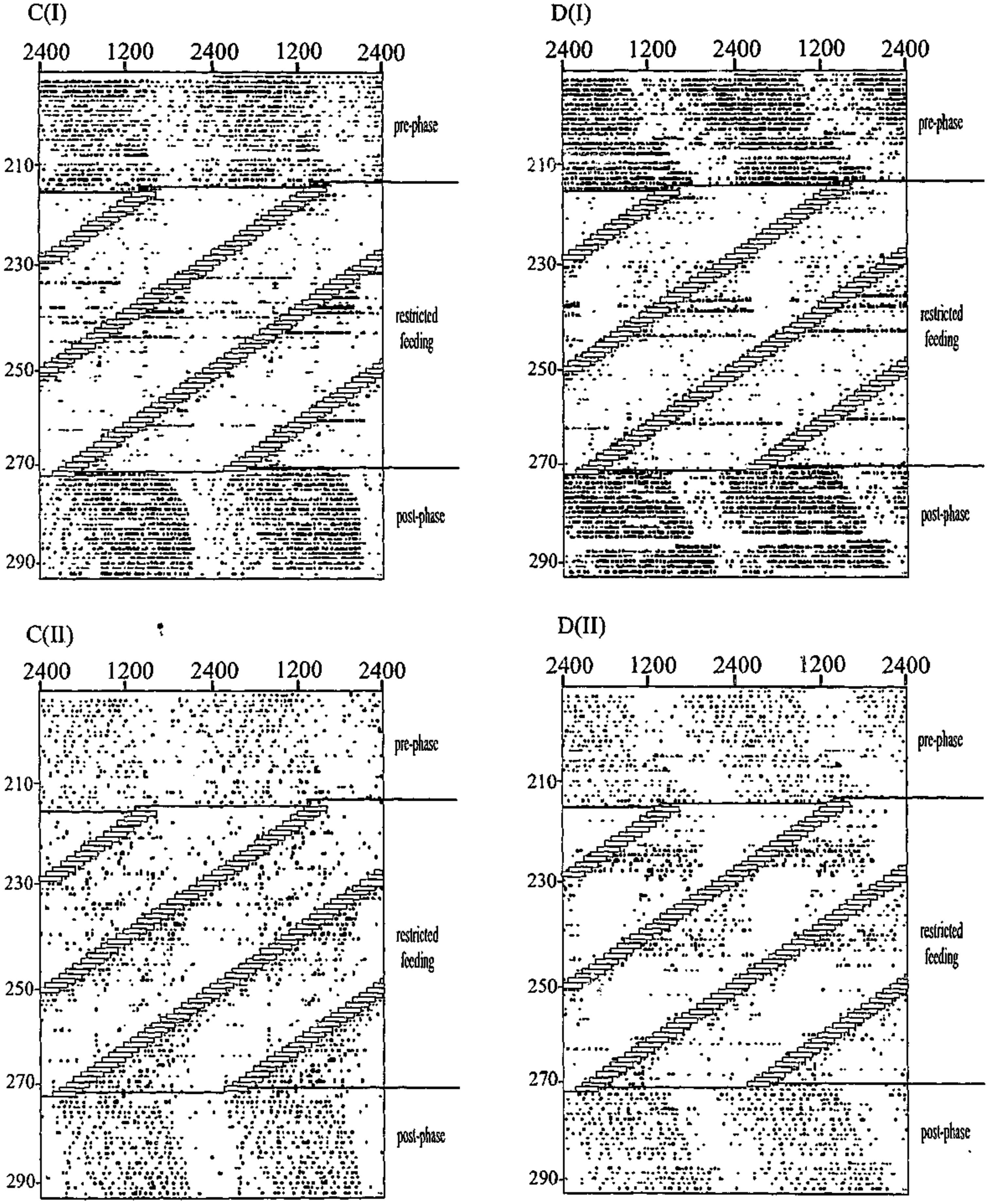


FIGURE 30 CONTINUED

TABLE 4.

TAU OF THE FREE-RUNNING LIGHT ENTRAINABLE RHYTHM BY PHASE FOR T<24H.

	Ad libtium 2	*Ad libitum* 3	*Ad libitum* 4
Mean	24.18	24.34	24.33
+SE	0.04	0.06	0.05

As seen in Table 4, changes in mean tau did occur. A repeated measures ANOVA, followed by Tukey's HSD *post-hoc* tests indicated that tau was significantly shorter during *ad libitum* 2 compared to the other phases, $F(2, 14)=4.89$, $p<.05$. In view of the fact that rabbits had been previously exposed to a 24h feeding schedule, this change in mean tau cannot be attributed to the feeding schedule with T=21h. This issue will be considered in more detail in section 8.4.

8.3.3. Entrainment to cycles of food availability with T>24h

Consideration of graphical distributions indicated that all rabbits exposed to T=27h satisfied the criteria for anticipatory activity. Actograms are presented in Figure 31. Anticipatory activity appeared after 28 to 40 days, with a mean of 34 days (*SD*=3.35). Despite the trend in the data, Pearson's product-moment correlation indicated a non-significant inter-rater correlation for estimates of PAD between activity onset and the onset of food access, $r=.60$, $p>.05$. The lack of significance observed in this parameter can most likely be attributed to the small sample size ($n=7$) and hence the decision was made to report the data from one rater. PAD ranged from 3h to 5.5h, with a mean of 3.79h (*SD*=0.86).

Actograms of rabbits exposed to feeding cycle T of 27h are presented in Figures 32 and 33. Activity free-runs were most likely to occur in general activity, followed by water counts, and least likely to occur in food counts. Visual inspection of the actograms indicated that the free-running light entrainable rhythm was less discernable during the feeding schedule than the pre-phase (*ad libitum* 2) and the post-phase (*ad libitum* 3). Forward extrapolation of regression lines between the pre- and post- phase for food counts indicated that phase changes of the free-running light

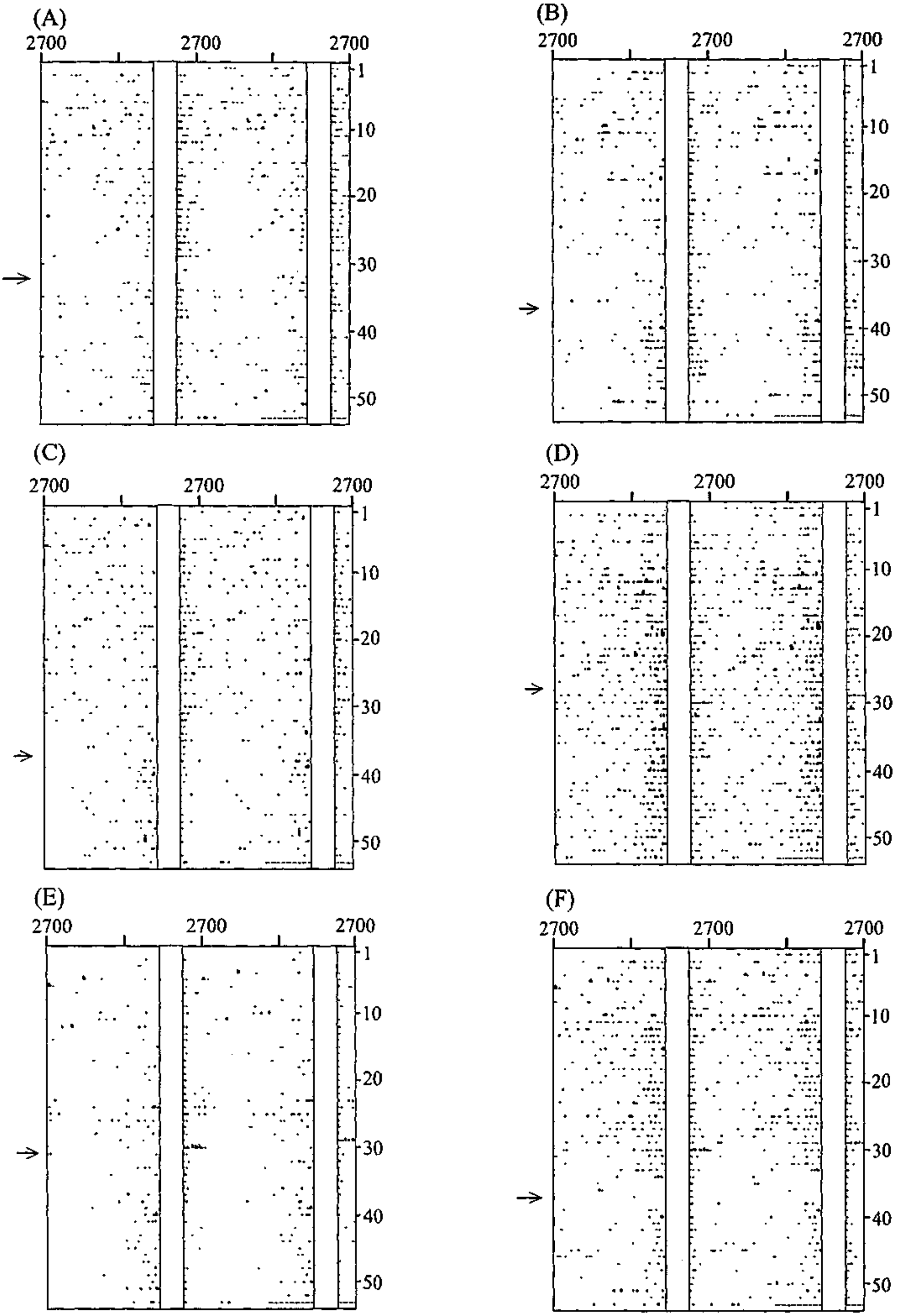


FIGURE 31. DOUBLE PLOTS OF FOOD COUNTS FROM SIX RABBIT EXPOSED TO T=27H. (A) SUBJECT #9. (B) SUBJECT #10. (C) SUBJECT #11. (D) SUBJECT #12. (E) SUBJECT #14. (F) SUBJECT #15. DATA ARE PLOTTED AT A DAY LENGTH CORRESPONDING TO THE FEEDING SCHEDULE. EXPERIMENTAL CYCLES ARE SHOWN ON THE RIGHT SIDE OF THE ACTOGRAMS. ARROW HEADS INDICATE EMERGENCE OF ANTICIPATORY ACTIVITY ON RIGHT SIDE OF ACTOGRAM.

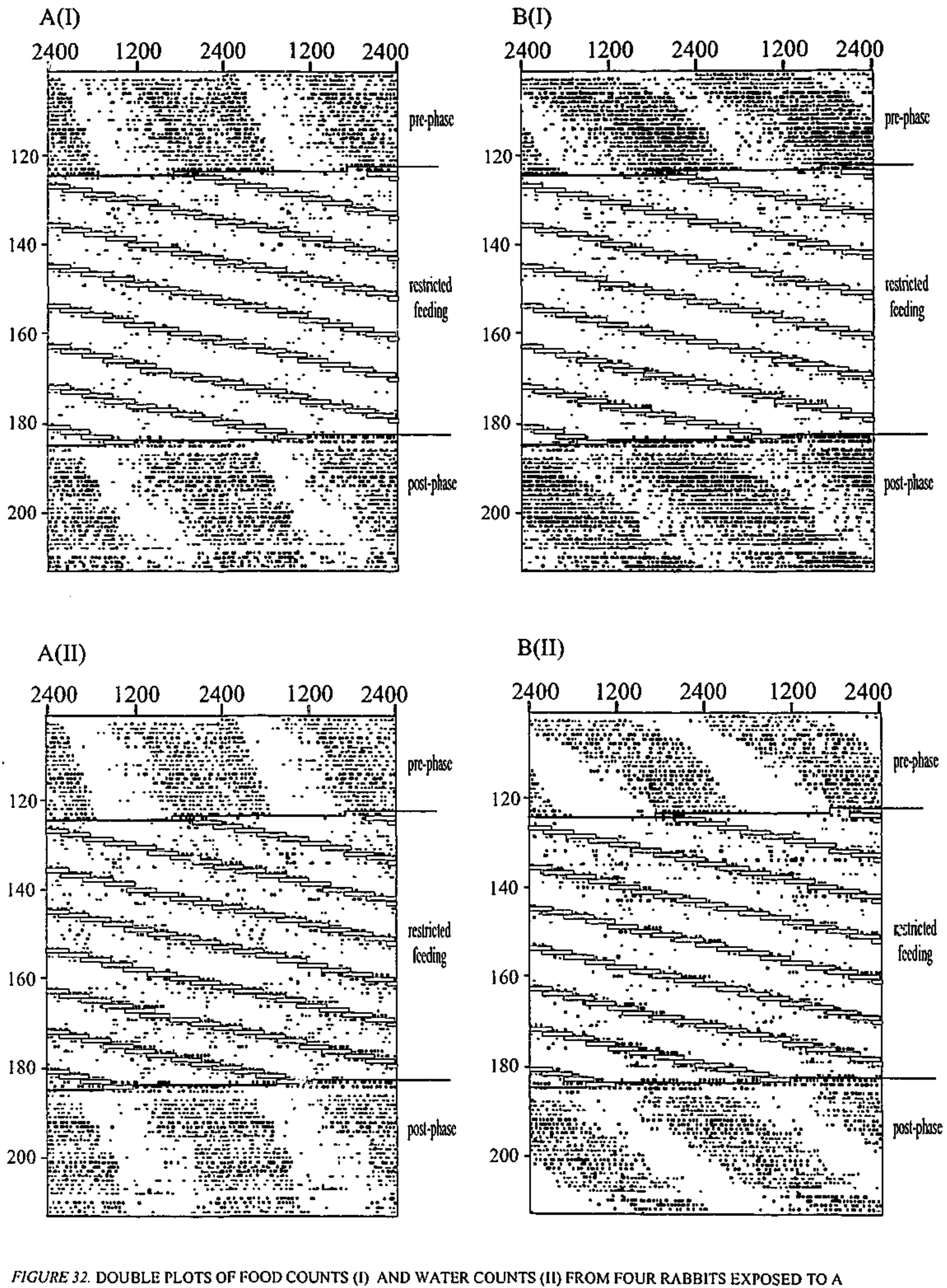


FIGURE 32. DOUBLE PLOTS OF FOOD COUNTS (I) AND WATER COUNTS (II) FROM FOUR RABBITS EXPOSED TO A FEEDING SCHEDULE WITH T=27H. (A) SUBJECT #9. (B) SUBJECT #10. (C) SUBJECT #13. (D) SUBJECT #15. CONVENTIONS AS FOR FIGURE 11. FOR SUBJECT #'S 9 & 10, POST-PHASE FREE-RUNS ARE LARGELY DETERMINED BY THE PHASE OF THE FEEDING SCHEDULE.

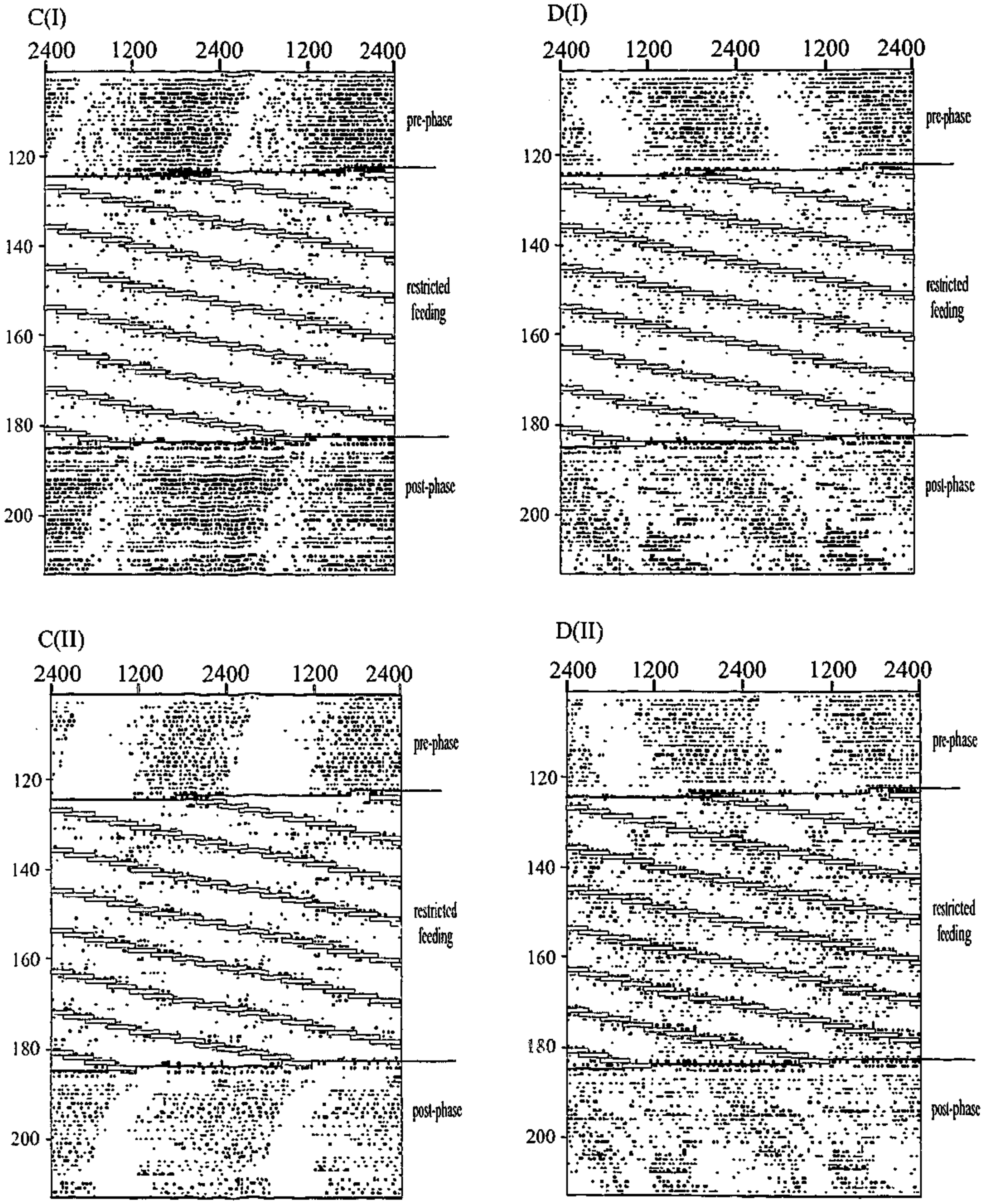


FIGURE 32 CONTINUED

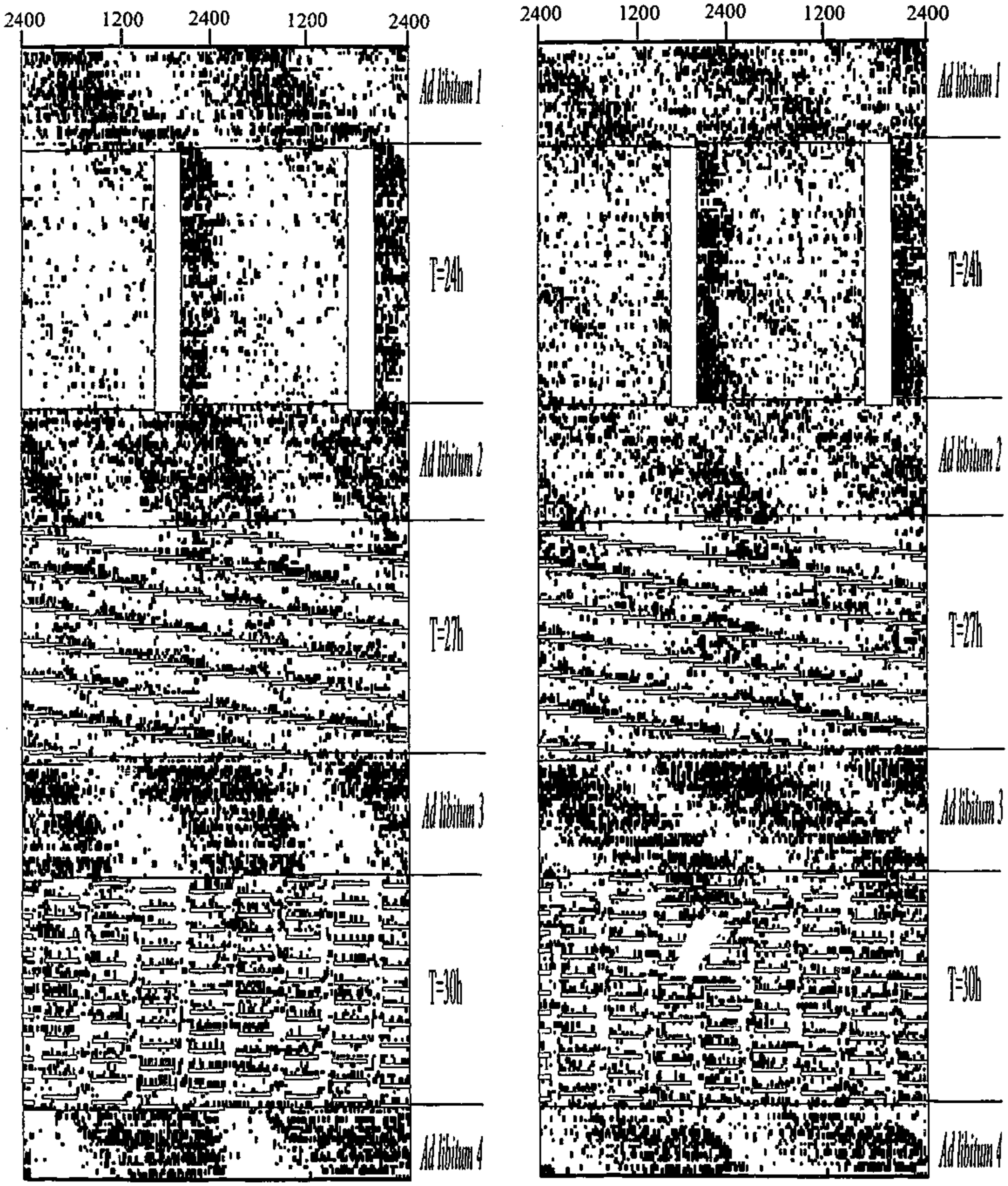


FIGURE 33. DOUBLE PLOTS OF GENERAL ACTIVITY FOR FOUR RABBITS. (A) SUBJECT #9. (B) SUBJECT #10. (C) SUBJECT #13. (D) SUBJECT #15. EXPERIMENTAL STAGES ARE INDICATED ON THE RIGHT SIDE OF THE ACTOGRAM. TIME OF DAY IS INDICATED AT THE TOP OF THE ACTOGRAM. FEEDING TIME IS INDICATED BY HOLLOW BARS.

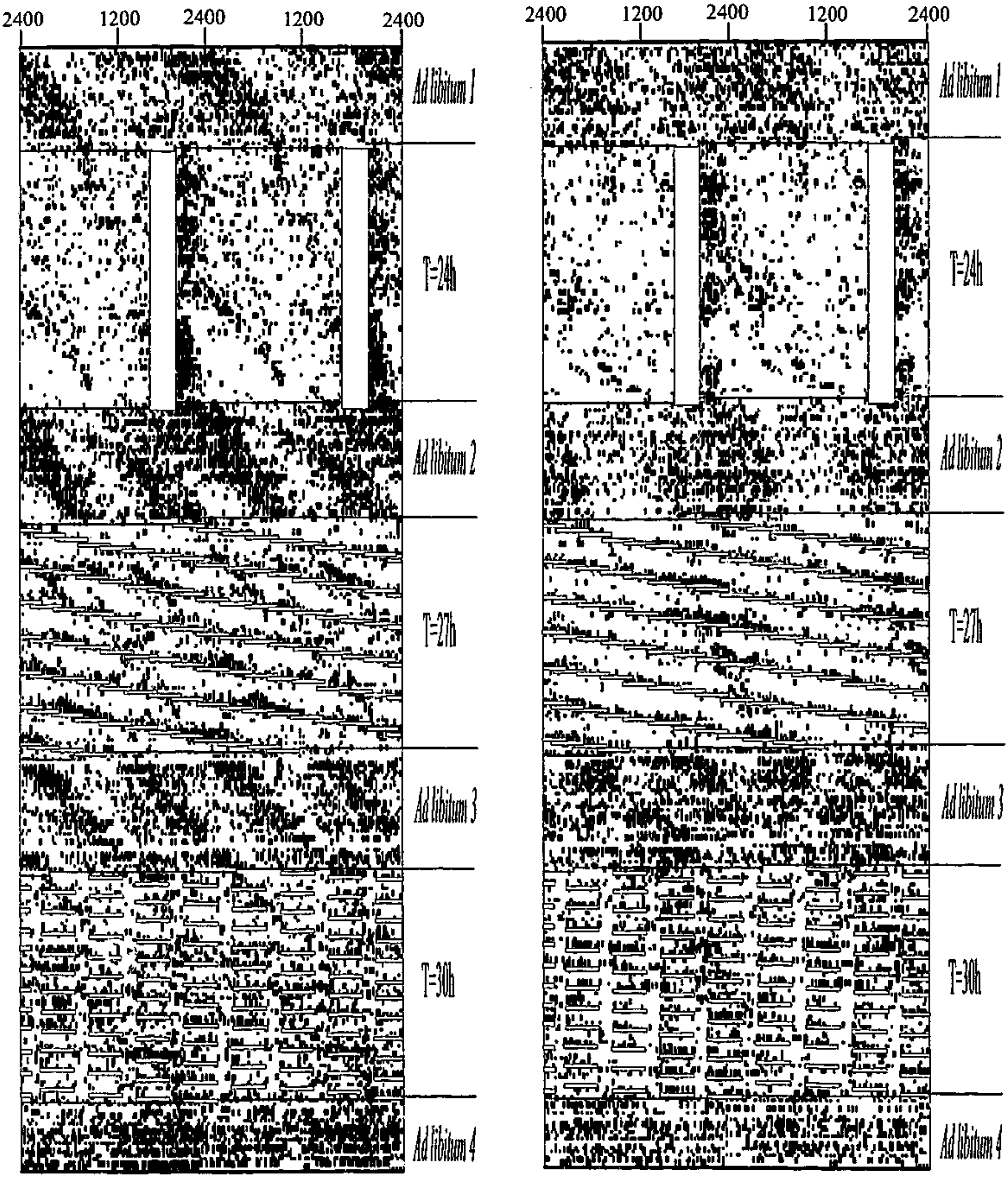


FIGURE 33 CONTINUED

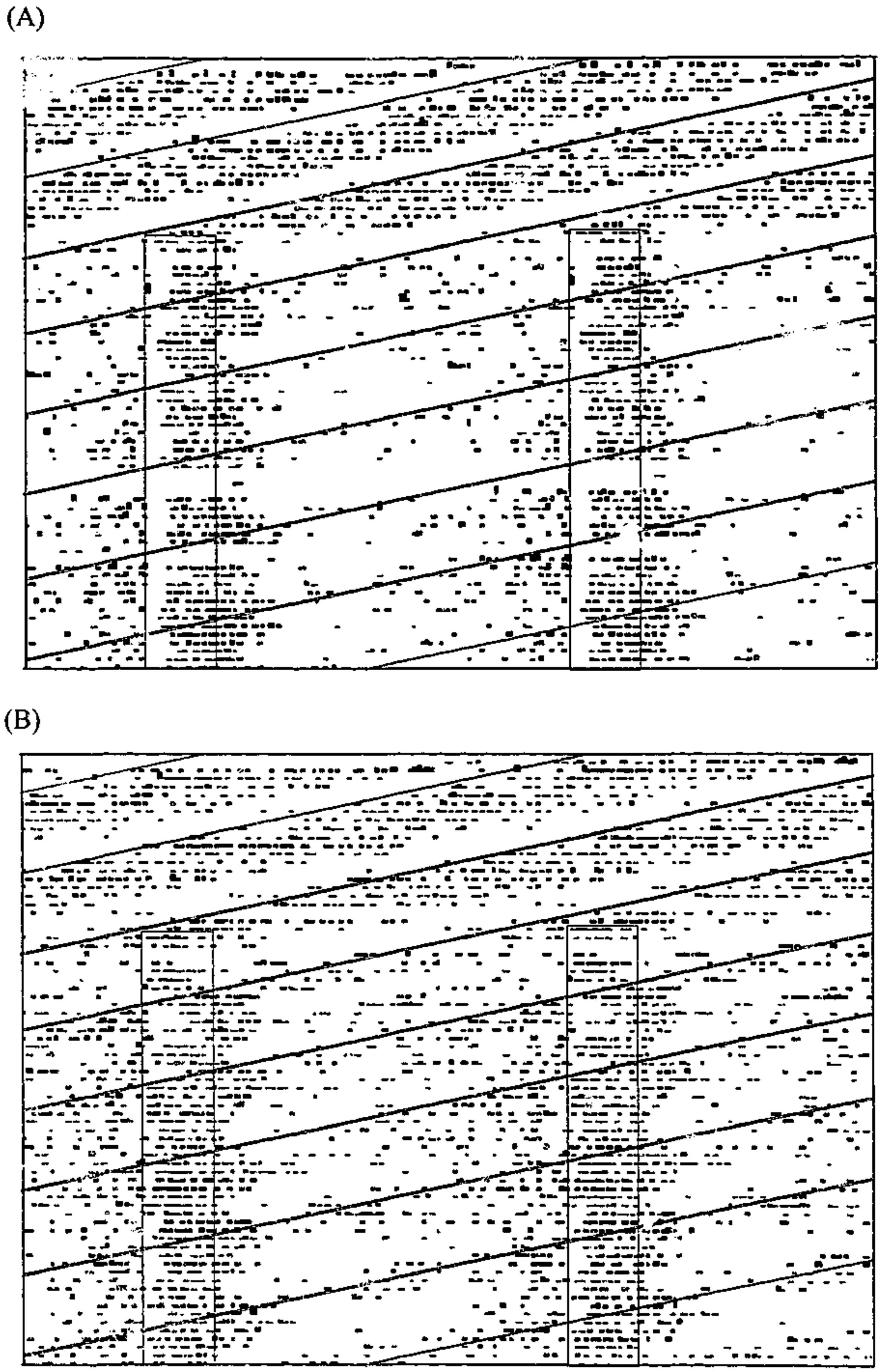


FIGURE 34. DOUBLE PLOTS OF WATER COUNTS FROM TWO SUBJECTS EXPOSED TO A FEEDING SCHEDULE WITH T=27H. (A) SUBJECT #1. (B) SUBJECT #8. DATA ARE PLOTTED AT A DAY LENGTH CORRESPONDING TO THE FEEDING SCHEDULE. FOOD ACCESS IS DEPICTED BY VERTICAL BARS. REGRESSION LINES HAVE BEEN DRAWN THROUGH ACTIVITY ONSETS DURING THE PRE-PHASE AND EXTRAPOLATED FORWARD TO THE FINAL DAY OF RESTRICTED FEEDING.

entrainable rhythm had occurred in all but one rabbit. As with previous schedules, some of the data indicated that the rhythm free-ran from a phase largely determined by the feeding schedule, and not by the phase of the rhythm during pre-entrainment. Representative actograms of these subjects are presented in Figure 32.

There was some, albeit weak, evidence in the actograms that the free-running light entrainable rhythm may alter the PAD between activity onset and the onset of food access. For three rabbits, a trend was apparent in that PAD increased as the free-running light entrainable rhythm crossed the feeding schedule. As seen in Figure 34, this tendency was observable as a phase advance of anticipatory activity in some cases. A tendency for succeeding activity to phase delay toward the free-running light entrainable rhythm was also noted. This effect occurred regularly, giving the appearance of relative coordination.

For T=30h, only one rabbit satisfied the criteria for anticipatory activity, but only when the analysis period was extended from 3h to 5h. Visual inspection of the actograms indicated that anticipatory activity was clearly expressed in water counts, but was less likely to occur in the other DVs. The decision was thus made to use water counts as the DV in subsequent analyses. The author acknowledges that comparisons across DVs may be tenuous.

The criteria used previously did not effectively identify anticipatory activity for water counts that had been previously identified by visual inspection. To overcome this problem, the actograms were examined visually in order to assess the effects of the feeding schedule. Data were plotted on a 30h time scale for easier visual assessment of anticipatory activity. The number of animals showing anticipatory activity was five. Actograms of these subjects are presented in Figure 35. Anticipatory activity appeared after nine to 23 days, with a mean of 16 days (*SD*=6.58). The inter-rater correlation for estimates of PAD was non-significant (r=.74, *p*>.05) however, the decision was made to report data from one rater for the reason described earlier. PAD ranged from 5.75h to 9.75h, with a mean of 7.45h (*SD*=1.90).

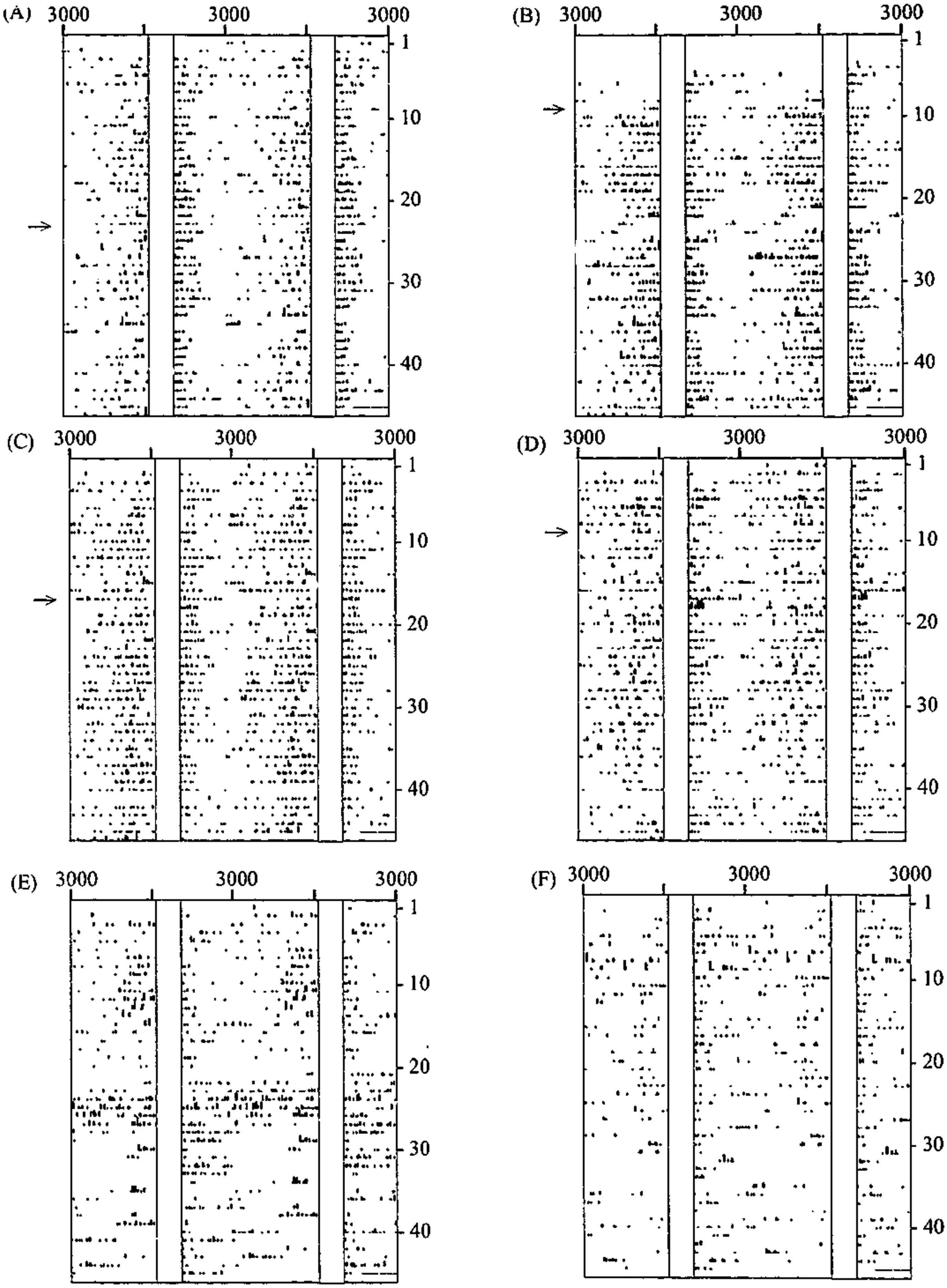


FIGURE 35. DOUBLE PLOTS OF WATER COUNTS ILLUSTRATING ANTICIPATORY ACTIVITY TO A FEEDING SCHEDULE WITH T=30H IN FOUR RABBITS. (A) SUBJECT #9. (B) SUBJECT #10. (C) SUBJECT #14. (D) SUBJECT #15. NO ANTICIPATORY ACTIVITY CAN BE SEEN FOR SUBJECT #'S 12 (E) AND 13 (F). DATA ARE PLOTTED AT A DAY LENGTH CORRESPONDING TO THE FEEDING SCHEDULE. RESTRICTED FEEDING CYCLES ARE INDICATED ON THE RIGHT SIDE OF THE ACTOGRAMS. ARROW HEADS INDICATE EMERGENCE OF ANTICIPATORY ACTIVITY.

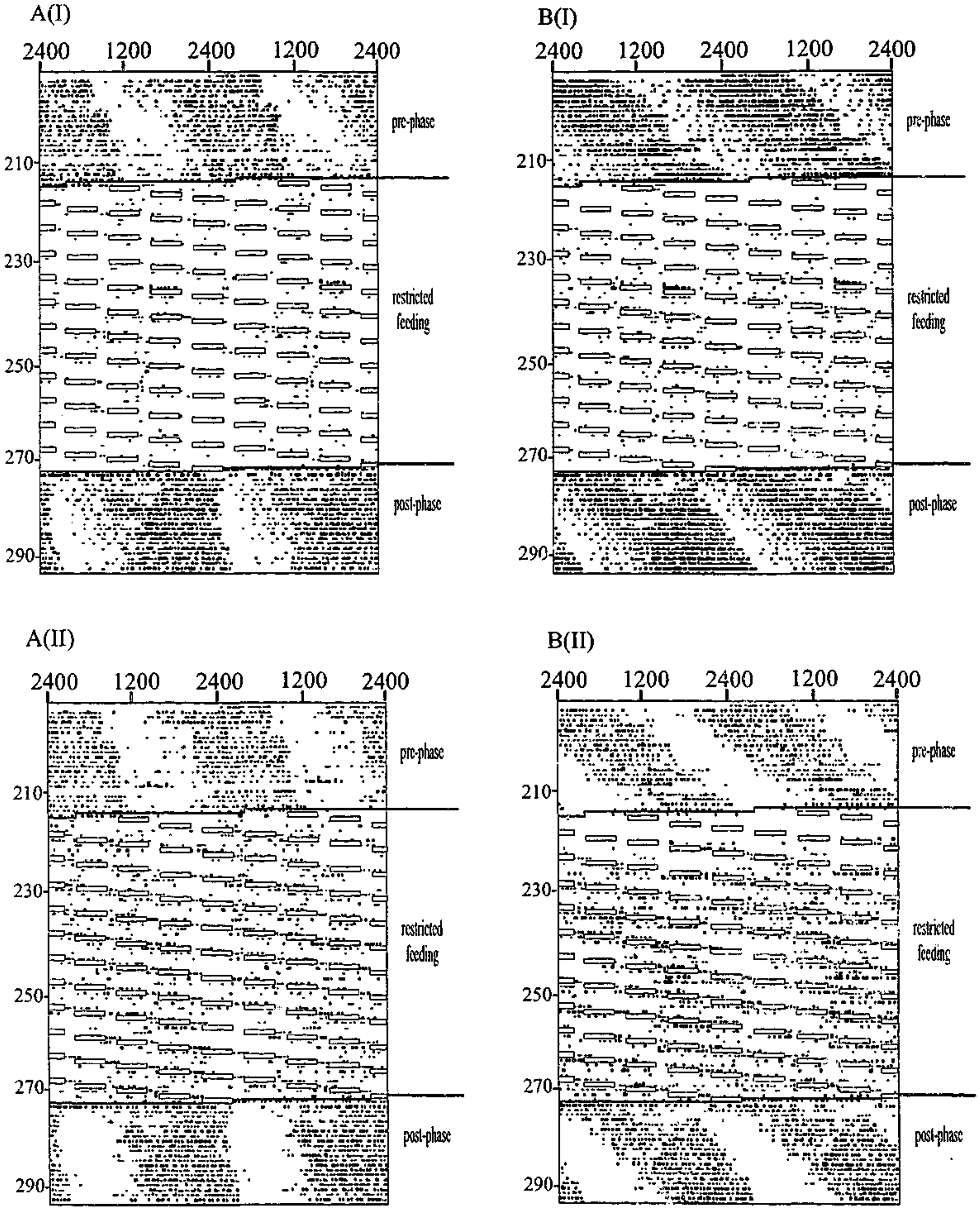


FIGURE 36. DOUBLE PLOTS OF FOOD COUNTS (I) AND WATER COUNTS (II) FROM FOUR RABBITS EXPOSED TO A FEEDING SCHEDULE WITH T=30H. (A) SUBJECT #9. (B) SUBJECT #10. (C) SUBJECT #13. (D) SUBJECT #15. CONVENTIONS AS FOR FIGURE 11.

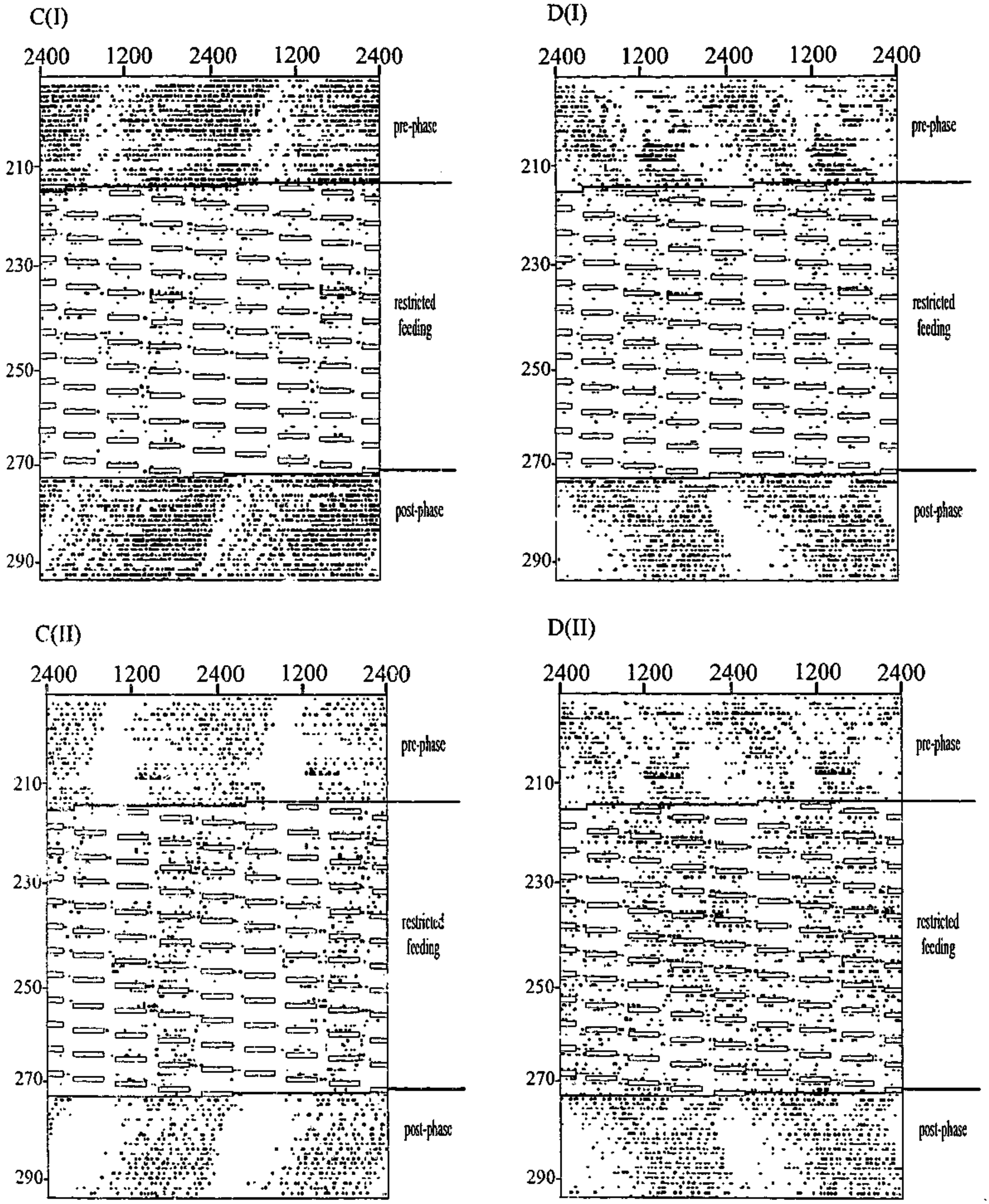


FIGURE 36 CONTINUED

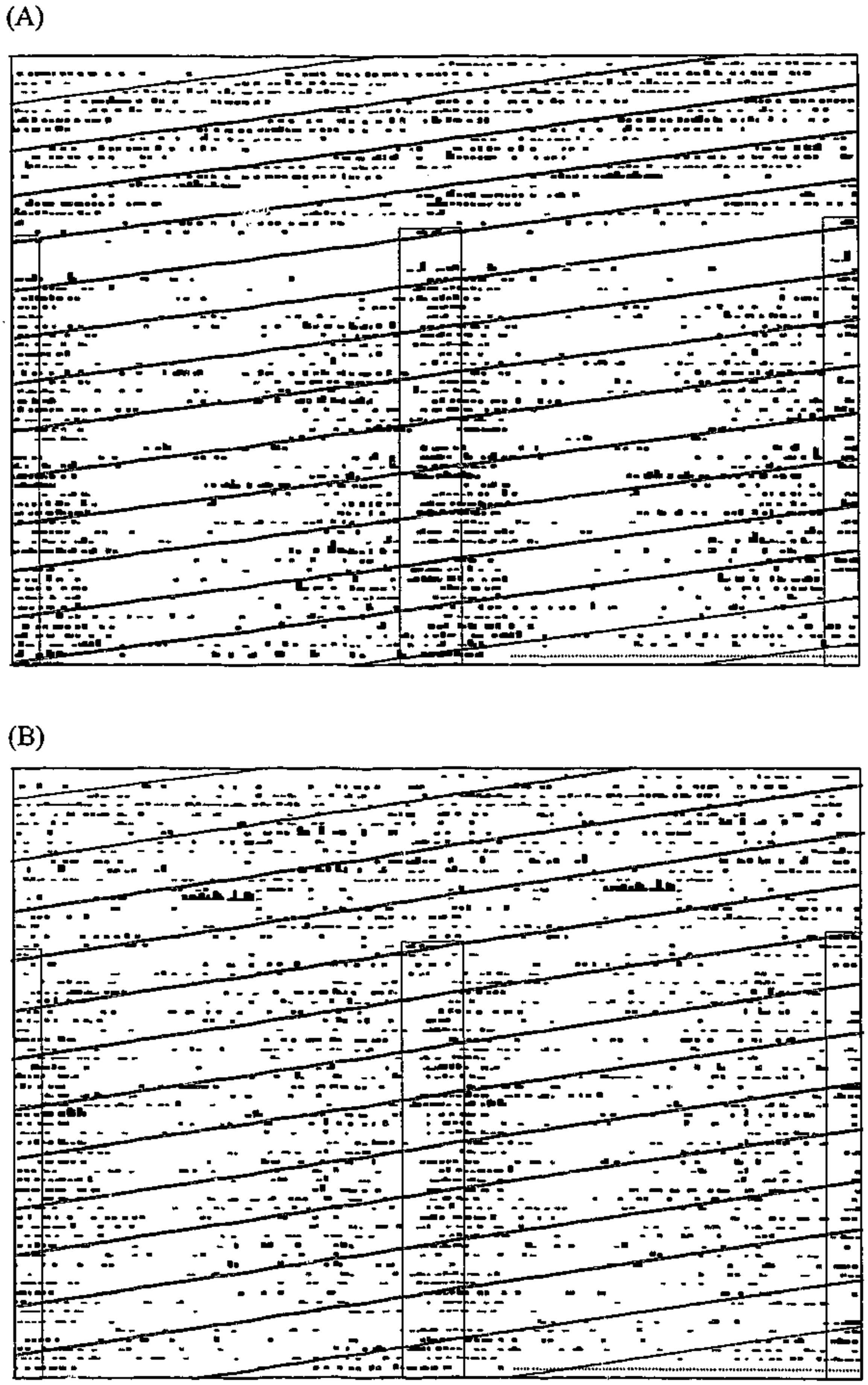


FIGURE 37. DOUBLE PLOTS OF WATER COUNTS FROM 2 SUBJECTS EXPOSED TO A FEEDING SCHEDULE WITH T=30H. (A) SUBJECT #2. (B) SUBJECT #8. DATA ARE PLOTTED AT A DAY LENGTH CORRESPONDING TO THE FEEDING SCHEDULE. FOOD ACCESS IS DEPICTED BY VERTICAL BARS. REGRESSION LINES HAVE BEEN DRAWN THROUGH ACTIVITY ONSETS DURING THE PRE-PHASE AND EXTRAPOLATED FORWARD TO THE FINAL DAY OF RESTRICTED FEEDING.

Free-running light entrainable rhythms were visible during the feeding schedule in general activity and/or water counts for each rabbit, but not in food counts. As seen in Figure 36, the free-running light entrainable rhythm was less discernable during the feeding schedule than the pre-phase (*ad libitum* 3) and the post-phase (*ad libitum* 4). Forward extrapolation of regression lines between the pre- and post- phase for food counts (the same process was used as described earlier) indicated that phase changes of the free-running light entrainable rhythm were common. Another common finding was that the PAD between activity onset and the onset of food access was affected by the free-running rhythm: PAD increased as the free-running light entrainable rhythm crossed the feeding schedule. Also consistent with earlier results was the finding that succeeding activity appeared to phase delay toward the free-running light entrainable rhythm in these subjects. This is illustrated in Figure 37.

In order to ascertain whether the free-running light entrainable rhythm was influenced by the feeding schedules, tau for *ad libitum* 2, 3, and 4 was calculated. A line of best fit was placed along activity offsets over the final 20 days of each phase. This was done separately by each rater and the scores were then correlated. Inter-rater reliability as determined by Pearson's product-moment correlations was highly significant ($r=.81$, $p<.01$). Data were averaged across both raters for use in the following analysis. Mean (+ SE) estimates of free-running tau by phase are shown in Table 5.

TABLE 5.

TAU OF THE FREE-RUNNING LIGHT ENTRAINABLE RHYTHM BY PHASE FOR T>24H

	Ad libitum 2	*Ad libitum* 3	*Ad libitum* 4
Mean	24.22	24.28	24.23
+SE	0.09	0.09	0.12

As shown in Table 5, mean tau changed little across the experiment. A repeated measures ANOVA indicated that mean tau did not differ across the experiment, $F(2,12)=0.29$, $p>.05$.

8.4. DISCUSSION

The aim of this study was to examine the effects of varying feeding cycle Ts on the circadian system of the herbivorous rabbit, with a view to satisfying the criteria for a zeitgeber. To this end, it must be shown that anticipatory activity is possible only if feeding schedule T does not deviate too far from 24h. The following discussion is divided into two parts. In the first part of the discussion, the effects of daily feeding schedules are considered with reference to both the feeding entrainable rhythm and the light entrainable rhythm. The purpose of part two is to consider the range of periodicities to which activity can be entrained to feeding schedules in the rabbit.

8.4.1. Entrainment to 24h cycles of food availability

The results of the study support the view that feeding schedules act as effective zeitgebers in the rabbit, with nine of 15 rabbits showing anticipatory activity. This figure is slightly lower than those cited in omnivores and carnivores, and suggests that anticipatory activity is more readily expressed in omnivores and carnivores compared to herbivores. This conclusion should be treated with caution however, since the criteria used to identify anticipatory activity in the present thesis has not been used before. As noted earlier, there is no consistent agreement as to how to identify anticipatory activity, and no attempt to establish uniform procedures has been made. The author acknowledges that, in the absence of an established criterion, comparisons between the three classes of species may be tenuous. It seems reasonable to expect however, that the criterion used has little effect on research outcomes, since the percentage of rats reported to entrain to feeding schedules is reasonably consistent across studies. Worthy of mention are the findings by Stephan and Becker (1989) who used a procedure similar to the one used here and reported anticipatory activity in 12 of 15 rats exposed to a feeding schedule with 4h food access.

These present results support those of Jilge and coworkers (1987, 1991, 1993) who reported that the FEP of the rabbit could be entrained to a feeding schedule, and the PAD was positive. These results are broadly comparable to findings with other species however, these researchers report PADs as long as 6h to 8h in both omnivores and carnivores (Aschoff et al., 1983; Davidson & Stephan, 1999; Kennedy et al., 1995; Lax et al., 1999; Mistlberger & Marchant, 1995; Ruis et al., 1989; Stephan, 1981, 1984; Stephan & Becker, 1989). This was not the case in the present study with activity onset occurring 1h to 3h prior to the meal, with a mean of 1h 37 minutes. One possible explanation for this inconsistency is related to species differences in tau of the FEP. Aschoff and coworkers (Aschoff & Wever, 1962; Aschoff, 1965a; Aschoff & Pohl, 1978) argued that both tau of the pacemaker and T affect PAD: the longer T is and the shorter tau is, the greater the postive PAD in the entrained state, and conversely PAD is more negative (or less positive) the longer tau is and the shorter T is. Therefore, it follows from Aschoff's work, in the rabbit, a longer tau of the FEP would make the PAD less positive than in other species. Another possible explanation for the discrepancies in PAD is related to the range of methodologies used. In the current study, PAD was derived by drawing a line of best fit along activity onsets over the final 10 days of the feeding schedule. Many researchers do not specify the method used to calculate PAD, but at least some researchers have used a different method (Aschoff et al., 1983; Ruis et al., 1989; Stephan, 1981). The problem is that no method has been used consistently and therefore, comparison to previous research is problematic. While the effects of different methods may be difficult to evaluate, the results of the present study suggest that the method used to quantify anticipatory activity may effect research outcomes; PAD estimates between activity onset and the onset of food access were found to be positively related to T, whereas the relationship was non-linear for the anticipatory ratio.

Results of the present study indicate that anticipatory activity developed within 46 days. This is generally consistent with previous research by Jilge (1991), although onset varied between 10 and 65 days. This did not occur in the present study with less variability being apparent. Other researchers have reported a shorter latency of two to seven days for omnivores and carnivores under LD conditions, and a slightly longer latency of 10 days in LL (Aschoff et al., 1983; Honma et al., 1983; Kennedy et al., 1991; Mistlberger & Marchant, 1995; Rossenwasser et al., 1984; Stephan, 1979a, 1986a,b). The differential effect of ambient lighting (i.e., LD cycles) could be explained by the internal coupling hypothesis detailed in the previous chapter. Although the difference between present findings and those of previous researchers is difficult to interpret, the much higher figure obtained in the present study is unlikely to be attributable solely to differences in methodologies. It should be noted that the present findings are not surprising given that similar results have been documented by Jilge (1991). These results support the view that a feeding schedule is a stronger zeitgeber in omnivores and carnivores than herbivores. It is notable that the latency to anticipatory activity did not appear to vary as a function of feeding schedule T for food counts. To the author's knowledge, there are no published data available on this topic.

Results from this study are suggestive of a multi-oscillator model of the circadian system in the rabbit. The results indicated that almost all rabbits expressed a non-24h free-running light entrainable rhythm in addition to anticipatory activity. The fact that the circadian rhythms separated into two distinct bands of activity with different periods can be taken as evidence for a dual FEP-LEP model. This is suggested by Stephan (1986a,b,c) who argued that a single pacemaker cannot readily express two or more periodicities. This finding is comparable to previous findings with omnivores and carnivores of multiple periodicities (Aschoff et al., 1983; Cambras et al., 1993; Edmonds & Adler, 1977a; Honma et al., 1983; Stephan, 1986a,b; Stephan & Becker, 1989).

Also relevant to the present study is the finding by these researchers that feeding schedules masked the overt rhythms in some cases. In the current study, masking effects appeared to be operating in some of the data, particularly for food counts. There were two examples of masking in the present study which are as follows. Firstly, most of the rabbits adapted to taking meals during the feeding schedule within one to two days. The immediacy of this effect suggests that masking was operating in the data. Similar results were obtained by Jilge (1991) who argued that an immediate adjustment to restricted feeding is advantageous for the rabbit which is highly susceptible to digestive disturbance caused by changes in food availability. The immediate masking of overt rhythms by the feeding schedule thus prevents digestive malfunction and weight loss. Secondly, rhythm free-runs were visible during restricted feeding in water counts and general activity, but not food counts. This finding suggests that activity associated with the free-running light entrainable rhythm may be suppressed by the feeding schedule (or some other aspect of the experimental situation) and hence the overt rhythm is not always an accurate indicator of the phase of the underlying pacemaker. That the free-running light entrainable rhythm is more readily suppressed in food counts than the other DVs is difficult to explain, but could reflect a greater tendency for masking to occur in events directly involved in the behavioural acquisition of food. Similar findings were obtained by Jilge and colleagues (Jilge, 1991; Jilge et al., 1987) who reported stronger masking by periodic feeding in some rhythms compared to others.

The free-running light entrainable rhythm appeared to be entrained to the feeding schedule in one rabbit only. This conclusion is tentative and should be treated with caution since tau modulation of the free-running light entrainable rhythm was not demonstrated. Nonetheless, this figure is lower than those reported in previous results based on omnivores and carnivores. The current results are similar to previous findings with the rat in which tau-T was less than 10 minutes when entrainment occurred. The present data show evidence in support of this relationship, with a free-running tau of (or very close to) 24h in the entrained rabbit. These results are generally consistent with the work by Jilge and colleagues (Jilge, 1991; Jilge et al., 1987) who observed entrainment of the free-running light entrainable rhythm in some rabbits but not others, and suggest that a feeding schedule is a weak zeitgeber for the LEP in this species. Stephan (1986a,b) has argued that this effect is mediated indirectly by coupling between the FEP and the LEP. Research consistent with this interpretation is reviewed in section 2.4.2. The differences between the results of the above-cited studies can be explained in terms of the strength of coupling between the FEP and the LEP. According to Stephan, entrainment of the free-running light entrainable rhythm occurs when coupling strength is sufficiently strong to entrain the LEP. Following this, coupling strength is postulated to be weaker in the rabbit than the rat, and is strongest in the carnivore.

The finding that changes in tau of the free-running light entrainable rhythm occur on removal of the feeding schedule is consistent with the above interpretation. Changes in tau were such that tau approached T in the post-phase. It is notable that tau close to T increases the likelihood of a *false-positive* identification that entrainment had occurred. Similar results have been previously reported in a large number of species, including omnivorous and carnivorous species (Kennedy et al., 1991; Mistlberger & Marchant, 1995; Stephan, 1981). This effect is likely to be due to aftereffects caused by exposure to the feeding schedule. The fact that aftereffects were found to persist throughout the post-phase is consistent with the work by Jilge and Stahle (1993) who reported that aftereffects typically last for up to 50 days in the rabbit. Other evidence in favour of the coupling hypothesis comes from the finding in this study of phase shifts of the free-running light entrainable rhythm on removal of the feeding schedule, occurring in all 15 rabbits. These results should be treated with caution, since the observed changes in tau may affect the phase of the free-running light entrainable rhythm. The present findings are consistent with past research. However, this figure is higher than previous results based on omnivores and carnivores. The reason for this discrepancy is unclear. Thus, despite the low incidence of entrainment of the free-running light entrainable rhythm by the feeding schedule, these results show that the pacemakers are not entirely independent.

The results of this experiment give further support to the hypothesis that anticipatory activity is an endogenously generated circadian rhythm in the rabbit. The finding that residual meal-associated activity persisted on removal of the feeding schedule is consistent with this hypothesis. This finding agrees with those of some researchers but not others; meal-associated activity is generally absent by the second day of *ad libitum* feeding or dissipates over three to four days when food restricted rats are fed *ad libitum* (see Mistlberger, 1994 for a comprehensive review; see also section 2.3.3.). This was not the case in the present study, with the majority of rabbits displaying two bouts of activity on removal of the feeding schedule that merged after approximately 10 days; meal-associated activity and a free-running light entrainable rhythm. The reason for this inconsistency is unclear. The fact that meal-associated activity persisted for 10 days supports the view that the FEP has a limited capacity to free-run under *ad libitum* conditions.

8.4.2. Entrainment to non-24h cycles of food availability

The results of this study support the view that anticipatory activity is an endogenously driven circadian rhythm. As expected, anticipatory activity developed only within a limited range of feeding schedule Ts. Although the present study was not designed to define precisely the range of entrainment, the results indicated that anticipatory activity developed to T cycles of 23h to 27h, but could not be observed at schedules of 21h. At 30h, anticipatory activity was found to occur in water counts, but not food counts. This suggests that 30h approaches the upper limit of entrainment in the herbivorous rabbit. These findings are similar to those of Stephan (1981), but inconsistent with other researchers who have found an upper limit <29h (Aschoff et al., 1983; Bolles & deLorge, 1962; Bolles & Stokes, 1965; Boulos et al., 1980; Mistlberger & Marchant, 1995; Stephan, 1979a, b). This discrepancy is most likely due to the different methods used. In the current study, feeding cycle T was changed sequentially from 24h to 27h to 30h. Gradual changes in feeding schedule T are known to extend the limits of entrainment (Stephan, 1981). This effect was attributed to aftereffects caused by prior exposure to feeding schedules; presumably because entrainment effects both tau and the PRC of the underlying pacemaker. The present findings suggest that external stimuli with a period of 24h are not necessary to establish anticipatory activity.

The large upper boundary and the substantial asymmetry of entrainment limits with regards to 24h is possibly the result of the phase response characteristics of the FEP. It is well established that the limits of entrainment to LD cycles depend on this feature; if the area under the phase delay section of the PRC is larger than the area under the phase advance section, some asymmetry in the direction of long periods is expected. It follows from this work, as stated by Stephan (1981), a PRC with a large delay section and a small advance section would account for the observed asymmetry in the rat. While this may be true, further studies are necessary to quantify PRC shape. Some studies have used rats as subjects and reported a PRC characterized by a large delay section and a small advance section (Coleman et al., 1989, 1991). This conclusion should be treated with caution however, since it is not clear at this stage whether the methods used are valid. This issue was discussed in detail in section 2.3.7 and will not be considered again here.

PAD between activity onset and the onset of food access varied as a function of feeding cycle T. A linear relationship between PAD and feeding schedule T was found; PAD became increasingly positive with increasing T within the limits of 23h and 30h. These results are consistent with those obtained in omnivores (Aschoff et al., 1983) and suggest that the onset of anticipatory activity reflects the phase angle at which the FEP is coupled to the mealtime. Given that activity onsets in the current study were not always precisely defined, interpretations based on PAD should be made with caution.

In order to maintain comparability to past research, anticipatory activity in the present study was quantified using Stephan's anticipatory ratio. The highest anticipatory ratio was found to occur for T=24h, followed by T=23h and T=27h. These results are inconsistent with the work by Aschoff et al. (1983) who found the anticipatory ratio to correlate positively with feeding schedule T. These results are comparable with those of Stephan (1981) in that the anticipatory ratio was highest for T=24h. However, unlike the Stephan study, ratios in the present study were lower for T=27h than T=23h. In comparing across studies, it is important to note that Stephan's work differed from the other studies in that he investigated SCN-lesioned animals. As in the study by Aschoff et al. (1983), estimates of anticipatory ratio in the present study may have been confounded by the free-running rhythm.

In all conditions chosen for this experiment, the free-running light entrainable rhythm was visible during the feeding schedule with few exceptions. These findings suggest that the free-running light entrainable rhythm did not entrain to cycles of food availability in the herbivorous rabbit, which is consistent with findings by Aschoff et al. (1983) who investigated the omnivorous rat. The pacemakers do not appear entirely independent however, as phase shifts of the free-running light entrainable rhythm were common. These findings are consistent with Stephan's coupled pacemaker hypothesis, and suggest that coupling strength between the FEP and the LEP is weak. Other evidence in favor of this hypothesis comes from the finding in this study of a dependence of the duration of anticipatory activity on the PAD between the LEP and the FEP; the duration of anticipatory activity increased as the free-running light entrainable rhythm crossed food access for feeding schedules with T=27h and T=30h. Given that the free-running light entrainable rhythm caused the bout of anticipatory activity to become more advanced (giving the appearance of relative coordination), a likely explanation for such an effect is that the FEP was temporarily forced out of entrainment because it is coupled to the LEP. The fact that activity succeeding the mealtime delayed towards the free-running rhythm when the two rhythms crossed also supports this theory, based on the assumption that succeeding activity represents the output of the trailing end of the FEP.

The present findings indicated a strong trend in the data; despite the fact that rabbits did not satisfy the criteria for entrainment of the free-running light entrainable rhythm to the feeding schedule, phase control of the rhythm by the feeding schedule in the post-phase was demonstrated. Such an effect is difficult to interpret, but the consistency of this effect would suggest a profound influence of feeding schedules on the light entrainable rhythm. One possible explanation is that the feeding zeitgeber had a differential effect on the multi-oscillatory LEP. As noted in section 1.2.3, there is strong evidence to suggest that the LEP is composed of two oscillators that normally oscillate in a coupled state. It is widely accepted that under certain circumstances the coupling breaks down and the two oscillators exist with different periods and phases. It is possible that feeding schedules cause a similar break down in the coupling between the two oscillators, such that one oscillator becomes entrained to the feeding schedule, while the other continues to free-run. This explanation is purely speculative; further research is required to resolve this issue. Interestingly, Lax and coworkers (1999) recently proposed a similar explanation to account for their results. This is an important issue and will be considered again in chapter 9.

With only one exception, tau of the free-running light entrainable rhythm remained virtually unchanged. Tau increased significantly between the pre- and post- phase for T=21h. One factor that limits the interpretation of these data is that the rabbits had been previously exposed to a 24h feeding schedule. The problem with this design is that tau in the pre-phase was most likely confounded by aftereffects caused by prior exposure to T=24h. Thus, subsequent lengthening of tau (and return to baseline values) in the post-phase may have reflected the influence of the previous entraining cycle. The fact that tau of the free-running light entrainable rhythm was generally unaffected across the experiment supports the view that feeding schedules do not entrain the LEP in this species.

While every attempt was made to identify and rectify problems quickly, mechanical failure of equipment could not be avoided. This resulted in days of *ad libitum* food access during restricted feeding. This occurrence was common for feeding schedules with T=23h and T=30h, but not for other T cycles. To the author's knowledge, similar occurrences have not been reported in the published literature. This is an important issue, since it is unclear whether the present results represent the true range of entrainment in the herbivorous rabbit. It is thus essential in future studies to replicate present findings.

8.4.3. Conclusion

The results of this study further support the view that feeding schedules act as effective zeitgebers for the circadian system in the herbivorous rabbit, by demonstrating that anticipation is possible only within a limited range of feeding schedule Ts.

CHAPTER 9. ENTRAINMENT OF CIRCADIAN RHYTHMS TO MULTIPLE FEEDING TIMES IN THE HERBIVOROUS RABBIT

9.1. RATIONALE AND AIMS

Multi-oscillatory models of the circadian system are widely accepted on the basis of functional evidence. For example, the most commonly held view that the LEP consists of two mutually coupled oscillators is based on findings of rhythm splitting, as well as the fact that most species show two major peaks at the beginning and at the end of activity (see section 1.2.3). The hypothesis that the feeding entrainable circadian system comprises two oscillators is also consistent with functional evidence. That the omnivorous rat entrains to two but not three daily meals is one line of evidence used to support this hypothesis. Relevant research is reviewed in section 2.4.3.

As noted in chapter 3, the necessity of a comparative approach has long been recognized. However, the rat is still the most commonly used research subject. This point is illustrated here. While findings in the rat might be best explained by postulating the existence of two feeding entrainable oscillators, the generalizability of the results is unclear. Such studies have not been conducted with other species; it remains to be established whether this effect is a generalized one, or whether it occurs specific to the omnivorous rat. It is possible that the FEP is not similar across mammalian species in the same way that the LEP is, and the rat is thus not representative of all mammalian species. The rabbit provides an opportunity to investigate the oscillatory properties of the feeding entrainable circadian system and to make comparisons to the omnivorous rat.

The aim of this study was to examine the effects of multiple feeding times on the circadian system in the herbivorous rabbit. Rabbits were exposed to two and three daily meals separated by 8h. The decision was made to limit daily food intake to 60g (2 meals x 30g; 3 meals x 20g) in order to avoid the possible confounding effects of meal calorific content (see section 2.4.4). Based on previous research with the rat, it was hypothesized that rabbits would anticipate two daily meals, but not three daily meals.

EXPERIMENT 1

9.2. METHOD

9.2.1. Animals and housing

Subjects were 12 female Dutch rabbits aged 1 year 2 months. Weight ranged between 3kg and 4.4kg at the start of the experiment. Rabbits were housed under the conditions described in sections 5.2.1 and 5.2.2.

9.2.2. Procedure

The experiment consisted of three phases. In the pre-phase, food and water were available *ad libitum* for 30 days. During restricted feeding which lasted for 89 days, rabbits received two daily meals separated by eight hours. Each meal consisted of 30g of pelleted mix and was 1.5h long. In the post-phase, rabbits were deprived of food for three days. This was followed by 30 days of *ad libitum* food access. In accordance with guidelines set by the Psychology Department Animal Experimentation Ethics Committee at Monash University, the laboratory was entered before food reinstatement in order to weigh rabbits and check their general well-being.

9.2.3. Design

Phase was the independent variable. Phase was a repeated measures variable consisting of three levels; pre-phase, restricted feeding, post-phase. The dependent variables included: PAD between activity onset and the onset of food access, and phase and tau of the free-running light entrainable rhythm. Activity was defined in terms of food and water counts. Data for general activity were not available due to equipment failure.

9.3. RESULTS

The process described in section 5.5.3 was used to identify anticipatory activity. Consideration of graphical distributions indicated that three rabbits satisfied the criteria for anticipatory activity to both feeding times. Actograms of these subjects are presented in Figure 38. Two rabbits demonstrated anticipatory activity to one

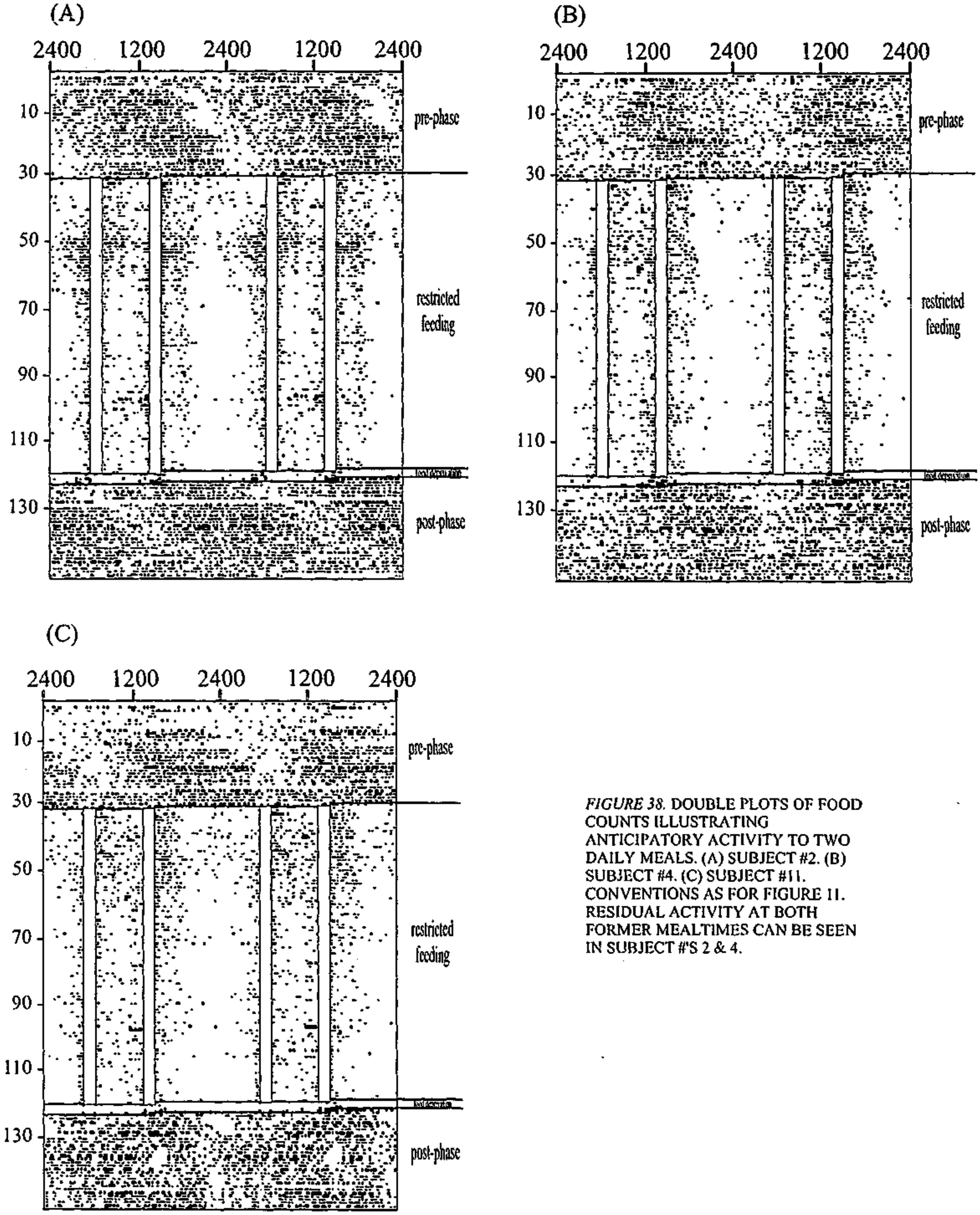


FIGURE 38. DOUBLE PLOTS OF FOOD COUNTS ILLUSTRATING ANTICIPATORY ACTIVITY TO TWO DAILY MEALS. (A) SUBJECT #2. (B) SUBJECT #4. (C) SUBJECT #11. CONVENTIONS AS FOR FIGURE 11. RESIDUAL ACTIVITY AT BOTH FORMER MEALTIMES CAN BE SEEN IN SUBJECT #'S 2 & 4.

feeding time, while the remaining rabbits showed no evidence of entrainment. Visual inspection of the actograms for food counts however, indicated that all rabbits showed some anticipatory activity to at least one mealtime during the 90 days in this condition. Data for water counts were similar to those for food counts, although anticipatory activity was less likely to occur in water counts.

Two raters were used to estimate PAD between activity onset and the onset of food access for rabbits showing anticipatory activity to both feeding times. Inter-rater reliability was examined using Pearson's product-moment correlations. The results indicated a highly significant inter-rater correlation ($r=.94, p<.01$) and no systematic differences between raters' estimates were found. The data from one rater was thus randomly selected and is reported here. Estimates of PAD ranged from 0.5h to 3h, with an overall mean of 1.54h (*SD*=1.01). Mean PAD was 1.08h (*SE*=0.38) and 2h (*SE*=1.3) to the leading (at the end of 16h) and trailing (at the end of 8h) meals, respectively.

In order to ascertain whether tau of the free-running light entrainable rhythm was influenced by aftereffects to the feeding schedule, tau for the pre- and post- phase was calculated for food counts. A line of best fit was placed along activity offsets over the final 20 days of each phase. This was done separately by each rater and the scores were then correlated. Inter-rater reliability was examined using Pearson's product-moment correlations. The inter-rater correlation was highly significant ($r=.87, p<.01$). As noted previously, the decision was made to use the average of the raters' estimates in statistical analyses. Mean (+*SE*) estimates of free-running tau by phase are shown in Table 6.

TABLE 6.

TAU OF THE FREE-RUNNING LIGHT ENTRAINABLE RHYTHM BY PHASE.

	Pre-phase	Post-phase
Mean	24.38	24.44
SE	0.45	0.26

A related samples *t*-test revealed that estimates of tau in the pre- and post- phase did not differ significantly from each other, $t(11)=0.36, p>.05$.

General patterns in the data were established through visual inspection of the actograms for food and water counts. The free-running light entrainable rhythm was not visible during the feeding schedule, but a well-defined rhythm was established in the post-phase. In the majority of the data, post-phase free-runs were predicted by one or both meals. This is illustrated in Figure 39. For some rabbits however, it could not be reliably determined whether or not the post-phase rhythm was consistent with pre-entrainment. The main problem was that activity onsets could not be determined in the pre-phase due to poorly defined onsets. In some cases, the reference phase of restricted feeding coincided by chance with the extrapolated phase of the free-running light entrainable rhythm during pre-entrainment. For at least five rabbits however, the free-running light entrainable rhythm appeared to become entrained to the feeding schedule. Figure 39 contains actograms of some of these subjects. Regression lines computed through activity offsets over the last 20 days of the pre-phase and extrapolated forward to the first day of the post-phase for food counts indicated that phase changes in the free-running light entrainable rhythm had occurred in all rabbits.

Visual inspection of the actograms indicated that eight of 11 rabbits displayed meal-associated activity to both feeding times during food deprivation for food counts. A general trend was apparent in the data for these rabbits. Activity associated with the leading meal appeared to merge with the onset component of the free-running light entrainable rhythm, while activity associated with the trailing meal showed what appeared to be a series of small advancing transients towards the offset component of the free-running light entrainable rhythm. The two rhythms then merged. This is illustrated in Figure 39. The results were similar for water counts.

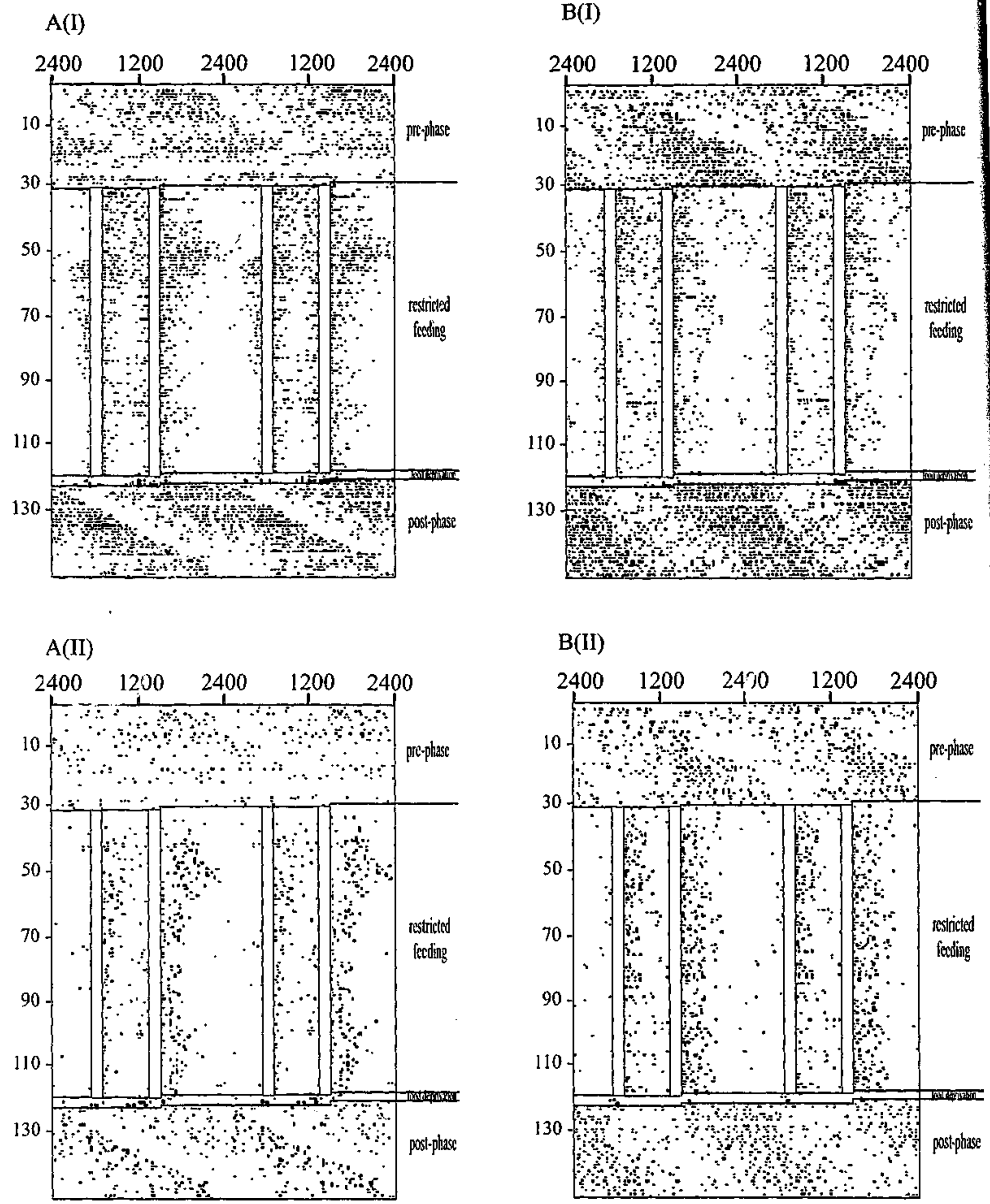


FIGURE 39. DOUBLE PLOTS OF FOOD COUNTS (I) AND WATER COUNTS (II) FOR SIX RABBITS EXPOSED TO TWO DAILY MEALTIMES. (A) SUBJECT #1. (B) SUBJECT #5. (C) SUBJECT #6. (D) SUBJECT #7. (E) SUBJECT 10. (F) SUBJECT #12. CONVENTIONS AS FOR FIGURE 11. ENTRAINMENT OF THE FREE-RUNNING LIGHT ENTRAINABLE RHYTHM BY THE FEEDING SCHEDULE CAN BE SEEN IN SUBJECT #'S 5, 6, 10, & 12.

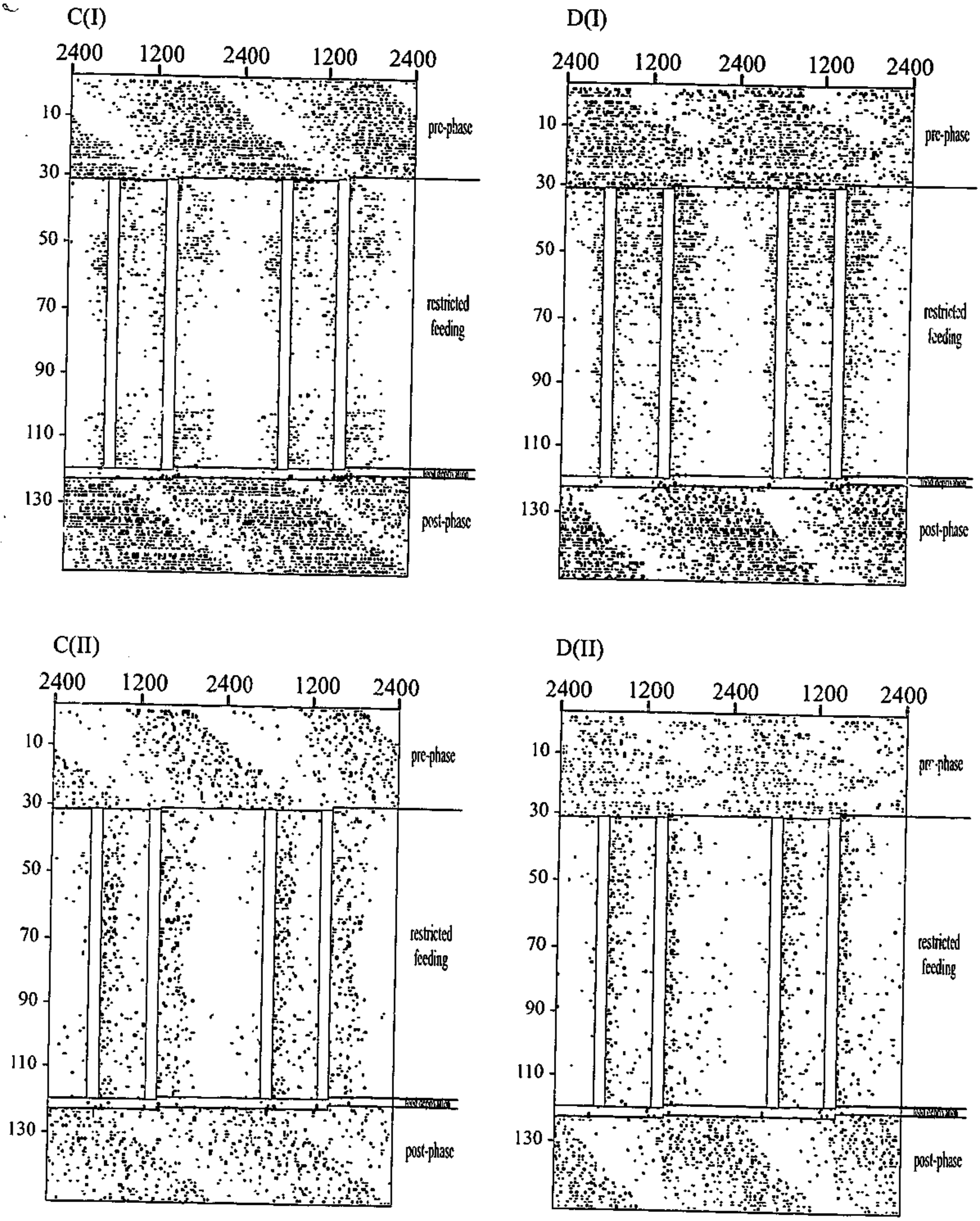


FIGURE 39 CONTINUED

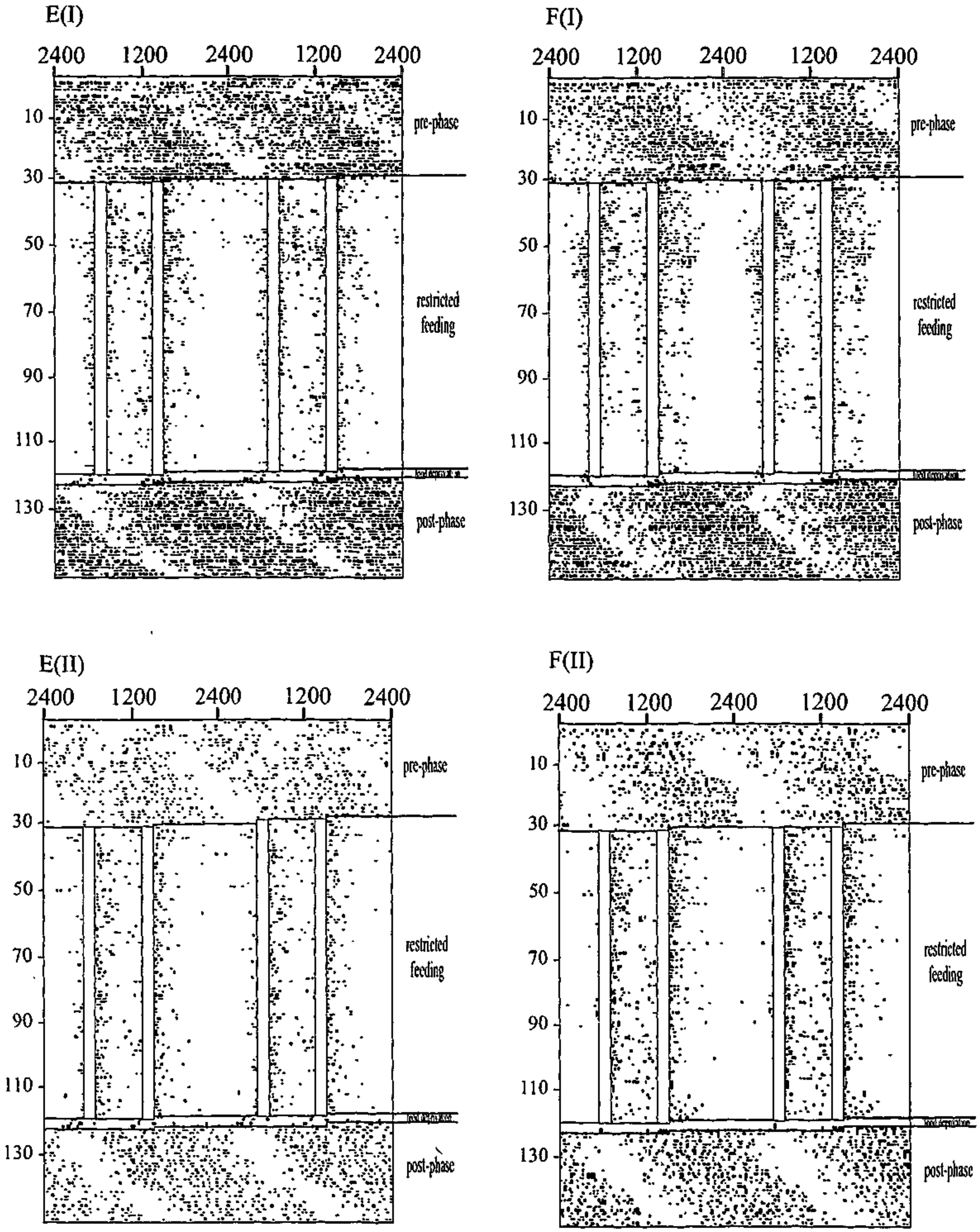


FIGURE 39 CONTINUED

EXPERIMENT 2

9.4. METHOD

9.4.1. Animals and housing

Subjects were 12 female Dutch rabbits aged 1 year 10 months. Weight ranged between 3kg and 5kg at the start of the experiment. Rabbits were housed under the conditions described in sections 5.2.1 and 5.2.2. These rabbits had been used previously in experiment 1.

9.4.2. Procedure

The experiment consisted of three phases. In the pre-phase, food and water were available *ad libitum* for 30 days. During the feeding schedule, rabbits received three daily meals, each consisting of 20g of pelleted mix. Food was made available for 1h at 0400h, 1200h, and 2000h over a period of 88 days. In the post-phase, rabbits were deprived of food for three days. This was followed by 30 days of *ad libitum* food access. In accordance with guidelines set by the Psychology Department Animal Experimentation Ethics Committee at Monash University, the laboratory was entered before food reinstatement in order to weigh rabbits and check their general well-being.

9.4.3. Design

Phase was the independent variable. Phase was a repeated measures variable consisting of three levels; pre-phase, restricted feeding, post-phase. The dependent variables were phase and tau of the free-running light entrainable rhythm. Activity was defined in terms of general activity, and food and water counts.

9.5. RESULTS

Visual inspection of graphical distributions for food counts, in addition to examination of actograms, indicated that rabbits failed to meet the criteria for anticipatory activity. Representative actograms are presented in Figure 40. With only one exception, there was no evidence of anticipatory activity at all. Subject #5 demonstrated anticipatory activity to the 1200h meal which persisted during subsequent food deprivation. The results were similar for water counts. The actograms for general activity were different from those for food and water counts in that anticipatory activity appeared when the free-running light entrainable rhythm crossed food access for most of the rabbits. This is illustrated in Figure 41.

Visual inspection of the actograms indicated that the free-running light entrainable rhythm was not apparent during the feeding schedule for food and water counts, but clearly discernable free-runs were evident in general activity for most rabbits. This is illustrated in Figure 41. No free-running activity could be seen in three rabbits: for subject #'s 9 and 10, post-phase free-runs appeared to be largely determined by the feeding schedule, and not pre-entrainment. Actograms of these rabbits are presented in Figures 42. For the remaining rabbit, post-phase control of the rhythm by the feeding schedule was not demonstrated. It is notable that activity onsets were difficult to identify due to poorly defined onsets in these rabbits. Regression lines computed over the last 20 days of the pre-phase and extrapolated forward to the first day of the post-phase for food counts indicated that phase changes of the free-running light entrainable rhythm were common, occurring in nine of 12 rabbits.

Residual meal-associated activity during deprivation was apparent in five rabbits for food counts, but was associated with the 1200h meal only. This activity however, is most likely due to laboratory entry which occurred during this time. In subject #'s 8 and 9, activity could be separated into at least two bands corresponding to two former mealtimes. The results for water counts were slightly different, with three rabbits showing residual activity to the 1200h and 2000h mealtimes. Furthermore, subject #4 exhibited meal-associated activity at all three mealtimes, but was also active between the 1200h and 2000h meals. The data for general activity were difficult to interpret due to the overlap between the anticipatory rhythms and the free-running light entrainable rhythm. It was therefore decided not to consider these data here.

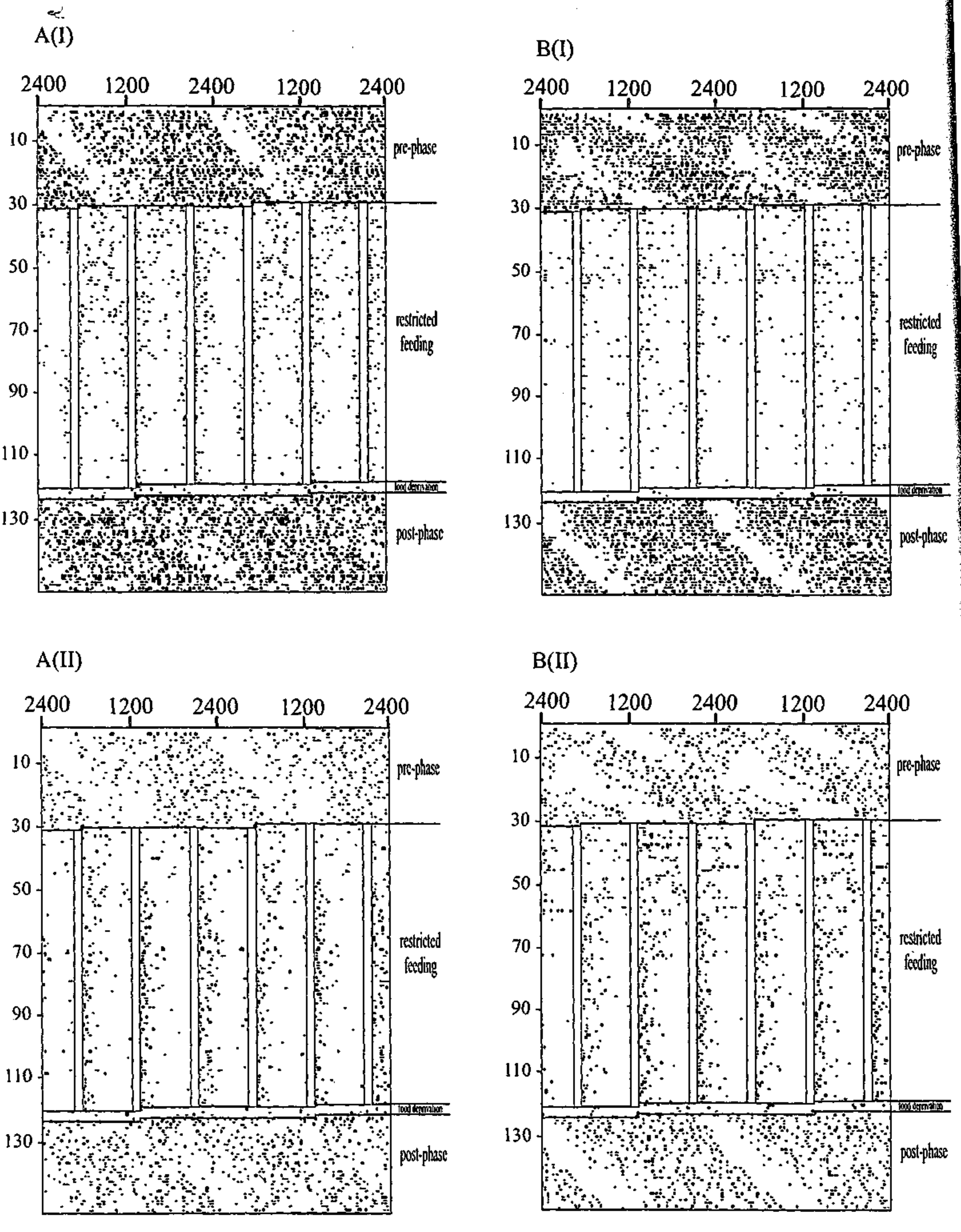


FIGURE 40. DOUBLE PLOTS OF FOOD COUNTS (I) AND WATER COUNTS (II) FROM SIX RABBITS FED THREE DAILY MEALS. (A) SUBJECT#1. (B) SUBJECT #3. (C) SUBJECT #4. (D) SUBJECT #5. (E) SUBJECT #11. (F) SUBJECT #12. CONVENTIONS AS FOR FIGURE 11.

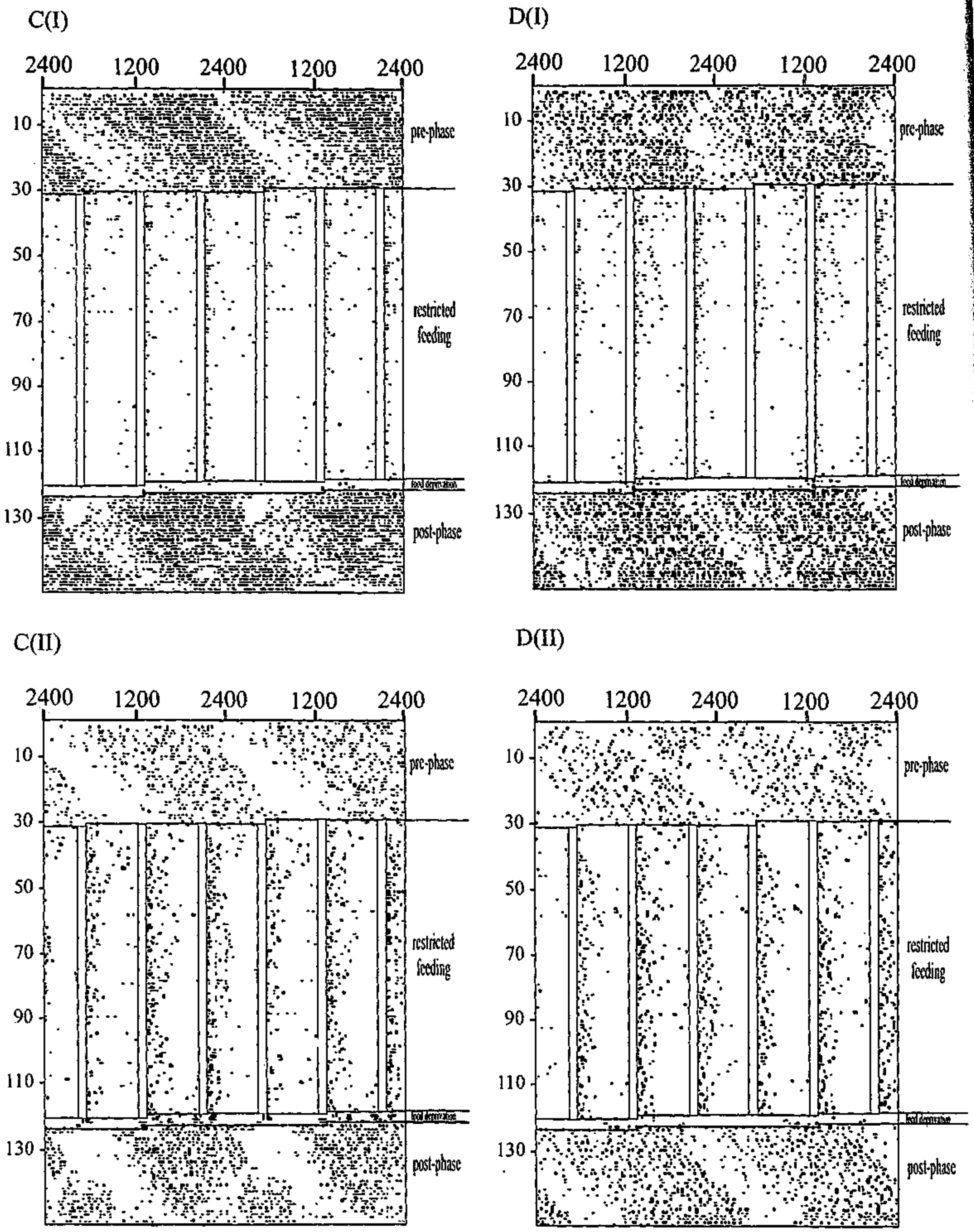


FIGURE 40 CONTINUED

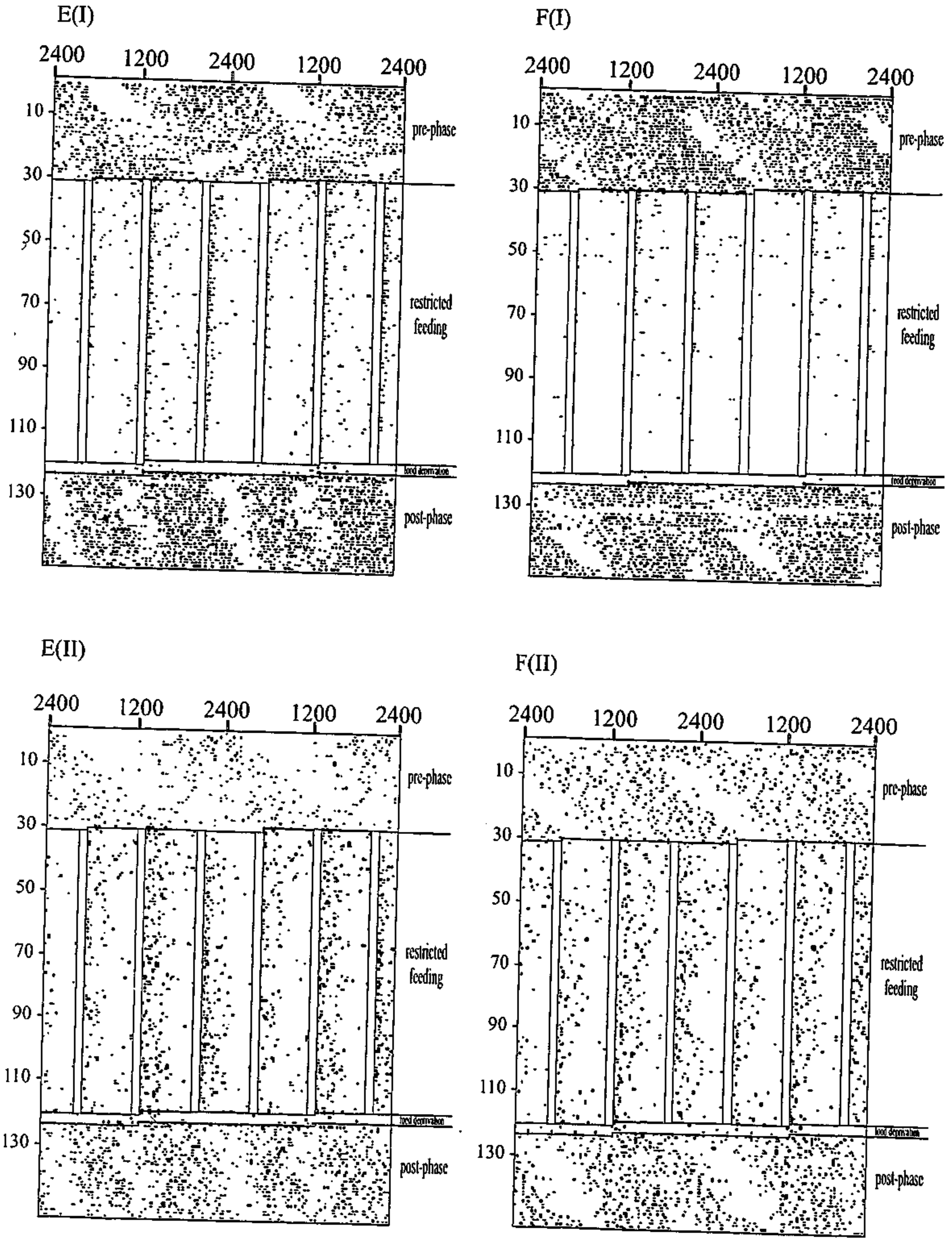


FIGURE 40 CONTINUED

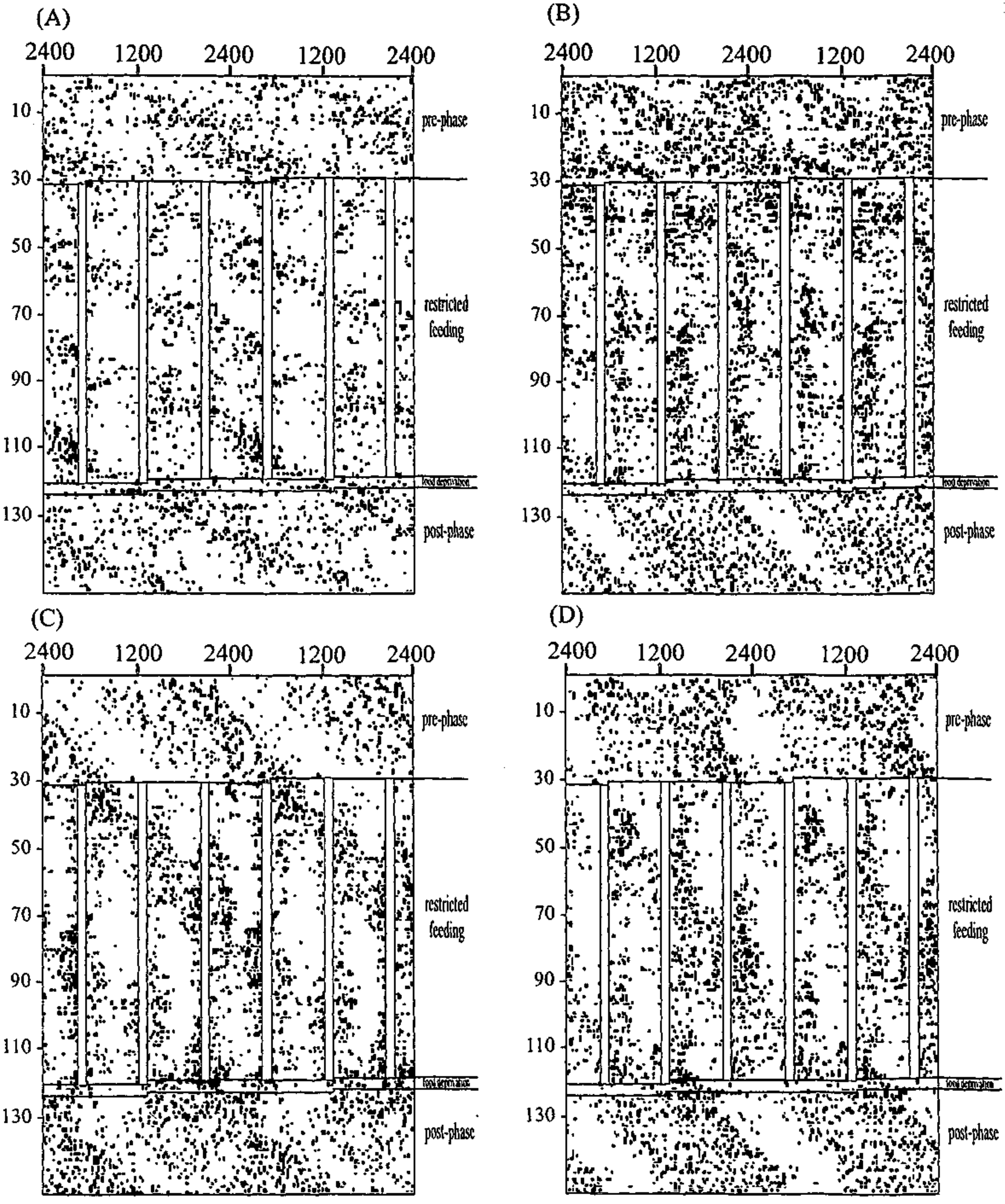


FIGURE 41. DOUBLE PLOTS OF GENERAL ACTIVITY FOR EIGHT RABBITS EXPOSED TO THREE DAILY MEALS. (A) SUBJECT #2. (B) SUBJECT #3. (C) SUBJECT #4. (D) SUBJECT #5. (E) SUBJECT #7. (F) SUBJECT #8. (G) SUBJECT #11. (H) SUBJECT #12. CONVENTIONS AS FOR FIGURE 11. FREE-RUNNING LIGHT ENTRAINABLE RHYTHMS DURING RESTRICTED FEEDING CAN BE SEEN IN ALL RABBITS.

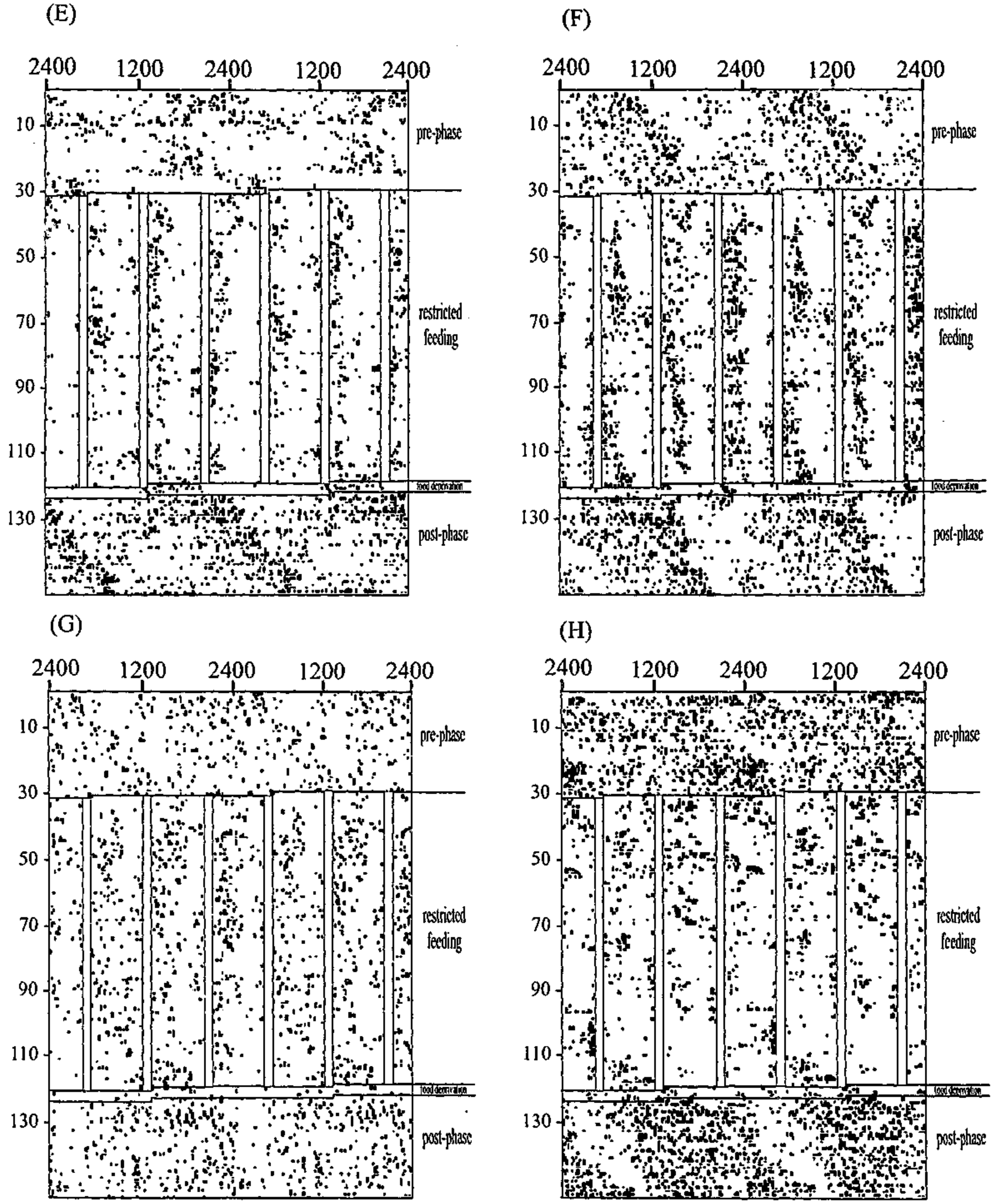


FIGURE 41 CONTINUED

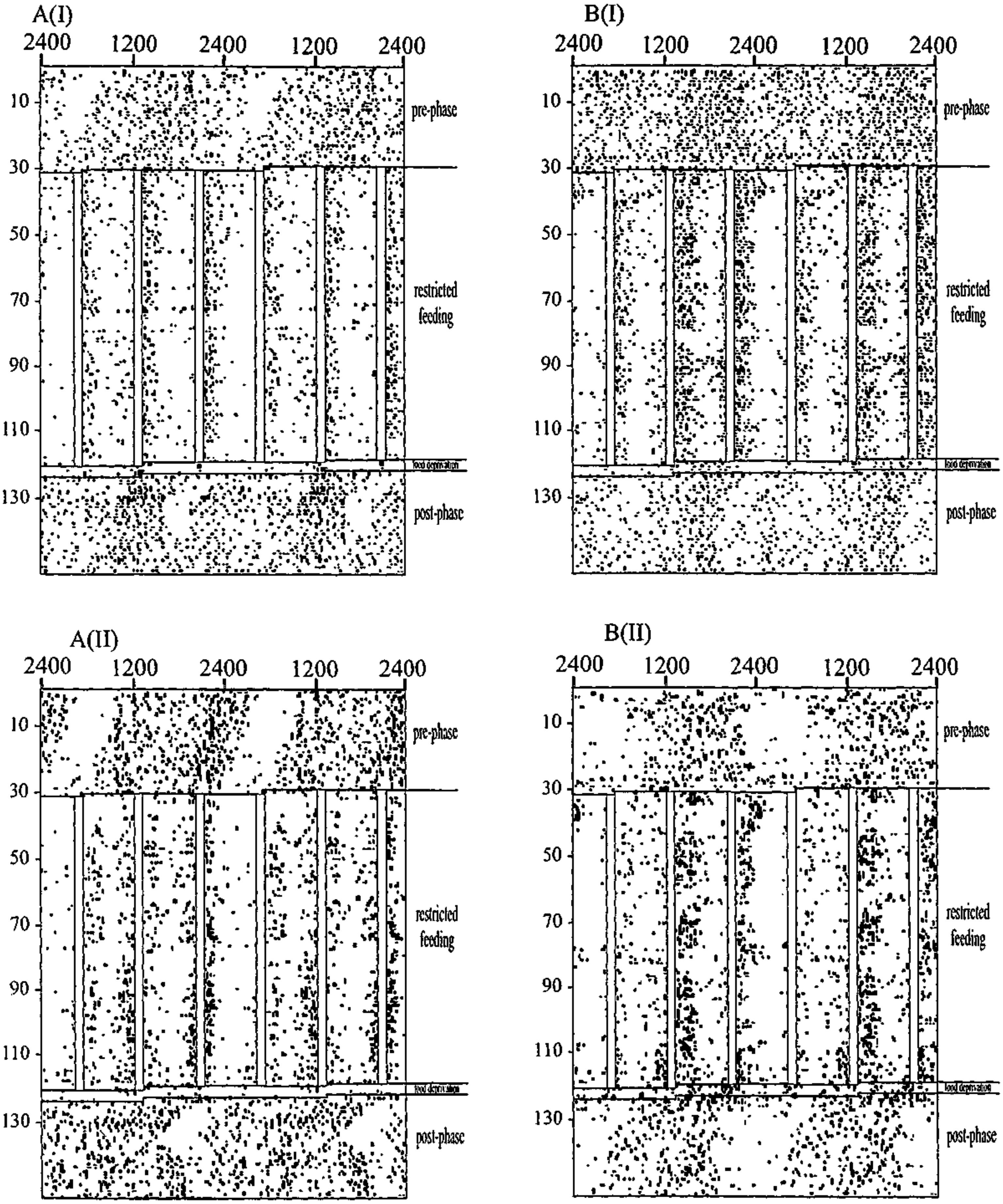


FIGURE 42. DOUBLE PLOTS OF WATER COUNTS (I) AND GENERAL ACTIVITY (II) DEMONSTRATING ENTRAINMENT OF THE FREE-RUNNING LIGHT ENTRAINABLE RHYTHM BY RESTRICTED FEEDING IN TWO RABBITS. (A) SUBJECT #9. (B) SUBJECT #10. CONVENTIONS AS FOR FIGURE 11.

In order to ascertain whether tau of the free-running light entrainable rhythm was influenced by aftereffects to the feeding schedule, tau for the pre- and post- phase was calculated for food counts. A line of best fit was placed along activity offsets over the final 20 days of each phase. This was done separately by each rater and the scores were then correlated. Inter-rater reliability was examined using Pearson's product-moment correlations. The inter-rater correlation was highly significant ($r=.88$, $p<.01$) and no systematic differences between raters' estimates of tau were found. Data were averaged for use in the following analysis. Mean (+*SE*) estimates of free-running tau by phase are shown in Table 7.

Table 7.

Tau of the free-running light entrainable rhythm by Phase.

	Pre-phase	Post-phase
Mean	24.46	24.42
SE	0.39	0.29

A related samples *t*-test revealed that estimates of tau in the pre- and post- phase did not differ significantly from each other, $t(11)=0.29, p>.05$.

9.6. DISCUSSION

9.6.1. Entrainment to two daily meal

The aim of this study was to examine the effects of multiple meals on the circadian activity rhythms of the herbivorous rabbit. The results of the study support the hypothesis, and suggest that the rabbit can anticipate two daily meals separated by eight hours. These results are consistent with previous findings (Bolles & Moot, 1973; Boulos & Logothetis, 1990; Edmonds & Adler, 1977b; Mistlberger et al., 1996; Stephan, 1989a,b). The present thesis supports past research in showing that fewer rabbits showed anticipatory activity to two meals compared to a single meal; three of 12 rabbits satisfied the criteria for entrainment to two daily meals, while nine of 15 rabbits anticipated a single daily meal in the previous study. This discrepancy is unlikely to be attributable to differences in energy balance, since food intake in the present study was limited to 60 grams per day. Our previous findings suggest that rabbits limited to a single daily meal consumed approximately 60 grams of food following a short period of adjustment of several weeks. It is thus unlikely that rabbits in the present study were less food deprived than those in our previous study. The above conclusion should be treated with caution however, since many rabbits displayed brief bouts of anticipatory activity to both mealtimes which did not persist over the final ten days of this condition. Similar findings were obtained by Stephan (1989a,b) who reported less stable and consistent anticipation of two daily meals compared to a single daily meal.

The present findings indicated that residual meal-associated activity persisted during subsequent food deprivation. Residual activity occurred at the former phase of feeding and could be separated into two bands corresponding to the two food access times. This finding supports the zeitgeber hypothesis which suggests that anticipation of two daily meals is mediated by the circadian system. Evidence of small advancing transients in activity onset during food deprivation is also consistent with this hypothesis. The finding that meal-associated activity advanced toward the onset/offset component of the free-running light entrainable rhythm before the two rhythms merged suggests that the FEP is tightly coupled to the LEP. Based on this finding, it seems reasonable to conclude that the LEP is the dominant pacemaker which normally drives the FEP, but dissociation may occur under certain conditions i.e., restricted food availability.

The results of the present study indicated a trend towards smaller PADs to the leading schedule (at the end of 16h) compared to the trailing schedule (at the end of 8h). This finding is however based on a small sample. The present findings are inconsistent with the results of some researchers, but not others. For example, Boulos and Logothetis (1990) recorded more lever pressing at the end of 16h than at the end of 8h in rats exposed to two daily meals separated by eight hours. Similar results were obtained by Stephan (1989a) who reported lower levels of anticipatory activity to the trailing schedule when the interval between two meals was reduced to 5h. In Stephan's study, anticipatory activity was reduced in both amount and duration. This finding is comparable to Stephan's other findings; rats exposed to two daily meals with different Ts showed a reduction in anticipatory activity to the trailing schedule as the interval between the two meals approached 5h to 7h (Stephan, 1989b). The results of Stephan's study, however, support the present findings in that the amount of anticipatory activity was not systematically related to the inter-meal interval for meals separated by six or eight hours.

The results of this study indicated that two daily meals entrained the free-running light entrainable rhythm in some rabbits. While there was no evidence of free-running light entrainable rhythms during restricted feeding, and the rhythm free-ran from a phase consistent with one or both meals in the post-phase, the phase of activity onset in the pre- and/or post-phase was difficult to assess due to poorly defined onsets in most cases. Despite these difficulties, the data suggested that entrainment of the free-running light entrainable rhythm by the feeding schedule had occurred in at least some rabbits. This finding is consistent with Stephan's coupled pacemaker hypothesis and suggests that the FEP and the LEP are not completely independent. Also consistent with this conclusion were the observed changes in phase of the free-running light entrainable rhythm on removal of the feeding schedule. These data also support the possibility that entrainment to the feeding schedules is mediated by the two light entrainable oscillators such that one oscillator is entrained to each mealtime, and highlight the need for research with SCN lesioned rabbits (see section 9.6.2). A similar explanation based on the functional model proposed by Pittendrigh and Daan (1976b) was proposed in chapter 8 and will be considered again in chapter 10.

Two theories have been proposed in the empirical literature to account for the present findings: multi-oscillator model versus continuously consulted clock (Stephan, 1986a,b,c). According to the multi-oscillator model, anticipation of two daily meals is mediated by two separate, but mutually coupled oscillators; one oscillator entrains to each meal. By contrast, the continuously consulted clock model postulates the existence of a single pacemaker that can be consulted at different phases. According to this explanation, each meal becomes associated with a different phase of the pacemaker. A similar mechanism involving a continuously consulted clock is postulated to underlie the time sense of bees and birds, with researchers noting that bees are capable of discriminating up to nine daily meals (Kolterman, 1974). While entrainment to two daily meals can be explained within the framework of a single pacemaker model, the present results provide support for a multi-oscillatory feeding entrainable circadian system, since rabbits failed to anticipate three daily meals (see section 9.6.2).

9.6.2. Entrainment to three daily meals

Consistent with the hypothesis, anticipatory activity to three daily meals did not occur. As noted above, these findings do not support the continuously consulted clock hypothesis. These findings are consistent with those of Stephan (1989a) and provide support for the view that there exist two separate feeding entrainable oscillators.

The majority of rabbits displayed robust free-running light entrainable rhythms during scheduled feeding in general activity. These results are consistent with our previous findings, and suggest that the LEP is not usually entrained by restricted feeding in the rabbit. The fact that tau of the free-running light entrainable rhythm did not change across the experiment is consistent with this view. Despite these findings, the present results provide some evidence for Stephan's coupling hypothesis, although coupling strength appears to be very weak in the rabbit. Two rabbits satisfied the criteria for entrainment of the free-running light entrainable rhythm by restricted feeding, and changes in phase of the free-running light entrainable rhythm across the experiment were common. Also relevant here is the finding that anticipatory activity appeared when the free-running light entrainable rhythm crossed food access in some rabbits. This finding is difficult to interpret. A possible explanation for such an effect is based on the hypothesis mentioned in section 9.6.1. According to this explanation, the bout of anticipatory activity is postulated to reflect the output of the FEP which resides in phase with the LEP and is engaged as the two pacemakers cross the feeding schedule. Based on current findings, it seems reasonable to assume that some degree of coupling exists between the two pacemaking systems.

While the results of the present study provide some support for the multi-osillator hypothesis, a number of limitations need to be considered. These results cannot be regarded as conclusive, as prolonged forced dissociation which has provided the most compelling evidence for the existence of two pacemakers has not been demonstrated in the rabbit. Future studies could examine the effect of two feeding schedules with different Ts on the circadian system in this species. Another issue that needs to be considered is related to the theoretical framework adopted in the present study. As noted by Stephan (1989b), the interpretation of results offered here depends heavily on inferences about the dynamics of the FEP that cannot be observed directly, since the locus of FEP is unknown. Alternative explanations could be advanced to explain the present findings. For example, as hypothesized by Edmonds and Adler (1977b), the dissociation observed in experiment 1 can be attributed to the uncoupling of neurons within a single neural centre such as the SCN. Or alternatively, the dissociation could be mediated at a more peripheral level. Given that many of the standard procedures used to examine the light entrainable system cannot be applied to the feeding entrainable circadian system, it is difficult to obtain direct empirical confirmation of the conceptual model reported here.

The present findings highlight the need for research with SCN lesioned rabbits. These studies were conducted in intact rabbits and hence it is impossible to rule out the possibility that entrainment to feeding schedules is mediated by the LEP. Use of SCN lesioned rabbits would provide an opportunity to eliminate the possible confounding effects of the LEP. It is worthy of mention that SCN lesion trials were conducted as part of this research program. Training trials were conducted over a period of six months with little success, and many difficulties became apparent during the course of stereotaxic surgery. The main problem was the inadequacy of the stereotaxic frame. The stereotaxic frame has been described in previous studies by Carpenter and Whittier (1952) and Rowland (1966) and will not be described again here. On several occasions the frame had to be modified to facilitate SCN surgery. Other problems could not be resolved. For example, it proved difficult to align the skull in the stereotaxic frame because the rabbit lacks internal meatuses as a reference point. Therefore, the clamps had to be positioned arbitrarily on either side of the skull, and adjusted symmetrically by free hand. Using this procedure, it was often difficult to judge how much the angle should be changed, and small errors in judgement made any stereotaxic surgery totally inaccurate. Other physical features of the rabbit, including the inter-individual variability in lambda and bregma reference points observed in our study, render this species unsuitable for any kind of stereotaxic surgery using currently available techniques.

9.6.3. Conclusion

The current findings indicate that the rabbit can anticipate two daily meals, but not three daily meals. These findings confirm previous findings by Stephan (1989a) and suggest that, as in the rat, the feeding entrainable circadian system of the rabbit consists of a maximum of two oscillators.

PART THREE. CONCLUSION

CHAPTER 10. MAJOR CONCLUSIONS

10.1. IMPLICATIONS OF MAIN FINDINGS FOR THE RABBIT FEEDING ENTRAINABLE CIRCADIAN SYSTEM

The present findings add to previous research by demonstrating that feeding schedules satisfy the criteria for a zeitgeber in the rabbit, which were developed by Enright (1981) and extended by others to include additional guidelines. The evidence in support of this proposition is summarized below.

- Feeding schedules were found to have a profound influence on the activity rhythms. Entrainment in the form of anticipatory activity of several behavioural variables was demonstrated, including food and water counts, and general activity. As expected, activity onset in the entrained state consistently preceded the onset of food access. PAD between activity onset and the onset of food access was found to correlate positively with feeding schedule T within the limits of 23h and 30h. Under these conditions, PAD ranged from less than 1h to 10h. Data showing that the crossing of the two pacemakers increased PAD were also provided.
- A 5h shift of food access resulted in a shift in the activity rhythms by the same number of hours. The feeding entrainable rhythms required one or more days to reentrain to the new feeding time. Reentrainment to a phase advance of the feeding schedule was achieved by advancing transients. No evidence of delaying transients was found.
- Meal-associated activity persisted in food deprived rabbits, but not when the deprivation trials were repeated after 15 days of *ad libitum* food access. Residual activity was found to persist at the former time of feeding when the feeding schedule was replaced with *ad libitum* food access.
- Anticipation was possible only within a limited range of feeding cycle Ts. Anticipatory activity developed to T cycles ranging from 23h to 27h, but could not be observed under T cycles of 21h. Water counts entrained to feeding schedules with T=30h, but there was little evidence of anticipatory activity in food counts. This discrepancy is unexpected; the most likely explanation for this result is that 30h approaches the upper limit of entrainment.

So far the discussion has focused on the efficacy of feeding schedules as zeitgebers in the rabbit. The present thesis also indirectly provides evidence concerning the functional organization of the circadian system of the rabbit. As stated in section 1.2.3, the generally accepted functional model of the circadian system of mammals proposes that multiple oscillators are coordinated in mutual coupling arrangements. The present results provide support for a multi-oscillator model of the circadian system, consisting of at least two, coupled pacemakers. Data demonstrating that the free-running light entrainable rhythm did not entrain to the feeding schedule, but co-existed with anticipatory activity were presented. It was proposed that a dual FEP-LEP model could account for these findings, on the assumption that a single pacemaker cannot express two or more periodicities. Entrainment of the free-running light entrainable rhythm by a single daily meal was found to occur in one rabbit suggesting that the two pacemakers may be coupled. As in the studies by Stephan (1986a,b), entrainment occurred when tau-T was less than 10 minutes. This finding raises the possibility that successful entrainment in the rabbit depends in part on the difference between tau of the respective pacemakers, and is consistent with a weak zeitgeber effect of feeding schedules on the LEP. Other evidence in favour of this hypothesis comes from the finding that the majority of rabbits showed changes in phase and tau of the free-running light entrainable rhythm in response to feeding schedules. Also relevant here is the finding that the crossing of the two pacemakers appeared to increase the PAD between activity onset and the onset of food access for feeding schedules with T=27h and T=30h.

The results of the present study support the existence of a multi-oscillatory FEP in the rabbit. Rabbits were able to anticipate two daily meals separated by eight hours. However, fewer subjects showed anticipatory activity to two meals compared to a single daily meal, and entrainment appeared to be less consistent. Meal-associated activity persisted at the former feeding times in food deprived rabbits, and merged with the onset/offset components of the free-running light entrainable rhythm when *ad libitum* food access was reinstated. Anticipatory activity to three daily meals did not occur. Based on these findings, it was postulated that there exist a maximum of two feeding entrainable oscillators. The importance of studies investigating SCN-lesioned rabbits was discussed in the context of these findings.

So far the present findings have been interpreted within the framework of widely accepted FEP theories, but other explanations could also account for research outcomes. For example, the hypothesis that entrainment to feeding schedules is mediated by the LEP accrued some support. Data were reasonably consistent in illustrating post-phase control of the rhythm by the feeding schedule in rabbits which displayed clearly discernable free-running light entrainable rhythms during restricted feeding. Such an effect was explained with reference to the multi-oscillator LEP model originally proposed by Pittendrigh and Daan in 1976. The model also accounts for the finding of a lack of free-running activity in rabbits which entrained to multiple meals. An attempt was made to lesion the SCN in the rabbit, with a view to shedding some light on this topic, however lesioning trials proved unsuccessful. It is important to note that this hypothesis is speculative and requires further study.

A surprising result in the present thesis was the finding that meal-associated rhythms persisted in rabbits which did not meet the criteria for anticipatory activity. While such an effect may be the result of the method used to identify anticipatory activity, the consistency of this finding argues against this interpretation. Rather, the unique digestive features of the rabbit were postulated to protect against a state of energy deficit during restricted feeding, thus masking the expression of anticipatory activity. This hypothesis is speculative and more research is required before any conclusions can be reached. In general, in the absence of more detailed cross-species studies, the extent to which this finding is specific to the rabbit is still unclear. The fact that this may not be a generalized effect, but may occur specific to the rabbit, has important implications for the validity of the current model of herbivore feeding entrainable circadian system, since data from the rabbit may not be representative of other herbivores.

10.2. IMPLICATIONS OF MAIN FINDINGS FOR THE HERBIVORE FEEDING ENTRAINABLE CIRCADIAN SYSTEM

The aim of the present thesis was to explore the effects of feeding schedules in the rabbit, with the aim of developing a model of the herbivore feeding entrainable circadian system. The first experiment reported in this thesis was designed to examine the suitability of the rabbit for use as a herbivore model of the feeding entrainable circadian system. The results indicated that the traditional method used to study the FEP can be applied to the rabbit. Food and water intake were lower during the feeding schedule compared to baseline, and significantly higher during the post-phase relative to baseline. A similar trend was apparent for body weight in the 2h group. All except one rabbit were able to maintain their body weight within 20% of baseline levels during the feeding schedule. It was concluded that the rabbit is suitable for use as a herbivore model of the feeding entrainable circadian system, because it provides an opportunity to investigate the FEP using the traditional paradigm involving time limited feedings, thus avoiding the potentially confounding effects associated with other paradigms.

The present section will discuss the relative effects of cycles of food availability for herbivores, omnivores, and carnivores. It should be noted, however, that definitive comparisons of zeitgeber effects across classes of species may be tenuous because the methods used to study the feeding entrainable pacemaker are varied. One of the primary problems is that there is no widely accepted criteria for establishing the occurrence of anticipatory activity. Other major difficulties relate to the range of methods used to assess other circadian parameters. It is clear that the methods used can have a considerable effect on research outcomes; hence, this is an important issue which will need to be addressed in future studies. Another issue that is worthy of mention is the type of activity under investigation. Food counts were selected as the most appropriate measure of activity in the present thesis for reasons outlined in section 5.5.3. Studies using rats as subjects frequently use wheel-running activity as the DV, but at least some investigators have used food counts (Lax et al., 1999; Ruis et al., 1989). It is possible that the results vary for different overt rhythms; the special position of wheel-running activity as compared to other activities was discussed in section 6.4.3 and will not be reiterated. Additional factors that can create variability or act as confounds have been considered throughout the thesis and will not be discussed again. Another issue worthy of mention includes the fact that limited data are available from herbivores; it remains to be determined whether the present results can be generalized to other herbivorous species. It would be desirable in future studies to explore the effects of feeding schedules in other herbivores. Nevertheless, the present section will broadly evaluate the characteristics of the rabbit feeding entrainable circadian system in order to make comparisons between herbivores, omnivores, and carnivores. A summary of the similarities across omnivores, carnivores, and herbivores, including present findings is presented in Table 7.

The present research with the rabbit confirms previous reports that herbivorous, omnivorous, and carnivorous species are basically similar in their general response to feeding schedules. The primary similarities in the results of different researchers are as follows: (i) when food access is limited to a few hours per day, anticipatory rises occur in activity several hours prior to feeding; (ii) following a phase shift of food access, anticipatory activity resets gradually in a series of transient cycles over several days; (iii) during subsequent food deprivation, activity persists at the former feeding time(s) for several days, and; (iv) if the feeding schedule deviates too far from 24h, no anticipatory activity occurs. It is interesting to note that these findings indicate a zeitgeber role for feeding schedules in herbivores, omnivores, and carnivores; their effectiveness is not limited to certain species or classes of species but rather all mammalian species examined so far respond to food zeitgebers. The present results further suggest that herbivores and omnivores (at least) are basically similar in their circadian organization; the circadian system appears to be comprised of at least two, coupled pacemakers; a FEP (consisting of two oscillating units) and a LEP.

Despite the above noted similarities, there appear to be some clear differences between the three classes of species. The effects of feeding schedules on the circadian system appear to be relatively weak in the herbivore relative to omnivores and carnivores. As expected, herbivores were less likely than omnivores and carnivores to exhibit anticipatory activity, and anticipatory activity took longer to develop and was greatly reduced in duration. 60% of rabbits satisfied the criteria for anticipatory activity to a single daily feeding schedule under LL conditions. This figure is lower than those cited for omnivores and carnivores and suggests that anticipatory activity is more readily expressed in the latter. Up to 46 days was required for the herbivorous rabbit to develop anticipatory activity; previous results based on omnivores and carnivores suggest that less than 10 days is necessary. PAD ranged from less than 1h to 3h in rabbits exposed to a single daily meal, whereas other researchers report PADs ranging up to 6h to 8h in both omnivores and carnivores. An interpretation of these effects suggests that the strength of feeding

TABLE 7.

A SUMMARY OF THE EFFECTS OF FEEDING SCHEDULES IN OMNIVORES, CARNIVORES, AND HERBIVORES, INCLUDING THE RESULTS OF THE PRESENT THESIS.

DV	Omnivorous rat	Carnivorous kowari	Herbivorous rabbit
Anticipatory activity	306 of 315 (97%) rats exposed to a single daily meal develop anticipatory activity.	19 of 22 (86%) kowari exposed to a single daily meal develop anticipatory activity.	14 of 20 (70%) rabbits exposed to a single daily meal in LL develop anticipatory activity. Note that Jilge (1991) did not provide sufficient detail to allow comparison.
PAD between activity onset and onset of food access	For a single daily meal, the PAD between activity onset and the onset of food access for locomotor activity and approaches to the food bin ranges from 2h to 4h, however, PADs as large as 8h have been reported.	For a single daily meal, the PAD between activity onset and the onset of food access for locomotor activity ranged from 0.25h to 3h in the study by Kennedy et al. (1991). Much larger PADs of up to 6h have been noted in other carnivores.	For a single daily meal, the PAD between activity onset and the onset of food access for locomotor activity and approaches to the food bin ranges from 1h to 3h.
Latency to anticipatory activity	Anticipatory activity to a single daily meal usually develops within 7 days.	Anticipatory activity to a single daily meal develops within 6 days.	Anticipatory activity to a single daily meal develops within 67 days.
Free-running light entrainable rhythm	Entrainment of free-running light entrainable rhythms to a single daily meal occurred in 12 of 69 (17%) rats.	Entrainment of free-running light entrainable rhythms to a single daily meal occurred in 12 of 20 (60%) kowari.	Entrainment of free-running light entrainable rhythms to a single daily meal occurred in 1 of 7 rabbits in the current study. These results are consistent with those obtained by Jilge et al. (1987).
Food deprivations	Meal associated rhythms persist in almost all rats (108/117) when a single daily meal is replaced with food deprivation. Meal associated rhythms are less likely to persist during subsequent food deprivations, following a period of *ad libitum* feeding.	Meal associated rhythms persist in all kowari (12/12) when a single daily meal is replaced with food deprivation. Meal associated rhythms are less likely to persist during subsequent food deprivations, following a period of *ad libitum* feeding.	Meal associated rhythms persist in almost all rabbits (9/11) when a single daily meal is replaced with food deprivation. Meal associated rhythms do not persist during subsequent food deprivations, following a period of *ad libitum* feeding.
Ad libitum food access	Meal associated rhythms have been shown to persist for up to two days during *ad libitum* food access following a single daily meal.	Meal associated rhythms have been shown to persist for up to three days during *ad libitum* food access following a single daily meal.	Meal associated rhythms have been shown to persist for up to 10 days during *ad libitum* food access following a single daily meal.
Transients	Reentrainment is achieved by delaying transients, although advancing transients have been observed following a 4h and 8h phase advance of food access.	Data not available	Delaying transients occur in response to a phase delay of food access. Advancing transients occur following a phase advance of food access.
T cycles	Anticipatory activity develops to feeding schedules with Ts ranging between 22h and 29h.	Data not available	Anticipatory activity develops to feeding schedules with Ts ranging between 23h and 30h
Multiple meals	Anticipatory activity develops to two but not three daily meals.	Data not available	Anticipatory activity develops to two but not three daily meals.

zeitgebers may depend on the ecological utility of this feature. As stated in the introduction (see below), the adaptive significance of a feeding entrainable circadian system, which allows all relevant bodily systems to prepare in advance for a meal, is clear in carnivores and omnivores where food may be available for a short time or in a small amount. The adaptive advantages in the case of the herbivore are less clear. It is unlikely to be more advantageous for the behavioural and physiological events involved in locating, handling, and assimilating food to occur at a particular time of day when food availability is most likely, since herbivores eat more or less continuously throughout the day and night. Thus, herbivores would be under little selection pressure to evolve such an ability. The above discrepancies also could be attributable to differences in the nutritional characteristics between omnivores, carnivores, and herbivores. As noted in section 3.1, the herbivore differs from the other two classes of species in that herbivores consume large amounts of low nutrient food. If anticipatory activity is mediated by food nutritive content, it is possible that high nutrient meals may produce a stronger zeitgeber effect in herbivores. Hence, the herbivorous rabbit provides an opportunity to explore the role of nutrient content in food entrainment, avoiding the potentially confounding effects associated with hypocalorific meals.

The herbivore differs from the omnivore and carnivore in that the free-running light entrainable rhythm is less likely to entrain to cycles of food availability. It was proposed that differences in the strength of coupling between the LEP and the FEP could account for these findings. It follows from Stephan's reasoning that coupling strength is weaker in the herbivore than the omnivore, and is strongest in the carnivore. As above, coupling strength may depend on the ecological utility of this feature and thus may be open to genetic selection in a strain or species or class of species. If coupling strength is important in fitting the animal for survival it may be selected for across successive generations. In carnivores, coupling strength may be stronger than herbivores as a result of ecological demands placed on predatory species. Carnivores must be able to adapt their activity programs to rapidly take advantage of food sources that might become available with daily periodicity for a few days or weeks. This is particularly relevant at certain times of the year when food is available at times of the day at which the animal is not usually active; thus, individuals that are able to rapidly change their activity program may be selectively favoured.

10.3. CONCLUSION

Over the last few years, the number of studies investigating the feeding entrainable circadian system has declined. The most likely explanation for this trend is that the feeding entrainable circadian system is proving to be intractable. This is not surprising, since most of these studies have adopted a similar line of investigation. More recently, researchers have adopted a broader approach to the problem, by studying a larger number of animal species. Species differences have been documented in recent comparative studies. Differences between species are likely to be related to the particular ecological niche that animals have adapted to through the course of evolution. The possibility that differences exist between omnivores, carnivores, and herbivores is consistent with this type of approach that focuses on ecological factors, since it likely that feeding patterns are related to a species habitat. The present thesis offers a new way of looking at an old problem; an analysis by class approach has the advantage of cutting across species lines.

Within this framework, the present thesis was designed to examine the effects of feeding schedules in the rabbit, with the aim of developing a comprehensive model of the herbivore feeding entrainable circadian system. Until now it has proven difficult to establish such a model, since the traditional method used to study the FEP cannot be applied to some herbivores. The experiments reported here indicate that the rabbit provides a promising herbivore model for investigating the properties of the feeding entrainable circadian system. The present thesis has yielded some inconsistencies between herbivores, omnivores, and carnivores; these data appear sufficient to warrant pursuit of an analysis by class approach.

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APPENDICES

APPENDIX A

Figure (i). Double plotted actograms showing the effect of laboratory entries on the recording of general activity. (a) empty cage. (b) laboratory entries. Days are shown on the right side of the actogram. Time of day is shown at the top of the actogram.

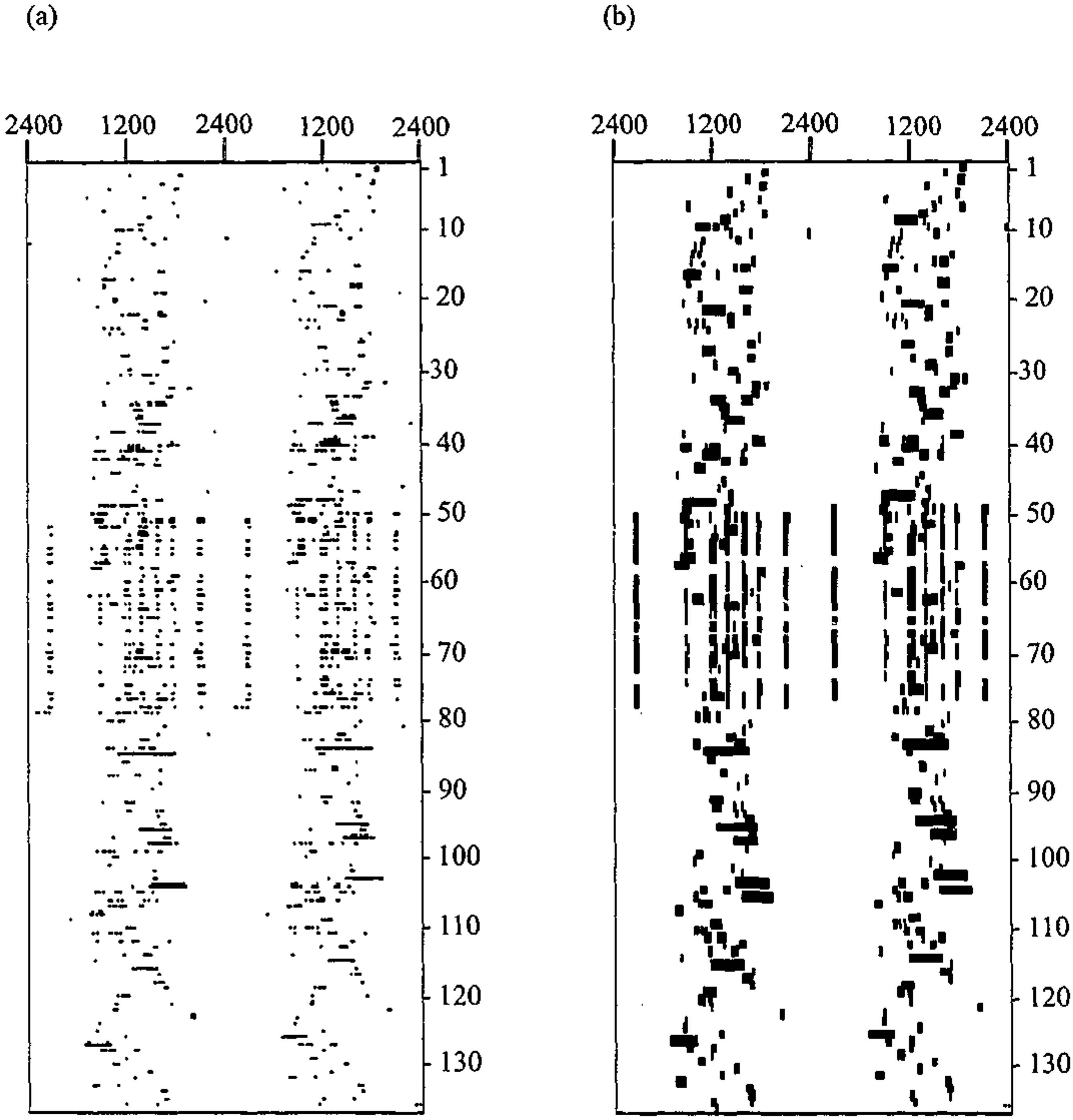

Table (i). Raw data: Tau estimates for rabbits housed under LL.

Rater 1	Rater 2	Enright's technique
24.17	24.11	23.9
24.19	24.15	24.2
24.52	24.54	24.4
24.13	24.20	24.2
24.52	24.42	24.4
24.36	24.31	24.3
24.45	24.42	24.2
24.32	24.16	24.2
24.24	24.13	24.1
24.04	24.01	24.0
24.00	24.21	24.2
24.13	24.26	24.2

APPENDIX B

Table (ia) *ANOVA summary table*

The independent variables were meal duration (2h, 3h, 4h) and phase (pre, restricted feeding, post). Meal duration was a between subject variable and phase was a within subject variable. Dependent variable: Body weight

Source of variation	SS	Df	MS	F	*P*
Meal duration	.62	2	.31	.64	.564
Within + residual	2.4	5	.48		
Phase	.58	2	.29	34.27	.000
Phase by meal duration	.23	4	.05	6.77	.007
Within + residual	.08	10	.00		

Table (ib). *Simple Main Effects summary table*

Source of variation	SS	df	MS	F
Phase at 2h group	.65	2	.32	38.69*
Phase at 3h group	.08	2	.04	4.76
Phase at 4h group	.03	2	.01	1.43
Within + residual	.08	10	.00	

*$p<0.05$

Table (ii) *ANOVA summary table*

The independent variables were meal duration (2h, 3h, 4h) and phase (pre, restricted feeding, post). Meal duration was a between subject variable and phase was a within subject variable. Dependent variable: Daily food intake

Source of variation	SS	Df	MS	F	*P*
Meal duration	4260.42	2	2130.21	7.81	.029
Within + residual	1363.19	5	272.64		
Phase	49117.25	2	24558.62	84.71	.000
Phase by meal duration	527.79	4	131.95	.46	.767
Within + residual	2899.14	10	289.91		

Table (iii) *ANOVA summary table*

The independent variables were meal duration (2h, 3h, 4h) and phase (pre, restricted feeding, post). Meal duration was a between subject variable and phase was a within subject variable. Dependent variable: Daily water intake

Source of variation	SS	Df	MS	F	*P*
Meal duration	3753.622	2	1876.81	.48	.647
Within + residual	19721.60	5	3944.32		
Phase	43291.47	2	21645.74	46.63	.000
Phase by meal duration	571.21	4	142.80	.31	.866
Within + residual	4641.91	10	464.19		

Table (iv) *ANOVA summary table*

The independent variables were meal duration (2h, 3h, 4h) and phase (pre, restricted feeding, post). Meal duration was a between subject variable and phase was a within subject variable. Dependent variable: Daily time spent feeding

Source of variation	SS	Df	MS	F	*P*
Meal duration	2435.18	2	1217.59	.01	.989
Within + residual	543493.09	5	108698.62		
Phase	68996.60	2	34498.3	5.01	.031
Phase by meal duration	15914.24	4	3978.56	.58	.686
Within + residual	68905.61	10	6890.56		

Table (v) *ANOVA summary table*

The independent variables were meal duration (2h, 3h, 4h) and phase (pre, restricted feeding, post). Meal duration was a between subject variable and phase was a within subject variable. Dependent variable: Daily time spent drinking

Source of variation	SS	Df	MS	F	*P*
Meal duration	666502.46	2	333251.23	5.24	.059
Within + residual	317783.38	5	63556.68		
Phase	2360365.96	2	1180182.98	13.01	.002
Phase by meal duration	72364.85	4	18091.21	.20	.933
Within + residual	907045.66	10	90704.57		

Table (vi) *ANOVA summary table*

The independent variables were meal duration (2h, 3h, 4h) and phase (pre, restricted feeding, post). Meal duration was a between subject variable and phase was a within subject variable. Dependent variable: General activity

Source of variation	SS	Df	MS	F	*P*
Meal duration	86639.32	2	43319.66	.26	.783
Within + residual	845109.42	5	169021.88		
Phase	26578.01	2	13289.01	1.37	.298
Phase by meal duration	33272.81	4	8318.2	.86	.521
Within + residual	97004.3	10	9700.43		

APPENDIX C

Table (ia) Raw data

Percentage nocturnality by phase (i.e., pre, early subjective day, late subjective day, and post) for general activity.

Rabbit no.	Pre	Early subjective day	Late subjective day	Post
1	59.95	49.15	52.39	59.02
2	51.87	54.84	58.08	61.19
3	59.29	46.23	52.82	67.00
4	43.94	40.09	41.20	45.95
5	53.20	44.45	48.74	51.22
6	61.11	52.05	57.09	59.34
7	62.19	57.68	68.08	60.04
8	51.00	40.87	52.67	53.41
9	56.64	47.56	56.08	65.43
10	55.91	45.23	55.00	54.96
11	59.69	42.70	52.60	55.78

Table (ib) ANOVA summary table

The independent variables were direction of the phase shift (phase advance vs phase delay) and phase (pre, early subjective day, late subjective day, post). Direction of phase shift was a between subject variable and phase was a within subject variable. Dependent variable: percentage nocturnality for general activity.

Source of variation	SS	Df	MS	F	*p*
Direction of phase shift	33.43	1	33.43	.30	.601
Within + residual	1021.57	9	113.51		
Phase	673.26	3	224.42	19.28	.000
Phase by direction of phase shift	56.27	3	18.76	1.61	.210
Within + residual	314.24	27	11.64		

Table (iia) Raw data

Percentage nocturnality by phase (i.e., pre, early subjective day, late subjective day, and post) for food counts.

Rabbit no.	Pre	Early subjective day	Late subjective day	Post
1	58.00	.73	.81	60.65
2	44.81	3.63	5.58	48.10
3	43.84	.32	2.27	66.17
4	32.35	3.24	3.29	38.17
5	33.47	.84	.65	48.94
6	60.23	.00	.00	65.04
7	49.57	.89	.53	60.70
8	63.53	.25	25.40	76.61
9	29.39	1.33	14.03	46.37
10	30.70	.53	1.41	41.25
11	50.59	.16	1.33	26.46

Table (iib) ANOVA summary table

The independent variables were direction of the phase shift (phase advance vs phase delay) and phase (pre, early subjective day, late subjective day, post). Direction of phase shift was a between subject variable and phase was a within subject variable. Dependent variable: percentage nocturnality for food counts.

Source of variation	SS	Df	MS	F	*p*
Direction of phase shift	.32	1	.32	.00	.97
Within + residual	1879.65	9	208.85		
Phase	23039.69	3	7679.90	91.60	.000
Phase by direction of phase shift	164.86	3	54.95	.66	.587
Within + residual	2263.77	27	83.84		

Table (iiia) Raw data
Percentage nocturnality by phase (i.e., pre, early subjective day, late subjective day, and post) for water counts.

Rabbit no.	Pre	Early subjective day	Late subjective day	Post
1	59.20	16.47	42.03	59.21
2	56.38	17.13	30.94	59.33
3	44.79	18.48	14.94	68.84
4	50.53	44.28	43.22	27.45
5	40.76	13.58	35.38	54.64
6	63.53	7.81	28.27	61.09
7	44.56	12.04	33.38	56.96
8	66.15	8.68	26.56	78.46
9	18.75	4.85	21.32	43.89
10	41.76	10.76	38.90	55.45
11	8.29	4.92	7.00	14.75

Table (iiib) ANOVA summary table
The independent variables were direction of the phase shift (phase advance vs phase delay) and phase (pre, early subjective day, late subjective day, post). Direction of phase shift was a between subject variable and phase was a within subject variable. Dependent variable: percentage nocturnality for water counts.

Source of variation	SS	Df	MS	F	*p*
Direction of phase shift	1103.24	1	1103.24	2.84	.126
Within + residual	3493.91	9	388.21		
Phase	9505.51	3	3168.5	20.60	.000
Phase by direction of phase shift	212.09	3	70.70	.46	.713
Within + residual	4152.00	27	153.78		

Table (iva) Raw data

PAD (hrs) between activity onset and the onset of LD transition by phase (i.e., pre, early subjective day, late subjective day, and post) for general activity.

Rabbit no.	Pre	Early subjective day	Late subjective day	Post
1	.75	1.00	3.75	.75
2	1.50	1.00	1.50	2.00
3	1.00	.75	4.50	.25
4	2.00	1.75	3.50	.50
5	2.25	2.00	3.75	3.50
6	1.75	2.50	2.5	.75
7	.75	1.00	1.50	.75
8	2.75	1.25	.00	3.00
9	.75	3.25	2.25	.75
10	1.25	1.75	2.00	3.00
11	1.00	2.25	4.75	2.50

Table (ivb) ANOVA summary table

The independent variables were direction of the phase shift (phase advance vs phase delay) and phase (pre, early subjective day, late subjective day, post). Direction of phase shift was a between subject variable and phase was a within subject variable. Dependent variable: PAD between activity onset and the onset of LD transition for general activity.

Source of variation	SS	Df	MS	F	*p*
Direction of phase shift	.06	1	.06	.05	.835
Within + residual	10.66	9	1.18		
Phase	10.13	3	3.38	3.04	.046
Phase by direction of phase shift	5.52	3	1.84	1.65	.200
Within + residual	30.02	27	1.11		

Table (v) Raw data

PAD (hrs) between activity onset and the onset of food access by phase of meal presentation for food counts.

Subject no.	Early subjective day	Late subjective day
2	1.25	2.50
4	1.50	3.00
5	1.50	1.50
9	2.00	2.00
10	2.00	2.50

APPENDIX D

Table (i) Raw data
PAD (hrs) between activity onset and the onset of food access and latency to anticipatory activity for feeding cycle T=24h.

Subject no.	PAD	Latency
2	1.50	36.00
5	1.25	46.00
8	3.00	37.00
9	1.00	41.00
10	1.50	45.00
11	1.00	45.00
12	1.75	32.00
13	1.50	45.00
15	2.00	16.00

Table (ii) Raw data
PAD (hrs) between activity onset and the onset of food access and latency to anticipatory activity for feeding cycle T=23h.

Subject no.	PAD	Latency
13	2.00	27.00
14	1.25	39.00
15	1.25	33.00

Table (iii) Raw data
PAD (hrs) between activity onset and the onset of food access and latency to anticipatory activity for feeding cycle T=27h.

Subject no.	PAD	Latency
9	3.00	31.00
10	3.00	36.00
11	3.50	37.00
12	5.50	28.00
13	4.00	40.00
14	3.50	31.00
15	4.00	36.00

Table (iii) Raw data

PAD (hrs) between activity onset and the onset of food access and latency to anticipatory activity for feeding cycle T=30h.

Subject no.	PAD	Latency
9	5.75	21.00
10	9.25	9.00
11	6.00	23.00
14	9.75	17.00
15	6.5	9.00

Table (iv) Raw data

Estimates of tau of the free-running light entrainable rhythm for the pre- and post-phase for feeding cycle T=24h.

Subject no.	Pre-phase	Post-phase
1	24.49	24.21
2	24.71	24.31
3	24.50	24.24
4	24.35	24.01
5	24.58	24.24
6	24.60	24.01
7	24.56	24.17
8	24.43	24.28
9	24.58	24.24
10	24.52	24.55
11	24.47	24.36
12	24.17	24.17
13	24.02	24.29
14	24.01	24.24
15	24.35	24.22

Table (v) Raw data
Estimates of tau of the free-running light entrainable rhythm by phase for food counts.

Subject no.	*Ad libitum* 2	*Ad libitum* 3	*Ad libitum* 4
1	24.21	24.16	24.55
2	24.31	24.38	24.29
3	24.22	24.39	24.42
4	24.00	24.00	24.10
5	24.24	24.51	24.27
6	24.01	24.32	24.29
7	24.17	24.51	24.28
8	24.28	24.45	24.39
9	24.24	24.21	24.29
10	24.55	24.67	24.59
11	24.36	24.42	24.03
12	24.17	24.22	24.39
13	24.29	23.93	23.77
14	24.24	24.21	24.59
15	24.22	24.27	24.19

Table (vi) ANOVA summary table
The independent variable was phase (*Ad libitum* 2, *Ad libitum* 3, *Ad libitum* 4) for animals exposed to T cycles<24h. Dependent variable: tau of the free-running light entrainable rhythm for food counts.

Source of variation	SS	Df	MS	F	*p*
Phase	0.13	2	0.07	4.89	0.02
Within + residual	0.18	14	0.01		

Table (vii) ANOVA summary table
The independent variable was phase (*Ad libitum* 2, *Ad libitum* 3, *Ad libitum* 4) for animals exposed to T cycles>24h. Dependent variable: tau of the free-running light entrainable rhythm for food counts.

Source of variation	SS	Df	MS	F	*P*
Phase	0.01	2	0.00	0.29	0.76
Within + residual	0.23	12	0.02		

APPENDIX E

Table (i) Raw data
PAD (hrs) between activity onset and the onset of food access for feeding schedules consisting of two daily meals.

Subject no.	Leading meal	Trailing meal
2	1.00	0.50
4	1.50	3.00
11	0.75	2.50

Table (ii) Raw data
Estimates of tau of the free-running light entrainable rhythm for the pre- and post-phase for feeding schedules consisting of two daily meals.

Subject no.	Pre-phase	Post-phase
1	23.10	24.95
2	24.43	24.43
3	24.60	23.99
4	24.50	24.00
5	24.53	24.53
6	24.50	24.50
7	24.05	24.40
8	24.50	24.35
9	24.93	24.60
10	24.50	24.50
11	24.60	24.65
12	24.33	24.43

Table (iii) Raw data

Estimates of tau of the free-running light entrainable rhythm for the pre- and post-phase for feeding schedules consisting of three daily meals.

Subject no.	Pre-phase	Post-phase
1	24.43	24.43
2	24.93	24.65
3	24.80	24.46
4	24.41	24.47
5	24.38	24.38
6	24.15	24.40
7	24.96	24.36
8	24.20	24.22
9	23.83	25.10
10	23.94	23.95
11	24.90	24.09
12	24.65	24.55