**Effects of Diet Composition and Length of Feeding Restriction on the Locomotor Rhythms of *Mus musculus***

by

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(ABSTRACT)

Changes in locomotor rhythms of *Mus musculus* as a response to combinations of lighting and feeding cues were quantitatively assessed in this study. Time allowed for feeding was varied (3, 5, and 7 hours) to examine effect of different zeitgeber strengths on locomotor patterns. The effect of temporal light/dark cue removal on locomotor rhythms was examined in conjunction with restricted feeding regimes. The potential to use blood glucose levels as a temporal feeding cue was examined by comparing the locomotor rhythms of mice consuming either a high glucose or high starch diet. Blood glucose curves for animals under restricted feeding regimes of 1 and 5 hour lengths were determined for the two diets.

Most of the locomotor activity records from animals in total darkness and a restricted feeding regime contained rhythm splitting. Only the anticipatory component of locomotor activity was synchronized by the 7 hour and 5 hour restricted feeding regimes in total darkness. Mice on a 3 hour restriction regime also exhibited a freerun component in locomotor activity. The two experimental diets did not produce statistical differences in plasma glucose concentrations when animals were placed on a 5 hour restricted feeding regime in total darkness, but did when they were placed in a light/dark 12:12 lighting cycle with a 1 hour feeding regime. The locomotor records indicated that freerunning locomotor components were synchronized only by light/dark transitions, while the anticipatory component was synchronized by food presentation. The results of this study are consistent with a Two-Oscillator Model for control of activity rhythms.

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# Introduction

Circadian rhythmicity in behavioral and physiological variables has been demonstrated in organisms as diverse as algae and human beings (Hastings, 1970). The use of the term circadian to refer to cycles whose periodicity approximates the 24-hour day was not introduced until the late 1950's (Halberg et al., 1954; Halberg, 1959). Since organisms constantly receive temporal information in the form of changing light to dark ratios, variations in the appearance and gravitational force of the moon, temperature changes, and fluctuations in food availability, it is natural to assume that there is a selective pressure for the formation of an internal timekeeping mechanism. In order to be effective, an internal clock needs to have an endogenous periodicity which approximates the 24 hour day and needs to run continuously in the absence of exogenous information (Pittendrigh and Daan, 1976b). It is now known that virtually every biological organism has an intrinsic ability to measure the passage of time, and thus to synchronize most of its biological rhythms with those of the external world.

The circadian clock consists of both neural and biochemical components and exhibits several characteristics that are common to all organisms. The clock mechanism is species specific and is passed genetically from one generation to the next (Moore-Ede et al., 1982; Richter, 1973). The clock is set prior to birth in mammalian young (Reppert and Schwartz, 1983), with the mother's temporal interpretation of the external world acting as a zeitgeber for her young. The internal clock

produces oscillations which are uniform both in timing and amplitude, temperature compensated, and endogenously generated when deprived of external temporal information (Kawamura and Ibuka, 1978; Moore-Ede et al., 1982; Pittendrigh and Daan, 1976a). Rhythms driven by the clock include, but are not limited to: activity, eating, drinking, body temperature, blood parameters (glucose, insulin, glucagon, growth hormone, corticosterone, and urea), intestinal enzymes, reproductive condition, cell growth and differentiation rates, cellular susceptibility to toxicity, and the rate of DNA replication.

One of the easiest and most accurate ways to determine the status of the internal clock in rodents is to record their locomotor rhythms (Richter, 1922). The activity wheel is one of the best devices for monitoring the influences of environmental variables on behavior since even animals in semi-captive conditions prefer wheels over more natural modes of expressing activity (Kavanau and Ramos, 1975). Quantitative activity records of wild, free-ranging animals are rare and often incomplete, but they are the first step in understanding the interaction between the environment and an animal's circadian system. Given the complexities involved in successfully quantifying activity in the wild, however, it is neccesary to perform controlled laboratory studies to evaluate clock function (Kavanau, 1971). Thus, the activity wheel has become the standard device for monitoring the internal clock in many different types of animals. As a generality, omnivores and herbivores tend to display relatively long continuous stretches of locomotor activity on activity wheels. Predators, on the other hand, tend to be active in bouts. Mice fall between these two extremes, with 99.5% of their activity occurring as discrete activity bouts during the dark phase of the light/dark (LD) cycle (Kavanau, 1969). Locomotor activity in the form of wheel running is a highly rhythmic function in most types of mice, and the automated data collection systems needed to monitor wheel running activity over time are relatively inexpensive to build and maintain. *Mus musculus* is a common experimental animal in the circadian literature, and its responses to external stimuli are well documented. Thus, *Mus musculus* were chosen for this study, and an automatic data collection system was designed to monitor their locomotor rhythms. The response of this rodent to various combinations of external stimuli thus could be monitored by quantifying changes in the overt running rhythms.

One point of interest in circadian research is the number and location of neural oscillators which are involved in temporal synchronization of mammals. Much evidence suggests that the suprachiasmatic nucleus (SCN) are involved in locomotor activity synchronization. If the SCN is acting in concert with one or more other neural oscillators, than it is possible that the anticipatory activity and the freerunning component of the locomotor rhythms of animals in continuous lighting (LL) or under conditions of severe food restriction are an overt expression of the different characteristics of these individual oscillators. Pittendrigh and Daan (1976c) postulated that two locomotory oscillators exist in the mammalian brain. They proposed that the morning oscillator was synchronized by the transition from dark to light (daybreak) and that the evening oscillator was synchronized by the transition from light to dark (dusk). If these two oscillators are driving the locomotor rhythms of animals in all experimental conditions, then studying the interaction between the two separate components when zeitgebers of varying strengths are applied will show how the circadian clock is reset by external temporal cues. In this study, the interaction of anticipatory and freerunning components of locomotor activity in total darkness (DD) with a feeding regime were examined, and the amount of time allowed for feeding was varied so that the effects of different zeitgeber strengths could be evaluated.

Laboratory mice and rats, like all mammals examined so far, exhibit circadian rhythmicity in plasma glucose and insulin levels (Gagliardino and Hernández, 1971; Jolin and Montes, 1973). It has been demonstrated that the first physiological step in the regulation of the circadian plasma glucose rhythm in mammals is the monitoring of plasma glucose levels via insulin glucoreceptors in the lateral hypothalamic nuclei of the brain (Gagliardino et al., 1984; Szabo and Szabo, 1975). These structures stimulate feeding and are tonically inhibited by the ventromedial hypothalamic nuclei (a satiety center). Both the lateral and ventromedial hypothalamic nuclei are directly innervated by the SCN, one of the major oscillators involved in the setting of mammalian circadian rhythms (Gagliardino et al., 1984). Thus, the neural system which drives the circadian rhythm of blood glucose and that which controls the acquisition of carbohydrates needed for the maintenance of the plasma rhythm share many common neural tracts and structures. It is possible, therefore, that blood glucose levels themselves may act as a temporal signal for the circadian system. This

hypothesis was examined by comparing the locomotor activities of laboratory mice which were on two different diets during a restricted feeding regime. If laboratory mice are able to reset the timing of their activity onset by monitoring the plasma glucose peak created by the act of feeding, then the use of two diets which create plasma glucose peaks that are temporally shifted from one another should cause an equivalent shift in the onset of wheel running activity. A high glucose and a high starch diet were chosen for this study because the time needed to break starch down to its base glucose units would create a temporal lag in the plasma glucose peak compared to that of the glucose-enhanced diet. The plasma glucose profiles created by the two diets were measured, and the timing of the plasma glucose peak was compared to the timing of the onset of wheel running activity.

# Literature Review

## *Early Circadian Research with Rodents*

The use of rodents in circadian research is documented extensively in the literature, perhaps due to the possibility that they can act as a model of the human circadian system in medical research. One of the first rhythms that was studied extensively was locomotor activity in the laboratory rat (Richter, 1922). Kurt Richter pioneered the use of the activity wheel coupled to a data collection system as a means of monitoring locomotor activity, a rhythm that is known to be endogenously driven. He found that locomotor activity is a cyclic phenomenon that can be influenced by both light/dark (LD) and eating/fasting (EF) cycles. When an animal synchronized its activity to coincide with the alterations of a cyclic external variable (or zeitgeber), Richter said that it had been entrained to the temporal signal. He found that rats could entrain their locomotor activity to both LD and EF cycles, with the onset of activity either directly preceeding the transition from light to dark (dusk) in the case of the LD cycle, or food presentation in the EF cycle.

Under constant conditions (such as LL or DD with food *ad libitum*), mammals exhibit spontaneous circadian cycles in both food intake and locomotor activity (Reinberg, 1974). Bouts of feeding and activity tend to be interspersed, but are usually confined to either the light or dark pe-

riod in an LD cycle. Under conditions of either total food deprivation or an EF regime, feeding and activity rhythms persist but often are altered. This is because metabolism itself is a cyclic phenomenon, dependent on a complex interaction of hormones and enzymes whose plasma levels vary throughout a 24 hour period. Normally, an LD cycle is considered to be a more powerful zeitgeber than an EF cycle, but an animal which must contend with a very short feeding interval that conflicts with his preferred rest period will shift many of his rhythms to accomodate the forced feeding time. The size of the feeding window is important in determining the overall strength of the forced interval feeding as a circadian zeitgeber, as well as the position of the window relative to other zeitgebers (such as the LD cycle). While most authors report entrainment of laboratory rats to feeding regimes with windows smaller than 5 hours, at least one study has found that "masking" of the underlying, true circadian periodicity of locomotor activity can occur with windows of 8 hour duration in otherwise constant conditions (Morimoto et al., 1979). Locomotor activity appeared to be synchronized to the EF regime, but a return to *ad libitum* feeding quickly resulted in the locomotor rhythm shifting in time to the point where it would be expected to appear if it were actually synchronized to the LD cycle. The physiological manner by which an animal synchronizes his internal rhythms to external feeding cues is a point of intense interest to medical researchers given the prevalence of jet lag, total parenteral nutrition (TPN), and shift work in our modern society.

The master clock, or oscillator, in the mammalian brain is believed to reside in the suprachiasmatic nucleus, or SCN (Moore and Eichler, 1976). The SCN interacts with one or more slave oscillators and drives the overt rhythms of feeding, drinking, etc., by means of a complex interaction of neural and/or hormonal factors. Temporal information from the external world is received by various receptors throughout the mammalian body, converted to a neural signal that can be received and interpreted by the brain, and used to fine-tune the timing of the internal clock. What is not known about this system is the exact number of oscillators involved, the manner in which they interact, the various routes by which temporal information can reach the mammalian clock, and the feedback mechanisms by which the brain controls the overt expressed rhythms.

# *Physiological Control of Circadian Rhythms*

Coordination of the hundreds of internal rhythms that are known to be driven endogenously in mammals is a complex task that involves a subtle interplay between neural and hormonal signals. It is now believed that a few master neural oscillators drive (or synchronize) the cycles present in many secondary slave oscillators which may be present even at the cellular level. Theoretically, therefore, individual rhythms could be produced which have a cycle length that is a function of a combination of oscillators driving the rhythm. This theory, called the Internal Coincidence Model when postulated by Pittendrigh in 1972, helps to explain why mammalian rhythms must have a periodicity that is either 24 hours or some simple function of 24 hours in length. The studies which have helped to clarify how individual rhythms are controlled at the physiological level in mammals can be placed into two main categories: lesion experiments and biochemical studies.

## Lesion Experiments

Lesions in various portions of the brain have been preformed in order to better understand the gross anatomy of the mammalian circadian system. These studies have helped delineate which portions of the brain are needed for rhythmicity and how those select areas are interconnected. One area which has been studied intensively is the suprachiasmatic nucleus (SCN). This region is located ventral to the hypothalamus and contains two main regions. The dorsomedial area receives no direct photic information and has no direct external projections. The ventromedial region, on the other hand, receives photic information both directly and indirectly and has projections into the hypophysiotrophic area of the hypothalamus (Moore, 1983). The SCN also has projections into the midbrain, contralateral SCN, lateral geniculate nuclei, and septal nucleus. Photic information from the external world is transmitted directly to the SCN via a retino-hypothalamic nerve tract.

Moore (1983) has suggested that the SCN acts in concert with other oscillators to serve as the main clock in mammals.

Many authors have reported the loss of locomotor, sleeping, eating, drinking, plasma corticosteroid, plasma thyrotropin, body temperature, photoperiodic sensitivity, and estrous cyclicity following SCN lesions in both hamsters and laboratory rats (Abe et al., 1979; Elliot, 1976; Kawamura and Ibuka, 1978; Krieger, 1974; Krieger et al., 1977; Stephan and Zucker, 1972; Stetson and Watson-Whitmyre, 1976). Hamsters with SCN lesions displayed arrhythmic activity patterns when placed in DD conditions. If placed in LL, however, they demonstrated a sporadic activity pattern (Stetson and Watson-Whitmyre, 1976). Loss of body temperature and plasma corticosteroid rhythms when laboratory rats received SCN lesions could be prevented if the animals were first entrained to a 2 hour EF cycle that corresponded to their rest period in the LD 12:12 light cycle (Krieger et al., 1977). Even locomotor activity in the form of prefeeding anticipatory activity (AA) could be recovered in laboratory rats with SCN lesions if a 2 hour restriction window was employed post-operatively (Stephan et al., 1979). The anticipitory activity present after lesions of the SCN has been shown to be endogenously generated: the rhythms persist for several days during food deprivation, and anticipatory activity cycles can be synchronized only by feeding regimes which have a periodicity that approximates 24 hours (Phillips and Milkulka, 1979). This evidence would seem to indicate that many mammalian rhythms normally are driven by the SCN, but that at least some of the rhythms can be driven by another oscillator or set of oscillators when they are temporally shifted by a feeding window (Moore and Eichler, 1976).

Studies on the ontogeny of the circadian system also support this view. The rhythm of body temperature in humans and laboratory rats is not present until the central nervous system has reached a high level of development, and the plasma corticosterone rhythm does not appear until much later still. It is at the time that the corticosterone pattern appears (21-30 days of age in the laboratory rat, 3-8 years of age in humans) that many other plasma rhythms also reach their adult status (Krieger, 1974). Obstructing the third ventricle in the brain leads to a loss of body temperature rhythmicity (Kawamura and Ibuka, 1978), pointing to a hypothalamic origin for the second driving oscillator. Some researchers postulated that the second driving oscillator was the

ventromedial hypothalamic nucleus (VMHN) since it is known to be involved in the synchronization of activity rhythms by feeding cycles. Lesions of the VMHN result in hyperphagia and hyperinsulinism (Martin et al., 1974), but it has been rejected as the possible site of a separate driving oscillator from the SCN based on other evidence (Inouye, 1983). With the information derived from lesioning experiments, it has been suggested that adult rhythm patterns can only appear once specific driving oscillators in the CNS reach a set level of maturity. Prior to that time, the variables are either arrhythmic, or are being driven by a different oscillator from that found in the adult mammal. Finding the exact locations of the secondary neural oscillators is now a prime research objective.

## Biochemical Studies

Circadian rhythms present in many mammalian blood plasma variables are affected by the feeding regime. It has long been known that the amount of weight gain experienced by an animal on a given diet is dependent on when that diet was administered (Halberg et al., 1976; Kahn et al., 1981). Weight gain in laboratory mice and rats is highest when the feeding window is located in the rodent's rest period. Shifting the feeding window affects both the position and amplitude of the cyclic plasma rhythms found in glucose, insulin, glucagon, and a host of other metabolic variables (Gagliardino et al., 1984). It has been suggested, therefore, that cyclicity of the plasma factors must be dependent on a complex interaction between the various metabolites and their relative phase-angles (or temporal positions relative to one another). When laboratory mice and rats are fed *ad libitum*, for instance, the circadian pattern found in plasma insulin concentrations is driven by the circadian ability of the pancreatic beta cells to release insulin (Pessacq et al., 1976). This rhythm remains independent of fasting (and thus the inherent rhythmicity in feeding) as long as the animal does not experience a large drop in serum glucose. Tissue energy demands, and not the actual feeding, influence the shape of the plasma insulin curve.

While the interaction between the feeding cycle and many metabolic rhythms has been tested extensively, the exact manner by which these rhythms are driven is not completely understood. Since an animal's locomotor rhythm can be synchronized by EF cycles even in the absence of the SCN, there must be some input to a separate master neural oscillator created by feeding. One hypothetical route by which this could occur is through the biochemical monitoring of specific plasma components whose concentrations are affected by feeding. Research in this area should be directed at determining how feeding rhythms are entrained.

Destruction of the ventromedial hypothalamic nuclei (VMHN) results in hyperinsulinism and hyperphagia in laboratory rats. Likewise, exposure of pancreatic tissue to secretions from the VMHN results in insulin release (Idahl and Martin, 1971). Electrical stimulation of the VMHN, however, creates a rapid rise in plasma glucose as a result of increased hepatic output without a concurrent change in the circulating insulin levels (Frohman and Bernardis, 1971). Insulin release in mammals decreases the circulating glucagon levels, increases glucose uptake by tissues (through changes in membrane transport), increases amino acid uptake, increases intracellular protein synthesis, increases glycogen synthesis, decreases hepatic gluconeogenesis, increases lipogenesis, and decreases lipolysis (Kahn et al., 1981; Page and Clibon, 1980). Insulin is known to interact with insulin-sensitive glucoreceptors in the hypothalamus, thereby triggering the reduction in hepatic glucose release that is associated with a feeding bout (Szabo and Szabo, 1975). The actions of insulin are counteracted by many hormones which include cortisol, growth hormone, the catecholamines, thyroid hormones, and glucagon. All of these metabolic hormones show cyclic rhythmicity in humans and laboratory rats (Moore-Ede et al., 1982). Cortisol dramatically increases the rate of protein degradation and shuttling of amino acids into gluconeogenesis. It also reduces the affinity of insulin receptors for insulin. The catecholamines block the release of insulin from the pancreatic beta cells and stimulate glycogenolysis (Gagliardino et al., 1984). Control of these metabolic hormones is mediated by the central nervous system. The lateral hypothalamic nuclei (LHN) stimulate food intake and are affected by plasma glucose levels, presumably through insulin glucoreceptors (adipocytes) located there. The VMHN acts as a satiety center which tonically inhibits the LHN. It is known that the SCN is directly innervated by the appetite control

areas (through the dorsomedial hypothalamic nucleus), raising the possibility that the blood glucose rhythm and the setting of locomotor rhythmicity by EF cycles might share a common mechanism. In any case, physical exercise and the feeding regime which an animal encounters have profound influences on the amplitude and position of the circadian changes in blood glucose concentrations.

Another facet of brain activity which is known to be directly affected by the ingestion of food is production of the neurotransmitter serotonin (Fernstrom and Wurtman, 1974). Laboratory rats that have ingested a high carbohydrate meal show an almost immediate increase in brain serotonin concentration. Concurrent with this is a drop in the plasma amino acid levels as protein construction by the tissues increases. Tryptophan, a precursor of serotonin, competes with five other neutral amino acids (leucine, isoleucine, valine, phenylalanine, and tyrosine) for transport through the blood-brain barrier. As the other amino acids are removed from the blood due to the action of insulin, plasma tryptophan levels begin to dominate. Increasing tryptophan availability in the brain thus leads to an increased production of serotonin. Protein-rich diets counteract this effect because tryptophan (an essential amino acid) is rare compared to the other neutral amino acids. The possibility that the circadian system could use serotonin levels as a signal to monitor feeding times is given added strength by the fact that serotonin, while an extremely common neurotransmitter in the brain, is produced in relatively large quantities by the pineal, an organ which has already been shown to be extremely important in the light cycle mediation of circannual reproductive rhythms. The pineal normally converts tryptophan to serotonin and then uses this compound to form melatonin. The enzymes utilized in this biochemical pathway are inhibited by light, so melatonin production is a cyclic phenomenon that is directly regulated by the length of light in an LD cycle. Whether or not this phenomenon is in any way associated with the setting of circadian rhythms by the ingestion of tryptophan has yet to be demonstrated in the laboratory.

## The Circadian System and Isotopic Tracer Studies

One of the most powerful reseach tools to be used in recent time for study of the mammalian circadian system is the use of [$^{14}$ C] labeling to trace the differential use of glucose by various structures in the brain. First used to measure blood flow in the brains of cats (Reivich et al., 1969), this method has since added a wealth of confirmational evidence to what had been only observational hypotheses in circadian research. Modified to better fit the needs of circadian research in the 1970's (Kennedy at al., 1975; Sokoloff et al., 1977), this technique has proven that the SCN changes its energy use as a function of external light levels. Increasing light levels result in increased energy use while decreasing light levels reduces the energy use (Schwartz et al., 1980). The amplitude and width of the energy use curve created by the SCN is thus a measure of the absolute phase position of the internal neural clock under any experimental conditions. In LL conditions, the SCN produces arrhythmic energy spikes which directly resemble the activity bouts recorded on the locomotor records of animals under the same conditions. In DD, the SCN utilizes energy in bouts which freerun through time, again corresponding to locomotor records for DD conditions (Schwartz and Gaines, 1977). No other brain area examined so far has shown a similar dependence on the external light/dark cycle. Carbon labeling was also used to prove that the phase of the mother's activity rhythm is transferred to the young in utero (Reppert and Schwartz, 1983).

# *Effects of Restricted Feeding Regimes*

## General Characteristics

Restricted feeding regimes can have profound effects on a wide variety of rhythmic physiological variables. The extent of the effect is dependent on the food restriction method chosen, the nature of the rhythmic variable being observed, the species under consideration, and the presence and strength of other potential zeitgebers such as LD cycles (Boulos and Terman, 1980). There are two main types of food restriction experiments: those in which an animal is presented with food *ad libitum* for a restricted time period, and those where the time allowed for feeding is unlimited, but food is present only in a limited quantity. When laboratory mice or rats are fed *ad libitum* during a set time interval early in their subjective rest period, weight gain is low. Conversly, feeding a set amount of food at the same time in their subjective day results in high weight gain. It appears that the two types of feeding restriction have differential effects on the underlying rhythmic variables of activity and metabolism and thus can create completely opposite effects (Boulos and Terman, 1980; Goetz et al., 1976; Halberg et al., 1976). Given food *ad libitum*, mammals feed in cyclic bouts (Boulos and Terman, 1980; Le Magnen, 1971; Reinberg, 1974) and produce a feeding peak during their subjective active period.

The type of LD cycle present also determines how feeding restriction affects an animal. Rhythms known to be synchronized by feeding windows in LD cyles include: locomotor activity, drinking, sleep stages, temperature, $O_2$ consumption, $CO_2$ emmission, plasma hormones (corticosterone, prolactin, thyroxine, adrenocorticotropin, insulin, and gastrin), plasma glucose, plasma amino acids, many hepatic factors (glycogen, tyrosine transaminase, RNA polymerase, tryptophan pyrrolase, phosphorylase, and PEP carboxykinase), brain neurotransmitters, and intestinal guananyl cyclase and disaccharidase levels (Boulos and Terman, 1980; Fernstrom and Wurtman, 1974; Gagliardino et al., 1984; Krieger et al., 1977; Le Magnen, 1971; Mouret et al., 1973;

Nelson et al., 1973; Phillipens et al., 1977; and Reinberg, 1974). When laboratory rats or mice are placed in constant lighting conditions (LL or DD), only the following rhythms have been shown to be synchronized by feeding cues: activity, drinking, sleep cycles, temperature, $O_2$ consumption, $CO_2$ emmission, plasma glucose, plasma corticosterone, hepatic glycogen, and intestinal guananyl cyclase and disaccharidase levels (Boulos and Terman, 1980; Krieger, 1974; Phillipens et al., 1977). Thus, it appears that the type of light cycle that is present can help determine which rhythms will be synchronized by a given EF regime. The ability of an animal to shuttle the control of various rhythmic functions among different driving oscillators apppears to be dependent on the strength and number of external zeitgebers present (Krieger et al., 1977). The idea that there is such plasticity in the circadian system, and that there may be several master oscillators in the mammalian brain acting in concert with the SCN, is slowly gaining credibility as more corroborative research data appear in the literature.

## The Phenomenon of Rhythm Splitting

One line of evidence which suggests that there must be several oscillators present in the mammalian brain which can act as independent clocks can be found in the phenomenon known as "rhythm splitting" in locomotor activity records (Albers et al., 1983; Daan and Pittendrigh, 1976; Jilge, 1980; Johnson et al., 1983). Animals are most susceptible to the effects of external zeitgebers when they are at the beginning of their subjective active period (Aschoff et al., 1973; De Coursey, 1964). It is at this point (dawn for diurnal mammals and dusk for nocturnal mammals) that wild animals normally reset the timing of their internal clocks. It is also at this point, however, that certain zeitgeber conditions can lead to the creation of two separate bouts of locomotor activity which respond differently to changing external stimuli. This phenomenon, called rhythm splitting, was first documented by Pittendrigh (1960) in the arctic ground squirrel. He named the two oscillators the morning and evening oscillators and noted that they appeared to be independently controlling the onset and end of the bulk of locomotor activity. Since that time, rhythm splitting

has also been documented in the locomotor records of tree shrews, golden hamsters, turkish hamsters, flying squirrels, striped palm squirrels, rabbits, and cats (Albers et al., 1983; Aschoff et al., 1973; Jilge, 1980; Johnson et al., 1983; Pittendrigh, 1960; Pittendrigh and Daan, 1976c). This phenomenon has been used by many authors as evidence that a multi-oscillator system must be operating during entrainment of circadian rhythms by external cycles in mammals.

When laboratory rats are deprived of food, water, or food and water, they exhibit a bout of anticipatory activity (AA) immediately preceding the time when food or water would normally be available (Bolles, 1968; Bolles and Duncan, 1969). Laboratory rats in LD 12:12 which are presented with two separate feeding windows within a 24 hour period respond by producing two separate bouts of locomotor activity which immediately precede the two windows (Boulos and Terman, 1980). When released into *ad libitum* feeding conditions (but still in LD 12:12), the rats continued to display two activity components. Rats in LL that are presented with two 1 hour feeding windows that have periodicities of 23 and 24 hours respectively (so that their relative phase-angle difference is constantly changing over time) will respond in one of three manners: they exhibit one distinct bout of activity preceding either the stable feeding time or the moving feeding time, they produce two bouts of activity which independently track each feeding time, or they have one large bulk of activity that freeruns (a condition resulting when the subjective 24-hour day is expressed at a constant rate that is different from 24 hours) between the two windows (Edmonds and Adler, 1977b). Release into *ad libitum* conditions resulted in maintenance of the two separate activity components by 10 out of 12 animals. Laboratory rats in LL which are fed within one 4 hour or one 2 hour feeding window per day also produce two separate peaks in both locomotor activity and plasma corticosterone (Honma et al., 1983). These peaks responded differently to the experimental regime, with one peak constantly preceeding the feeding window and the other freerunning in real time. Release of the animals into *ad libitum* conditions resulted in a rejoining of the components by phase delaying transients. Similar results were found when laboratory rats were given a 2 hour window in LD 12:12 (Edmonds and Adler, 1977a; Gibbs, 1979), a 2 hour window in DD (Meyer-Lohmann, 1955), or when drinking windows were used instead of feeding windows (Boulos et al., 1980). From studies such as these, it has become obvious that the partic-

ular oscillator or combination of oscillators that is responsible for driving any given overt rhythm is a function of the combination of external stimuli to which an animal is exposed and how that combination is perceived. All of these data (and the internal desynchronization of rhythms which can sometimes accompany large temporal phase shifts) can be explained by a two-oscillator model, in which one oscillator is synchronized by feeding regimes (producing the anticipatory activity), and one oscillator is unaffected by the cyclic presentation of food or water (producing the freerun component). This general theory has been presented in different forms by many authors (Albers et al., 1983; Bolles and Moot, 1973; Boulos et al., 1980; Boulos and Terman, 1979; Coleman et al., 1982; Daan and Pittendrigh, 1976; Honma et al., 1983; Moore-Ede et al., 1982; Pittendrigh, 1960; Pittendrigh and Daan, 1976c; Wiedemann, 1984) but is still not universally accepted (Johnson, 1983; Morimoto et al., 1979). Further research into the manner by which organisms convert external stimuli into temporal signals will require restricted feeding studies to clarify the mechanics of the circadian system.

## Informational Pathways Involved in Feeding

One important area of circadian rhythm research involving feeding regimes is the exact manner by which organisms convert a multi-faceted stimulus like a feeding response into a single temporal cue which can be interpreted and used by the internal timekeeping mechanism. Instrumental in this endeavor are studies involving circadian rhythms and the presentation of food either orally, intravenously (IV), or intragastrically (IG). Researchers in this area hope that breaking the act of feeding down into its base components will allow them to understand how the circadian system responds under more normal circumstances. Much of this research is applied, with the ultimate goal being the benefit of humans that must be placed on long-term parenteral feeding for medical reasons.

Laboratory rats fed continuously either orally or IG gain weight faster and produce a higher plasma insulin peak than when given an equivalent diet IV (Lickey et al., 1978). This seems to

indicate that metabolic responses to continuous food administration are mediated through intestinal and hepatic factors. These types of food presentation are known to differentially affect various metabolic rhythms: continuous IV feedings abolish the circadian plasma glucose rhythm as well as the cyclic hepatic weight and glycogen content rhythms, while cyclic discontinuous IV feedings abolish only the serum insulin and corticosterone rhythms (Lanza-Jacoby et al., 1982; Saito et al., 1981; Sitren and Stevenson, 1980). In addition, squirrel monkeys on cyclic IV feeding regimes gain weight faster when the infusion is placed in their subjective rest period as opposed to either in their subjective active period or applied continuously (Finn et al., 1982). When animals are cyclically infused with insulin to mimic the body's response to a high carbohydrate diet, it is found that the infusion timing is important. A dose-response curve for the increase in both number and size of spontaneous feeding bouts is found for animals infused during their subjective rest period, while animals infused during their subjective active period show only a small increase in the number of feeding bouts, a response which is independent of the dose of insulin used (Larve-Achagiotis and Le Magnen, 1979). Obviously, the overt response of a mammal to the presentation of food is dependent on temporal as well as physiological parameters.

# Materials and Methods

## *Experimental Animals*

The experimental animals used in this study were inbred male laboratory mice (*Mus musculus*, BALB/cAnN/NIH strain) purchased from Dominion Laboratories (Dublin, Va.). Starting weights were between 13 and 15g. Before shipping, the animals were given hard lab chow and water *ad libitum*, and were housed in communal cages under an LD 12:12 (lights on at 0700 and off at 1900). Shipping time never exceeded several hours, and upon arrival at Virginia Polytechnic Institute and State University, they were singly housed in standard plastic shoebox tubs (28cm × 16.5cm × 13cm). Animals were housed continually in environmental chambers and prior to the experimental period were provided with hard lab chow and water *ad libitum*. Twenty animals were placed in activity cages at the beginning of both the long-term and short-term studies. There was no selection in the animals for running ability. Animals number 1, 3, 6, 13, and 18 died early in the long-term study. Animals number 21, 24, 26, 27, and 30 died early in the short-term study. The animals that died lost as much as 50% of their body mass in three days as a result of not eating. It is not known why some of the animals suddenly refused to eat.

# *Experimental Diets*

There were four basic diets used in this research. The first consisted of Prolab 3000 (Agway Inc., Syracuse, N.Y.) in hard chow form. The second consisted of powdered Prolab 3000. Hard chow was crushed with a meat grinder and then sieved with a fine mesh screen (63μm) to ensure particle uniformity. The last two diets consisted of powdered Prolab 3000 mixed 50/50 by weight with either powdered glucose (Domino Brand, Amster Corporation, New York, N.Y.) or corn starch (Argo Brand, Best Foods, Englewood Cliffs, N.J.). All powdered diets were prepared in 1000g batches as needed and were stored in sealed plastic containers to retard hydration and spoilage. Diets were never allowed to sit unused for more than ninety days. Weighed food packets of $15 \pm 0.1$g were prepared as required on a day-to-day basis. Manufacturers specifications for Prolab 3000 state that the chow contained 22% protein, 5.5% fat, 0.9% linoleic acid, 4.5% fiber, 5.5% ash, and 52% nitrogen-free extract by weight.

Manufacturers specifications for Prolab 3000 state that it contained 18.41 kJ/g total energy, 15.48 kJ/g digestible energy, and 14.64 kJ/g metabolizeable energy. For the purposes of this study, only gross energy evaluations of the diets were performed. Determination of the energy content of the powdered chow and two mixed diets was carried out with a Phillipson Microbomb Calorimeter (Gentry Industries, Aiken, S.C.) following the method outlined by Phillipson (1964). Benzoic acid was used as the standard for these determinations. The samples to be analyzed were dried in an oven for 24 hours at 80°C. Randomly sized pellets between 20 and 40mg were prepared with a pellet press (Gentry Industries, Aiken, S.C.) for each determination, and were burned in the presence of 0.05ml of water and $O_2$ at a pressure of thirty atmospheres. Four trials were made for the powdered chow energy determination, and ten trials were made for each of the experimental diet determinations. Since the Prolab 3000 gross energy value of 18.41 kJ/g (as reported by the manufactorer) fell within two standard deviations of the experimentally determined value of $17.36 \pm 0.79$ kJ/g, the diet energy values were assumed to be correct.

## *Construction of Activity Cages*

The twenty activity cages used in this study were designed and built at Virginia Polytechnic Institute and State University (Figure 1). Each cage consisted of a standard plastic shoebox tub (28cm × 16.5cm × 13cm), and a rectangular wire mesh box (28cm × 13cm × 10cm) anchored to a wire mesh tub lid. A running wheel (16.5cm diameter, 8cm wide) was suspended so that it extended halfway into the wire mesh box above and halfway into the plastic tub below. The wheel was suspended towards the back of the box so that a wire mesh wall could be placed 8cm from the front of the box. This wall had a hole (5cm × 5cm) cut in the lower right corner so that the mouse had full access to the front of the cage. Along the left-hand side of the chamber created by the wall, a metal food tin (8cm × 5cm × 4cm) was secured, and a hinged lid was constructed so that access to the food cup could be regulated from outside the cage. A second hinged door was placed at the front of the wire mesh box to give the investigator access to the box itself. Finally, a 0.5g micro-switch was anchored to the left side of the wire mesh box so that each revolution of the running wheel triggered the microswitch. Animals were provided with water *ad libitum* by an automatic gravity-feed system. Plastic piping above each shelf connected a large reservoir to individual sipper tubes protruding directly in front of the food tins within each cage.

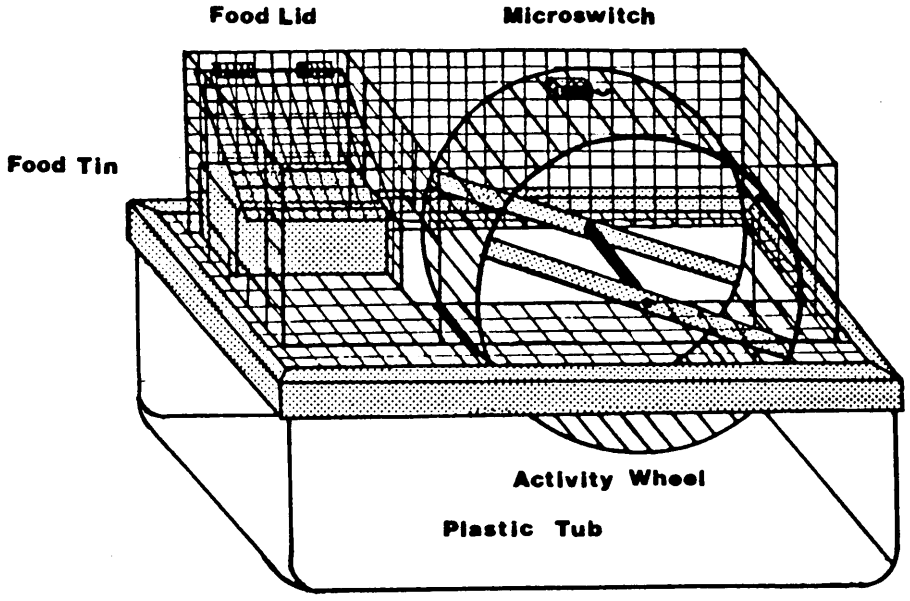



**Figure 1.** **Schematic of the animal cages:** The top and front wire-mesh panels, the investigator's access door, and the water sipper tube have been removed for clarity. When in operation, access to the food bin was controlled from outside the cage by means of a small wire connected to the center of the food lid.

# *Experimental Protocol*

Control of the potentially cyclic environmental variables of light, temperature, and noise level was provided by housing all experimental animals in environmental chambers. A large walk-in chamber (2.03m × 2.15m × 2.20m) was used during the acquisition of the locomotor records and the 5 hour feeding restriction curve (Warren/Sherer, Division of Kysor Ind., Marshall, Mich., Model PER-88-MH). A smaller reach-in chamber (0.68m × 1.33m × 1.40m) was used only during the acquisition of the 1 hour feeding restriction curve (Warren/Sherer, Division of Kysor Ind., Marshall, Mich., Model RI-48-LP). Lighting cycles in both chambers were controlled with time clocks. Chamber temperatures were regulated at 19± 1°C.

The twenty activity cages in the large chamber were placed on two free-standing racks, 5 cages per shelf. In order to reduce sound transmission between adjacent cages and eliminate visual contact between animals, cages were surrounded completely by partitions made of 5mm thick sheets of packing foam sandwiched between 5mm thick sheets of cardboard. Cages in the smaller environmental chamber were not separated by partitions in this manner because locomotor rhythms were not being monitored and social synchronization of the locomotor activity of the animals was desired to reduce interindividual differences. Cage substrate in all cases consisted of sterile wood shavings obtained from the woodshops at Virginia Polytechnic Institute and State University (V.P.I. and S.U.). Lighting in the large chamber was provided by four ceiling-mounted flourescent tubes (91cm length) during the light cycle and by a single small red-filtered flourescent tube (30cm length) during the dark cycle. The red light normally was kept in a position that provided equal illumination to all of the cages. The mice used in this study can not see light in the red region of the light spectrum (Michael Menaker, University of Virginia, personal communication), so constant dim red light was perceived by the animals as DD conditions. Personal observation of these mice under red-light conditions confirmed their lack of visual perception as mice only responded to auditory and tactile cues.

Cages were cleaned as needed at randomized times over the 24 hour day, but not during restricted feeding periods. When animal weights were required, all animals were weighed within a 1 hour period. Animal weights were obtained at the beginning and end of each new combination of lighting/feeding regimes, and at approximately 30 day intervals within any given combination. Animals were weighed by placing each mouse inside a pre-weighed plastic container, and then weighing him on an Ohaus triple-beam scale (Ohaus Scale Corp., Florham Park, N.J.; Model 311; 0.1gm accuracy). Animals always were weighed in conjunction with cage cleaning to minimize disturbance.

The large chamber was used for one long-term (eight month) study and one short-term (two month) study. Energy intake of six randomly selected mice during the long-term study was measured by weighing their powdered food daily over the course of the experiment. When the powdered chow was replaced by experimental diets, the six mice were split between the two experimental diet groups and food measurement was continued. The food weight data were used in conjunction with energy values derived for each powdered diet to give an estimate of the energy use of the mice during the long-term study. The combination of deep food tins and a restricted time period allowed for feeding effectively eliminated food spillage. Thus, no correction for food loss was necessary in the energy intake calculations.

Different lighting and feeding regimes were used in the long-term (Table 1) and short-term (Table 2) studies. It should be noted that the day numbers in these two tables are relative to the beginning of the experiment, with the original conditions of LD 16:8 and unrestricted hard chow in the long-term study and unrestricted hard chow and DL 12:12 in the short-term study acting as acclimation periods for the animals. When the mice were fed *ad libitum* in total darkness (DD) at the end of the long-term study, the investigator continued to enter the chamber at the times that food was presented and removed in the earlier feeding restriction regime. This was done to determine if the animals were entraining to the investigator's presence rather than food presentation.

The original twenty animals used in the long-term study were sampled near the end of the study to generate the 5 hour feeding restriction blood glucose curve. These same animals then were transferred to the smaller chamber and used for the 1 hour blood blood glucose curve determi-

nation. Protocol for operation of the smaller chamber was similar to that used in the large chamber except animals were kept in standard plastic shoebox tubs (28cm × 16.5cm × 13cm) instead of activity cages and were synchronized to a DL 12:12 light cycle (lights on at 2100 and off at 0900). Water was provided in water bottles which were cleaned weekly at randomized times, and the experimental diets were presented in metal food cups (7.5cm × 5cm × 4cm) suspended above the substrate on wire-mesh shelves.

**Table 1. Experimental conditions used in the long-term study.**

Day one was designated as the beginning of the experiment. Light cycles consisted of a LD 16:8 (lights on at 0700 and off at 2300), a DL 12:12 (lights on at 1900 and off at 0700), and DD (total darkness). Restricted feeding (RF) regimes were either seven hours in length (food from 0730 to 1430) or five hours in length (food from 0830 to 1330). The experiment ended on day 228..

| Condition (hours) | Day | Light Cycle | Diet | RF Length |
|---|---|---|---|---|
| 1 | -23 | LD 16:8 | Hard Chow | None |
| 2 | 1 | DL 12:12 | Hard Chow | None |
| 3 | 38 | DL 12:12 | Powdered Chow | 0700 |
| 4 | 50 | DD | Powdered Chow | 0700 |
| 5 | 67 | DD | Powdered Chow | 0500 |
| 6 | 99 | DD | Experimental | 0500 |
| 7 | 192 | DD | Experimental | None |

**Table 2. Experimental conditions used in the short-term study.**

Day one was designated as the beginning of the experiment. Light cycles consisted of a DL 12:12 (lights on at 1900 and off at 0700) and DD (total darkness). Restricted feeding (RF) regimes were either five hours in length (food from 0730 to 1230) or three hours in length (food from 0730 to 1030). The experiment ended on day 50.

| Condition | Day | Light Cycle | Diet | RF Length (hours) |
|---|---|---|---|---|
| 1 | -8 | DL 12:12 | Hard Chow | None |
| 2 | 1 | DL 12:12 | Powdered Chow | 0500 or 0300 |
| 3 | 35 | DD | Powdered Chow | 0500 or 0300 |

# *Data Acquisition*

## Recording of Locomotor Rhythms

Activity rhythms were monitored with an Esterline-Angus 20-Channel Event Recorder (Esterline-Angus Corp., Indianapolis, In., Model A). Activity cages were wired independently to a connector board mounted on the back of each animal rack. The connector boards were wired to an Esterline-Angus event recorder mounted directly outside the environmental chamber. This arrangement permitted individual activity cages to easily be removed for cleaning or repair. Wheel revolutions within the activity cages produced pen deflections within the recorder, thereby creating a continuous locomotor record on paper moving through the recorder at 2cm/hr. Power to the strip recorder was regulated by a Cynex Voltage Regulator (Model PS 77A). Reference time marks were made manually on the paper strip each day. Data were removed from the recorder at approximately one week intervals. Reference marks were used to divide the paper strip into 12 hour intervals which were then reduced on a zerox machine and recombined into 24 hour long strips. The individual strips representing locomotor activity for each cage were separated then and pasted sequentially on sheets of paper (each sheet thus constituted a single activity record for one animal over time). Since time was recorded manually, no attempt was made to reset the paper strip on the recorder to any set mark when data was removed from the machine. Thus, the original time units found on the paper can be ignored in the final activity records. Whenever there was a loss of data due to a mechanical failure, the loss was noted on the final activity record by white blocking. Loss of data was due to severed wires, wheels which had become temporarily immobilized, faulty pens in the recorder, or a lack of paper in the recorder.

## Construction of Blood Glucose Curves

Blood glucose concentrations were determined with a hexokinase plasma test kit (Sigma Chemicals, Kit 16UV, St. Louis, MO.). Blood samples were taken from the infraorbital sinus with heparinized capillary tubes (Fisher Scientific, Pittsburgh, Pa.) by the method described by Harkness and Wagner (1983). No anesthesia was used. Three capillary tubes were filled during each sampling. Each capillary tube held approximately 60µl of blood. Mice were handled for no more than ninety seconds each during a sampling session. Care was taken not to disturb the mice prior to each bleeding. Bleedings were timed so that a blood glucose curve over a 24 hour time period could be generated. Two blood glucose curves were constructed: one for a restricted feeding regime of 5 hour length, and one for a restricted feeding regime of 1 hour duration. The 5 hour blood glucose curve was constructed with blood samples taken from mice running in activity cages near the end of the long-term experiment. They were kept in constant darkness (DD) and had free access to water only. Blood samples were randomly drawn from animals within each experimental diet. Each animal was sampled no more than four times, with sampling periods separated by one week. After the long-term experiment had ended, the animals were transferred to the smaller environmental chamber and allowed to synchronize their locomotor activity to a reversed DL 12:12 light cycle (lights on at 2100 and off at 0900). Blood samples for the 1 hour blood glucose curve were then taken under red-light conditions as previously described. In this case, however, the mice were placed on a 1 hour RF regime for three days prior to each bleeding but were otherwise fed *ad libitum*. The beginning of food presentation was timed to coincide with lights off in the small chamber (0900).

Colorimetric determinations of hexokinase test results were made with a Gilford Model-260 Spectrophotometer. All of the tests were run at 460 nm with a blue filter (per manufacterers recommendations). Test results were read from a calibration curve constructed with glucose/urea standards (Kit 16-11, Sigma Chemicals, St. Louis, Mo.). The capillary tubes were centrifuged in a Clay-Adams Autocrit II centrifuge (Model 0574, Becton, Dickinson, and Comp., Parsippany,

N.Y.) to isolate the blood plasma, and then the three plasma samples from each animal were mixed on a small watch glass until uniform. Three separate 10µl aliquots were tested per animal per sampling time with the glucose assay solution. The average of these three tests was designated as the blood glucose concentration for that animal at that sampling time. Points on the blood glucose curves were then obtained by averaging the means of the tests for three different animals per diet group per sampling time.

## *Data Evaluation*

Statistical evaluation of the blood glucose curves was carried out with the SAS computer package at Virginia Polytechnic Institute and State University. A General Linear Models (GLM) procedure was used to analyze the significance of model main effects in both blood glucose determinations with animals not sampled at any given time point treated as empty cells in the evaluation. The GLM test was designed as a two-way crossed analysis of variance in which the independent variable of time represented repeated measurements on animals. When a particular parameter was found to be significant, two-sample t-tests were used to evaluate the data further.

In order to analyze food intake data from the long-term study, a 5 day mean of the daily food intake masses surrounding each animal weighing period was calculated. This value was multiplied by the energy value for that diet to form average daily energy intake figures. Next, the diet energy values were corrected for differential hydration created by air exposure in the chamber. Once divided by the mass of the mouse to make the energy values mass specific, these figures for the six representative mice were then used to test for changes in overall energy intake over time. Since the data were balanced, an analysis of variance (ANOVA) procedure designed to account for repeated measurement of animals over time was employed to test for significance of the individual model parameters. The model consisted of a two-way crossed analysis of variance in which the independent variable of day represented repeated measurement of animals over time.

In those cases where an animal had at least one week to adjust to a new combination of experimental conditions, and at least fourteen consecutive days where the activity onset was apparent, the locomotor data were analyzed in the following manner. When a component of an animal's locomotor activity rhythm was synchronized by the experimental LD cycle and/or RF regime, the timing of activity onset for that animal was determined by plotting the first indication of activity on a daily basis. The mean time $\pm$ SEM (standard error of the mean) for the daily activity onsets was then calculated in military time. In records where the data were too erratic to reliably determine the SEM, the best approximation of the mean daily activity onset time was determined by eye-fitting.

When any portion of an animal's locomotor activity rhythm was not entrained by the experimental conditions, it would freerun in real time at a rate determined by the difference between an animal's subjective 24 hour day and the real 24 hour day. The animal's subjective day length (designated $\tau$) was determined from locomotor activity freeruns by a variation of the center of gravity method (Kenagy, 1980) whenever data met the qualifications set for the activity onset determinations, or by line-fitting by eye whenever the first method was not feasible. These methods have been previously shown to be comparable (Kenagy, 1980). The geometric mean between activity onset and the end of activity was determined by eye on a day-to-day basis. The means were then used to construct a line with a linear-least-squares program. The inverse slope of the line (in $h*d^{-1}$) the amount by which the length of the subjective day differed from real day length. The $\tau$ values were computed either by subtracting the inverse slope from 24 hours (in the case of a freerun to the left in the records) or adding the inverse slope to 24 hours (in the case of a freerun to the right in the records). Correlation coefficients obtained from the linear-least-squares program were used as a variance estimate in the $\tau$ determinations.

When animals were shifted to a 7 hour RF regime during the reversed DL 12:12 portion of the long-term study, only 14 days elapsed before they were shifted to DD conditions, so this portion of the activity records was evaluated only in a qualitative manner. Only 15 days was allowed for the DD and food restriction portion of the short-term study, so eye-fitting alone was used to evaluate the locomotor trends present in this region of the records. In some cases, portions of the

locomotor records were arrhythmic or simply missing, so no evaluation of the data was possible by any of the methods previously described.

# Results

## *Locomotor Activity Records*

Locomotor activity and and concurrent mass records were compiled for fifteen animals in the long-term study (Appendix A, pp. 59-74) and fifteen animals in the short-term study (Appendix B, pp. 75-83). Long-term locomotor activity records are presented as a triple-plot (Figure 2) while those from animals in the short-term study are presented in the standard double-plot form (Figure 3). Locomotor patterns found in both the long and short-term records will be discussed in this report using the following working definitions. *Activity onset* is defined as the beginning of daily locomotor activity. An *entrained state* is produced by an external zeitgeber (temporal cue) when the circadian system responds by producing a relatively continuous activity band which has low daily temporal variance in activity onset. When a zeitgeber (or combination of zeitgebers) produces two distinct components in the daily locomotor record, the phenomenon is referred to as *rhythm splitting*. During rhythm splitting, a *freerunning* component appears in the locomotor activity record. The activity onset of the freerunning component changes daily by a set amount of time in a set direction. The elapsed time between the cyclic activity onset appearances of a freerunning component is designated tau ($\tau$) and usually approximates 2400 hours. The second

locomotor activity component found during rhythm splitting is called *anticipatory activity* (AA) and directly precedes daily food presentation in a restricted feeding regime. Daily timing of the AA component also exhibits low temporal variance. Each of the animals used in this study produced a distinctive locomotor activity record. Thus, each record may or may not contain all of the described locomotor activity patterns (Figure 4).

Long-term locomotor activity measurements demonstrated that a great amount of inter-animal plasticity existed in the responses of the circadian system to different zeitgeber combinations (Table 3). Of the fifteen animals in the 7 hour restricted feeding (RF) regime, fourteen had interpretable records and were entrained by the DL 12:12 lighting regime. Five animals began their activity before the transition from lights-on to lights-off (Appendix A; numbers 2, 7, 8, 17, and 20), four animals became active exactly at lights-off (Appendix A; numbers 4, 10, 12, and 16), five animals became active following the transition to lights-off (Appendix A; numbers 5, 9, 14, 15, and 19), and one animal had no data (Appendix A; number 11). The light/dark cycle was a strong enough zeitgeber to entrain the animals.

With the transition in DL 12:12 from food *ad libitum* to a 7 hour RF regime, seven animals responded by shifting their activity onset to earlier in the day (Appendix A; numbers 2, 4, 5, 10, 12, 17, and 19), seven animals were unaffected (Appendix A; numbers 7, 8, 9, 14, 15, 16, and 20), and one animal had no data (Appendix A; number 11). With the transition to total darkness (DD), more complex patterns began to appear. Three of the fifteen animals displayed a freerun in their locomotor rhythms with a $\tau$ value that was less than 24 hours in length (Appendix A; numbers 4, 15, and 20). Six animals exhibited rhythm splitting with an anticipatory activity component (AA) directly preceeding the feeding window and a freerunning component with a $\tau$ value greater than 24 hours (Appendix A; numbers 8, 12, 14, 16, 17, and 19). In three animals, there was a shift in the onset of activity to earlier in the day (Appendix A; numbers 2, 5, and 7), and three animals were unaffected (Appendix A; numbers 9, 10, and 11). The loss of the strong zeitgeber of light resulted in rhythm changes in most of the animals, even though the RF regime was still present.

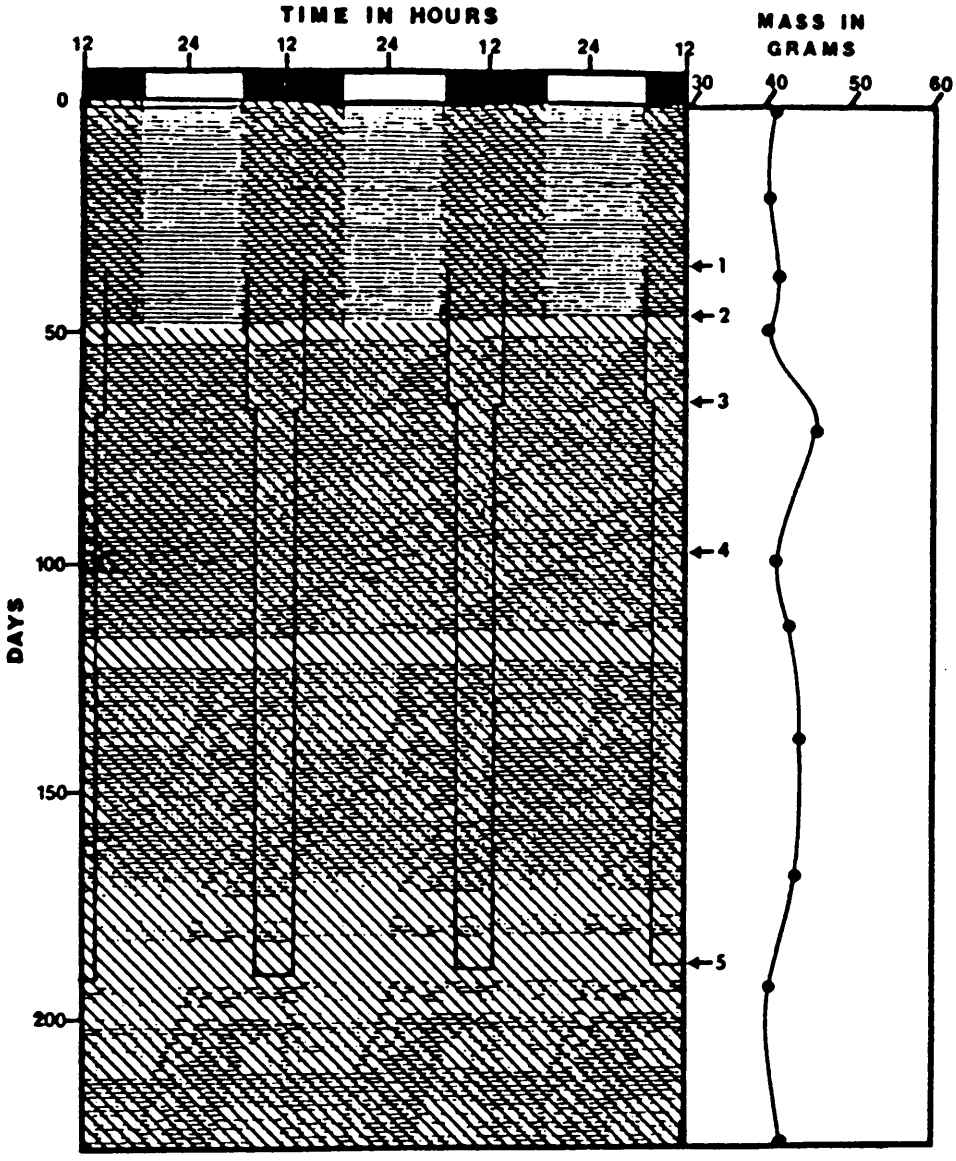
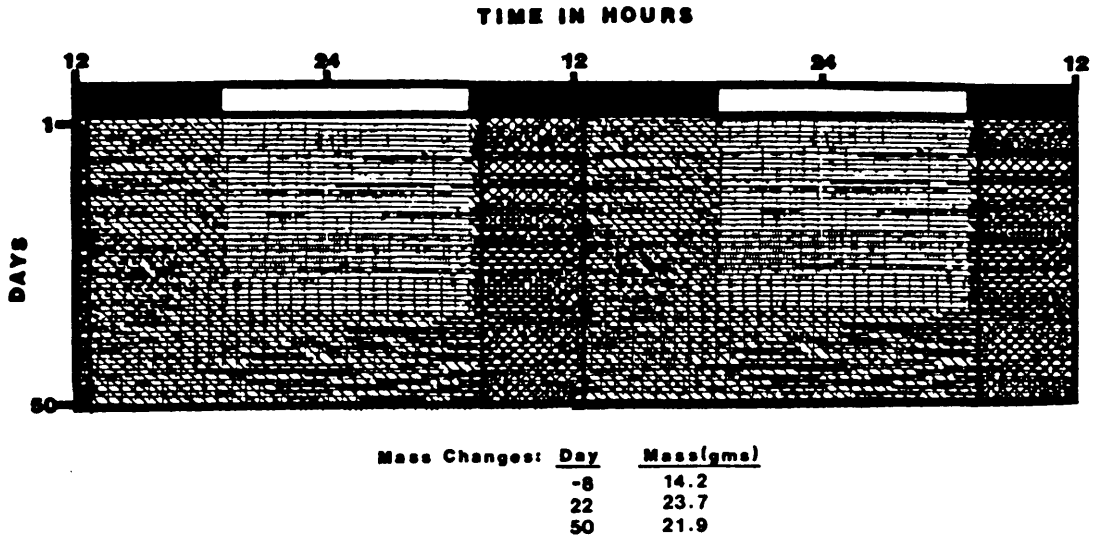



**Figure 2. Example of long-term record with experimental conditions superimposed - Animal number 4:** This figure shows changes in locomotor activity and mass for animal number 4 over time. The left side of the abcissa is marked in time units (hours) with the original reversed dark/light (DL) cycle indicated. The right side of the abcissa is marked in weight units (grams). The ordinate is valid for both plots and is marked in days. The locomotor record is a triple-plot. The superimposed slanted parallel lines represent parts of the record created while the animal was in darkness. White blocks in the record indicate a loss of data. Arrows indicate changes in the experimental conditions: 1 = beginning of 7 hour food restriction, 2 = total darkness, 3 = beginning of 5 hour food restriction, 4 = beginning of experimental diets, 5 = beginning of *ad libitum* feeding.




| Mass Changes: | Day | Mass(gms) |
|---|---|---|
| | -8 | 14.2 |
| | 22 | 23.7 |
| | 50 | 21.9 |

**Figure 3. Example of short-term record with experimental conditions superimposed - Animal number 22:** This figure shows the changes in locomotor activity and mass for animal number 22 over time. The abcissa is marked in time units (hours) with the original reversed dark/light (DL) cycle indicated. The ordinate is marked in days. The locomotor record is a double-plot. The superimposed slanted parallel lines represent parts of the record created while the animal was in darkness. Cross hatching denotes the temporal feeding window. Mass changes over time are presented below the figure.

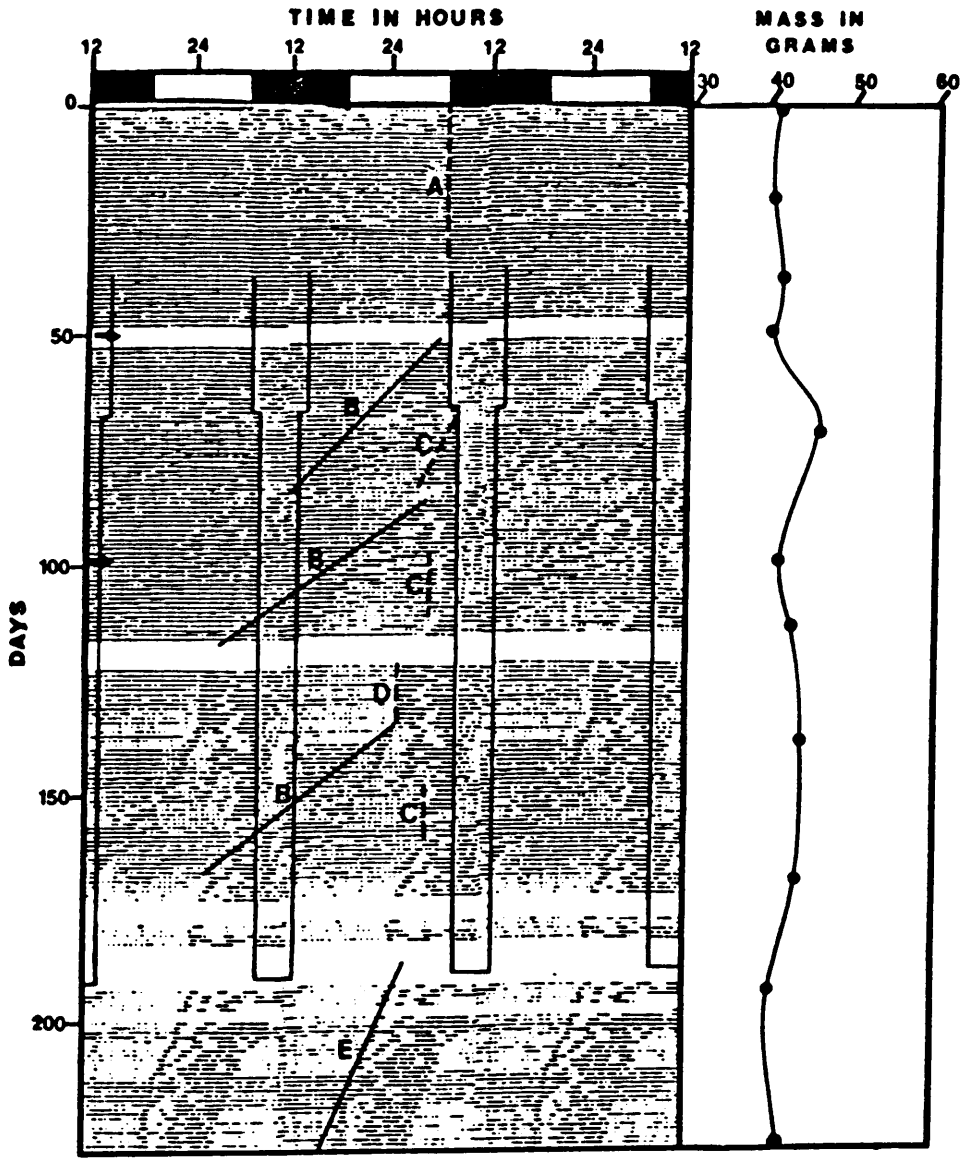



**Figure 4.** **Example of patterns seen in locomotor activity records - Animal number 4 in the long-term study:** This figure contains examples of all the locomotor activity patterns seen in the animal records. The left side of the abcissa is marked in time units (hours) with the original reversed dark/light (DL) cycle indicated. The right side of the abcissa is marked in mass units (grams). The ordinate is marked in days and is valid for both plots. The locomotor record is a triple-plot. White blocks in the record indicate a loss of data. The small arrow at day 50 denotes a transition to total darkness (DD). The second arrow at day 99 indicates where the powdered chow was replaced by experimental diets. The solid vertical lines denote the temporal feeding window. Lines associated with letters were fit by eye through daily activity onsets for each locomotor activity component (solid = freerunning; dash = entrained). A = total entrainment; B = freerun component in rhythm split; C = anticipatory activity (AA) in rhythm split; D = coupling of the freerun (B) and anticipatory activity (C) components with the anticipatory activity component entrained; E = freerun of the coupled components (B and C).

With a reduction in feeding time from seven hours to five hours, many of the locomotor rhythm patterns which had appeared in the records were altered. In five of the fifteen animals, the locomotor activity component which had begun to freerun with release into DD became synchronized to the removal of food (Appendix A; numbers 12, 14, 16, 17, and 19). In the records of two animals, the entire activity rhythm simply shifted to later in the day and entrained to the new feeding time (Appendix A; numbers 2 and 11). The record of animal number 4 showed continuation of an earlier freerun with the appearance of an AA component. Animal number 9 began a split pattern with a $\tau$ value greater than 24 hours. Two animals began to freerun with a $\tau$ value greater than 24 hours and eventually showed an AA component (Appendix A; numbers 7 and 20). Animal number 8 continued the split pattern with a reduction in the $\tau$ value of the freerunning component, while animal number 15 synchronized the end of his activity bout to the time of food presentation. The remaining two animals showed more complex patterns (Appendix A; numbers 5 and 10). These data indicate that the circadian system driving the locomotor rhythms of mice during DD and food restriction conditions is more complex than a single oscillator system.

In many of the animals, the freerunning component and the AA component of the locomotor rhythms interacted several times while the animals were in DD with a 5 hour feeding window (Appendix A; numbers 4, 9, 10, 12, 14, 16, 17, and 20), while in other animals the rhythm splitting did not occur until late in the experiment (Appendix A; numbers 2, 11, and 15). The only record during the long-term study which showed no indications of rhythm splitting was produced by animal number 5.

**Table 3. Locomotor activity measurements for all animals from the long-term study.**

The following table contains estimates of the mean daily time of locomotor activity onset (AO) in cases where rhythm components were synchronized by the experimental regime, and freerun τ estimates in cases where rhythm components were not synchronized by the experimental regime. Values are in military time. Numbers smaller than 2000 are mean AO estimates and are accompanied by the standard error of the mean in parentheses where available. The numbers between 2300 and 2500 are τ estimates and are accompanied by the linear-least-squares correlation coefficient in parentheses where available. Values with no variability measure in parentheses were obtained by eye-fitting. The symbol (----) represents places where the data were too erratic to be quantified. Asterisk denotes animals fed chow-glucose experimental diet. Animals without an asterisk were fed chow-starch experimental diet. Information regarding the experimental conditions can be found in Table 1. Freerunning or entrained components which lasted for at least 14 days and were distinct enough to be evaluated sometimes occurred without a change in the experimental conditions. In those cases, multiple measurements are listed in the order in which locomotor activity changes occurred in the record. Other abbreviations: DL (dark/light cycle), DD (total darkness), RF (restricted feeding regime).

| Animal # | Experimental Conditions | | | |
|---|---|---|---|---|
| | DL 12:12 | DD & 7hr. RF | DD & 5hr. RF | DD |
| *2 | 0630(0041) | 0430 | 0645(0028) | 2425(0.992) |
| *4 | 0700(0025) | 2330 | 2305(0.995)<br>0115(0011)<br>2310(0.982) | 2349(0.997) |
| *9 | 0730(0050) | 0700 | 0545 / 2429 | 2439(0.994) |
| *11 | No Data | 0630 | 0730 / 2438 | 2455(0.992) |
| *14 | 0730(0034) | No Data | 0700 / 2440(0.960) | Dead |
| *16 | 0700(0030) | 2430 | 0727(0041) / 2450 | 2447(0.995) |
| *17 | 0645(0018) | 0630 / 2420 | 0703 / 2436 | No Data |
| *20 | 0545(0029) | 2330 | 2420 / 0730(0036)<br>2432 | 2429(0.987) |
| 5 | 0730(0016) | 2336 | ---- | No Data |
| 7 | 0500(0122) | 0545 | 0645(0032) | Dead |
| 8 | 0545(0035) | 0645 | ---- | Arrhythmic |
| 10 | 0700(0029) | 0615 | 0630 / 2431 | 2447 |
| 12 | 0700(0032) | 0615 | ---- | 2405 |
| 15 | 0715(0033) | 2320(0.990) | 1830 / 2417(0.958)<br>0650(0033) | ----<br>2430 |
| 19 | 0715(0025) | 2424 | 0700 and 1130 | 2429(0.990) |

Release of the surviving twelve animals into DD and *ad libitum* feeding conditions resulted in a fusion of the two activity components and a subsequent freerun of the entire activity bout with a $\tau$ value greater than 24 hours in nine of the animals (Appendix A; 2, 9, 10, 11, 12, 15, 16, 19, and 20), and with a $\tau$ value less than 24 hours in only one case (Appendix A; number 4). Two animals either ceased running or became arrhythmic (Appendix A; numbers 5 and 8). The last animal had no data (Appendix A; number 17). Since most of the animals exhibited a freerun in activity during this phase of the long-term study, it is clear that the investigator's entrance into the chamber did not serve as a zeitgeber for the animals.

In the long-term locomotor activity records, there were many places where anticipatory activity and freerunning components of a locomotor rhythm interacted. In animal number 4 (Appendix A; number 4), the freerunning component broke away from the entrained state when the lighting during the 7 hour RF regime was changed from a DL 12:12 to DD conditions. The freerun component had a $\tau$ value which was less than 24 hours in length. It crossed through the RF window three times during the study. The AA was initially observed following reduction in the length of the restricted feeding window from 7 hours to 5 hours. As the freerunning locomotory component initially crossed through the feeding window, locomotor activity within the window dramatically increased and the AA began to phase delay. As the freerunning component passed the feeding window, locomotor activity disappeared within the window, and the AA component returned to its prior entrainment state. On the second transit of the freerunning component through the feeding window, locomotor activity again increased dramatically within the window, but this time the end of activity was synchronized to the time of food removal. This entrained state continued for approximately 14 days, at which point the freerunning component reappeared. As the freerunning component moved away from the feeding window, the anticipatory activity component became apparent. The last pass through the feeding window resulted in locomotor activity entrainment in a manner similar to what was observed before. The increase in locomotor activity during feeding as the freerunning locomotory component crossed the feeding window, and the phase changes of the AA component when the phase-angle between it and the freerunning component decreased,

were common characteristics of most of the long-term locomotor activity records (Appendix A; animals 4, 7, 8, 9, 10, 11, 12, 14, 15, 16, 17, 19, and 20).

The locomotor record of animal number 16 had many characteristics which were similar to those found in the record of animal number 4, but did not show relative coordination (Appendix A; number 16). Again, the freerunning component began immediately upon release into DD conditions, but was greater than 24 hours in this case. The AA component appeared following transition from a 7 hour to a 5 hour RF regime. This time, however, the $\tau$ value of the freerunning component increased dramatically as the two components separated (from 2430 to 2450), and the AA became erratic. As the freerunning component crossed through the window three separate times, the locomotor activity within the window changed as previously described, and the AA scalloped as a function of the phase-angle between it and the freerunning component. Overall, nine of the fifteen animals produced a split in their locomotor activity records as a result of the transition to DD, three produced a split with the reduction of the restricted feeding window from seven hours in length to five hours, two animals exhibited a split late in the DD and 5 hour RF portion of the experiment, and one animal was continuously entrained.

The transition in DD conditions from a 5 hour RF regime to feeding *ad libitum* produced complex rhythm patterns in four locomotor records. In animal 9 (Appendix A; number 9), the peak of activity present at the time of food presentation persisted for several days following the transition to *ad libitum* feeding, and then shifted to the leading edge of the freerunning component by means of a phase delay. In animal 10a (Appendix A; number 10), the AA merged after several days with the freerunning component following release into feeding *ad libitum*, but no phase shifts were involved in this case. Animal 14 (Appendix A; number 14) responded to the change in experimental conditions by shifting the bulk of AA to the rear edge of the freerun component by means of a large phase advance over two days. The record for animal number 15 (Appendix A; number 15) contained two separate freeruns. The original AA and freerunning components remained 180° out of phase with one-another for several weeks, at which point a single cohesive locomotor rhythm appeared. The new rhythm had many of the characteristics of an entrained rhythm, including a cyclic onset of activity which was only slightly phase-advanced from the mean

anticipatory activity onset time produced during the 5 hour restricted feeding regime conditions. If animals had responded to opening and closing of the food bins during the feeding restriction, than they would have produced an entrained locomotor pattern when this practice continued with food *ad libitum*. This was not the case in the long-term activity records (Appendix A).

Locomotor rhythm measurements for the short-term study animals can be found in Table 4. Two of the eight animals kept in LD 12:12 and given a 5 hour feeding window produced a highly erratic onset in activity which preceeded lights-off (Appendix B; numbers 25 and 29). The other six animals had an activity onset which fell between lights-off and the time that food was presented (Appendix B; numbers 22, 31, 34, 36, 38, and 39). In the animals given a 3 hour feeding window instead of a 5 hour feeding window, two animals exhibited an activity onset which slightly preceeded lights-off (Appendix B; numbers 32 and 35). Four animals had an activity onset which fell between lights-off and the time at which food was presented (Appendix B; numbers 23, 33, 37, and 40). The last animal had no data recorded (Appendix B; number 28). The change from a DL 12:12 to DD in the five-hour restriction animals resulted in an immediate phase delay of at least 0342 hours and a subsequent freerun with a $\tau$ value less than 24 four hours in five of the eight animals (Appendix B; numbers 31, 34, 36, 38, and 39). Two animals displayed no phase delay but a freerun with a $\tau$ value less than 24 hours (Appendix B; numbers 22 and 29). The last animal was arrhythmic (Appendix B; number 25). An equivalent change in the lighting cycle for animals in the 3 hour feeding regime resulted in a phase delay of at least 0148 hours and a subsequent freerun with a $\tau$ value less than 24 hours in three of the seven animals (Appendix B; numbers 28, 37, and 40). No phase delay but a freerun with a $\tau$ value less than 24 four hours resulted in one of the animals (Appendix B; number 35). The last three animals were either arrhythmic (Appendix B; numbers 32 and 33) or displayed a bimodal pattern (Appendix B; number 23). It is apparent that at least some component of locomotor activity was not synchronized by RF regimes of either 3 or 5 hour duration when the lighting cycle was removed as a zeitgeber.

**Table 4. Locomotor activity measurements for all animals from the short-term study.**

The following table contains estimates of the mean daily time of locomotor activity onset (AO) in cases where rhythm components were synchronized by the experimental regime, and freerun $\tau$ estimates in cases where rhythm components were not synchronized by the experimental regime. Lengths of locomotor phase delays created by a change in the experimental conditions are denoted by an asterisk. Freeruns following phase delays originate from a point determined by the phase delay. Values are in military time. Numbers smaller than 2000 are mean AO estimates and are accompanied by the standard error of the mean in parentheses where available. The numbers between 2300 and 2500 are $\tau$ estimates. Values with no variability measure in parentheses were obtained by eye-fitting. The symbol (----) represents places where the data were too erratic to be quantified. Information regarding the experimental conditions can be found in Table 2. Freerunning or entrained components which lasted for at least 14 days and were distinct enough to be evaluated sometimes occurred withiout a change in the experimental conditions. In those cases, multiple measurements are listed in the order in which locomotor activity changes occurred in the record. Other abbreviations: RF (restricted feeding regime), DL (dark/light cycle), and DD (total darkness).

| Animal # | RF Length | Experimental Conditions | |
|---|---|---|---|
| | | DL 12:12 & RF | DD & RF |
| 23 | 0300 | 0701(0017) | ---- |
| 28 | 0300 | No Data | *0418 / 2240 |
| 32 | 0300 | 0658(0054) | 2304 [6 days early] |
| 33 | 0300 | 0713(0039) | Arrhythmic |
| 35 | 0300 | 0656(0035) | 2247 |
| 37 | 0300 | 0714(0034) | *0330 / 2320 |
| 40 | 0300 | 0705(0012) | *0148 / 2337 |
| 22 | 0500 | 0709(0032) | 2230 |
| 25 | 0500 | 0554 | ---- |
| 29 | 0500 | 0600 | 2226 |
| 31 | 0500 | 0706 | *0342 / 2314 |
| 34 | 0500 | 0707(0019) | *0600 / 2300 |
| 36 | 0500 | 0703(0018) | *0400 / 2332 |
| 38 | 0500 | 0712(0021) | *0359 / 2331 |
| 39 | 0500 | 0700(0013) | *0354 / 2323 |

The long-term study 5 hour feeding window was not a strong enough zeitgeber to entrain mice locomotory rhythms. Freerunning locomotory activity existed in thirteen out of fifteen mice under DD lighting conditions and a 5 hour restricted feeding regime. Mice in the short-term 5 hour restricted feeding regime also had freerunning components in their locomotor activity records while in continuous darkness (DD). Reducing the length of the feeding window, as in the 3 hour restricted feeding regime in the short-term study, did not prevent a freerun of the locomotor rhythm in DD. Thus, it appeared that the 3 hour and 5 hour restricted feeding regimes did not provide a strong enough zeitgeber to totally entrain the locomotory rhythms. At least one component of the locomotor activity continued to freerun regardless of the strength of the feeding zeitgeber. This is in agreement with what was found by Meyer-Lohman (1955).

Mass records for animals from the long-term study indicate that there were no significant changes in mass associated with the experimental conditions (F-value of 1.62; $df = 31,22$; $p = 0.1448$). These animals had already attained adult mass by the time that the experiment began. Subsequent fluctuations in mass were variable between individuals over time. The only nonsignificant trend in the weight records associated with the experimental conditions was the loss of weight by some of the animals for a short time directly after entering the 7 hour RF regime (Appendix A; numbers 2, 4, 7, 11, 14, and 20). There were no consistent changes in either the mass or locomotor activity records associated with the shift from powdered chow to the experimental diets.

The fifteen animals in the short-term study gained weight over the course of the study. Weight gain in these animals was a function of increasing age. Response of the mice to the change from a DL 12:12 to DD conditions while in a RF regime was varied: nine of the animals continued to gain weight (Appendix B; numbers 23, 31, 34, 35, 36, 37, 38, 39, and 40). Three of the animals began to stabilize their weights (Appendix B; numbers 28, 29, and 32). The last three mice actually lost a small amount of weight (Appendix B; numbers 22, 25 and 33).

# *Energy Evaluations*

Gross energy evaluations for the three diets produced the following data: powdered Prolab 3000 contained $17.36 \pm 0.79$ kJ/g, the chow-glucose diet contained $16.82 \pm 0.33$ kJ/g, and the chow-starch diet contained $16.15 \pm 0.42$ kJ/g. These values were 16.32 kJ/g, 16.32 kJ/g, and 15.50 kJ/g, respectively, when corrected for hydration.

Comparison between the mean mass specific energy intake values for the six mice randomly chosen in the long-term study show that there was no significant difference created by the two experimental diets (F-value of 1.50; $df = 13,16$; $p = 0.2195$). However, the animals did vary their weight specific energy consumption while consuming powdered chow (Figure 5). There is a significant change in energy intake over the course of the long-term study (F-value of 3.37; $df = 21,32$; $p = 0.0010$). The mice had a higher daily energy intake at the beginning of the 7 hour RF regime than they did during the 5 hour RF regime or subsequent *ad libitum* feeding. The temporary response to food limitation was to initially increase consumption. Subsequent energy intake means represent more normal levels of adult dietary intake. From day 70 till the end of the long-term experiment, no significant differences in mass specific energy intake rates occurred due to food restriction or diet type.

# *Blood Glucose Curves*

Circulating blood glucose levels of animals on a restricted feeding regime of 5 hour length were statistically unaffected by diet or sampling time (F-value of 1.25; $df = 29,18$; $p = 0.3179$). All points on the curve were thus statistically equivalent (Figure 6). Circulating blood glucose concentrations of animals given a feeding window of 1 hour length did differ between diets and sampling times (Figure 7). Analysis of this curve indicates that the model was statistically significant at $\alpha = 0.05$

(F-value of 4.39; df = 30,23; p = 0.0003). The model parameters of diet type (F-value of 16.08; df = 1,23; p = 0.0005), interaction between diet type and sampling time (F-value of 3.07; df = 8,23; p = 0.0166), and animals within a diet group (F-value of 3.77; df = 13,23; p = 0.0027) were also statistically significant at $\alpha = 0.05$. The two experimental diets produced different blood glucose concentrations only at the 0915 sampling time (t-value of 3.110, df = 4, $p < 0.0250$).

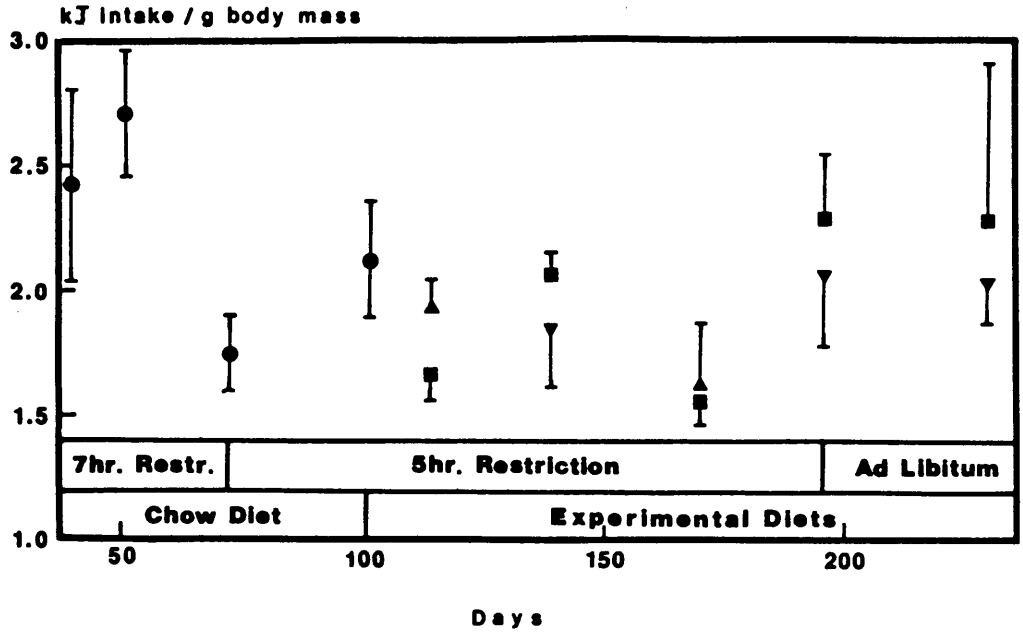



**Figure 5.** Mean weight specific daily energy intake values for six mice during the long-term study: The first four points on the curve were formed by pooling the mean weight specific daily energy intake values for all six representative mice while consuming powdered chow. Subsequent points were formed from the mean of only three mice as the initial mice were randomly divided between the chow-glucose and chow-starch experimental diet groups. The first four points consist of mean ± SD (1 standard deviation). Standard deviations of subsequent points are represented by a bar either above or below the symbol. Symbol base is the mean. Symbols: ● = powdered chow, ■ = chow-glucose diet, ▲ = chow-starch diet.

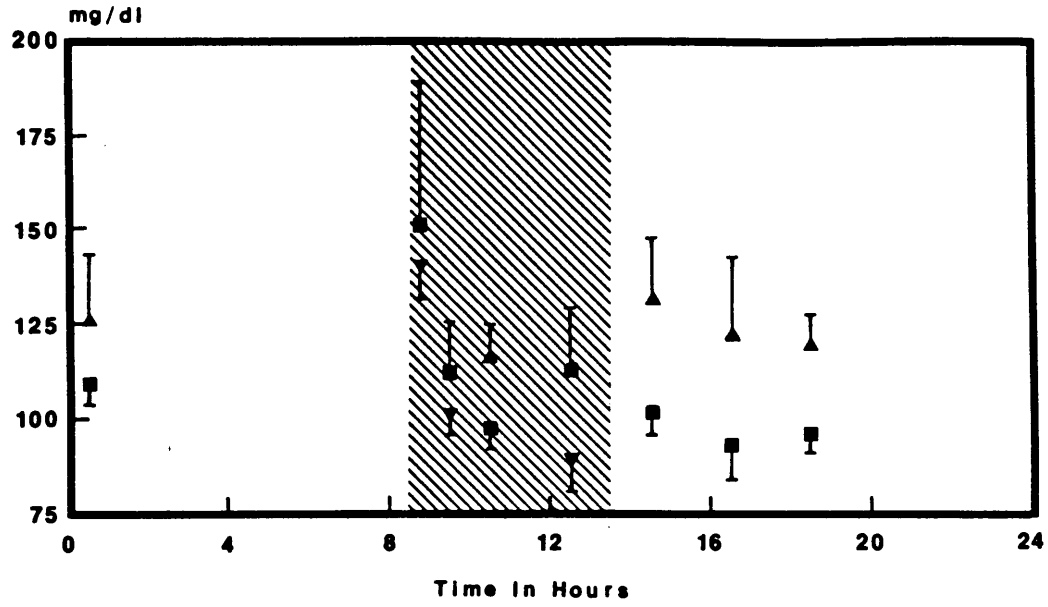



**Figure 6.** **Blood glucose curve for animals under the 5 hour restricted feeding regime:** Animals were sampled while in total darkness and a restricted feeding regime of 5 hour length (food from 0830 to 1330). Symbols represent mean ± SD (1 standard deviation). Standard deviations are represented by a bar placed either above or below each symbol. Symbol base is the mean. Feeding window is indicated by slanted parallel lines. Symbols: ■ = chow-glucose diet, ▲ = chow-starch diet.

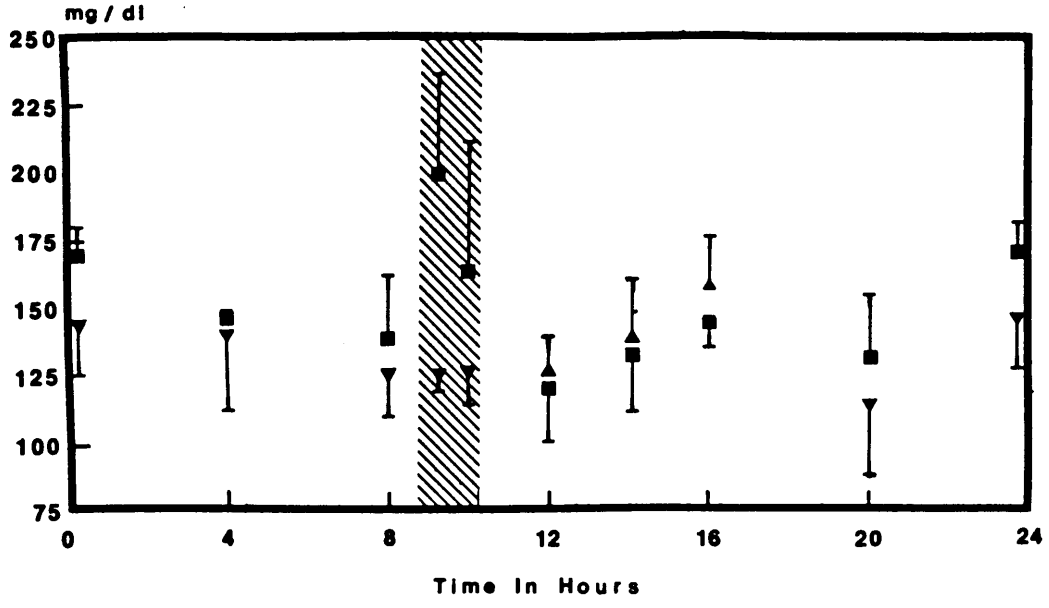



**Figure 7. Blood glucose curve for animals under the 1 hour restricted feeding regime:** Animals were sampled while in an LD 12:12 (lights on at 2100 and off at 0900) and a restricted feeding regime of 1 hour length (food from 0900 to 1000). Symbols represent mean ± SD (1 standard deviation). Standard deviations are represented by a bar placed either above or below each symbol. Symbol base is the mean. Feeding window is indicated by slanted parallel lines. Symbols: ■ = chow-glucose diet, ▲ = chow-starch diet.

One of the hypotheses of interest in this study, namely that blood glucose levels could be used as a means of monitoring feeding cues for resetting the circadian clock, was not effectively tested. It was initially believed that the 5 hour RF regime used in the long-term study would create a pulse of plasma glucose during the time that feeding occurred. Statistical analysis of the blood glucose concentrations of the mice during this time, however, showed that there were no differences in plasma glucose levels over the time period encompassing feeding (F-value of 1.25; $df = 29,18$; $p = 0.3179$). The small sample size of three animals per time point limited the possibility of detecting very minor differences in the blood glucose concentrations. It is possible that the combination of a relatively long feeding interval (5 hours) coupled with the temporal asynchrony of energy use by individual animals in DD led to the lack of a detectable glucose peak within the feeding window. With the wide variation in the timing of peak activity, it is unlikely that peaks in energy use were temporally coincident. Given a feeding interval larger than a few hours, mice are reported to intersperse feeding bouts with running bouts, leading to the maintenance of rather constant blood glucose concentrations (Johnson et al., 1983). The large amount of variation present in the locomotor rhythms of the mice during the bleeding period is readily apparent, and may help to explain why the blood glucose concentration curve for the 5 hour restricted feeding regime did not at least demonstrate an endogenous circadian rhythmicity in plasma glucose concentrations. It has previously been shown that the blood glucose levels in mice are highest during their subjective rest period and lowest during their subjective active period (Gagliardino and Hernandez, 1971). This trend is not at all apparent in the 5 hour restricted feeding curve. However, when the time interval allowed for feeding was reduced to 1 hour in duration and a DL 12:12 light cycle was used to synchronize animal locomotor activity, the mean blood glucose concentrations created by the two experimental diets were significantly different in the first fifteen minutes of feeding (t-value of 3.110, $df = 4$, $p < 0.0250$).

# Discussion

This study has demonstrated the importantance of long-term data collection when quantifying changes in locomotor activity patterns as a function of zeitgeber combinations. Interactions between the locomotor activity components present in the long-term study could only be observed after several months. The short-term study gave information regarding when certain zeitgeber combinations could create entrainment of the locomotor rhythms, but did not provide enough time for full study of the emerging locomotor rhythm patterns. Thus, care must be exercised when stating conclusions regarding the amount and type of entrainment experienced by an animal exposed to a given set of zeitgebers.

During the course of the long-term and short-term studies, the two zeitgebers of light and food were used to attempt to synchronize the locomotor activity rhythms of the mice. The LD cycle was shown to be a much better zeitgeber than food presentation because the locomotor records produced with light as a zeitgeber contained less variability both within and between records. This is consistent with what has been reported in earlier studies (Pittendrigh and Daan, 1976a). However, when only cyclic food presentation was used as a zeitgeber there was a high degree of variability both within and between records. Animals in the long-term study mainly produced freerunning locomotor activity components which had a $\tau$ value greater than 24 hours, in direct opposition to "Aschoff's Rule" for nocturnal rodents in DD conditions (Pittendrigh and Daan,

1976b). This could result from after-affects of one of the prior experimental combinations or age effects on the pacemaker (Pittendrigh and Daan, 1976a). On the other hand, animals in the short-term study only produced locomotor activity freeruns in DD which had $\tau$ values less than 24 hours, a finding which was in agreement with "Aschoff's Rule". Thus, it appeared that animals in the short-term study were affected differently by changes in the experimental regime than were animals in the long-term study. Aging also affected the absolute $\tau$-values of freerunning locomotor activity components in this report. Normally, the $\tau$-value of freerunning locomotor components in animals' activity records decrease as animals age (Pittendrigh and Daan, 1976a). The animals in the long-term study were full adults when the experiment began. However, animals in the short-term study were still maturing at the beginning of the experiment. Since the two groups of animals were different ages during equivalent portions of the studies, the absolute $\tau$-values were not comparable between the long and short-term studies. This would not affect comparisons between animals within each study, however, because mice were approximately the same age at each stage in the experimental protocol.

Almost all of the locomotor records for mice in DD lighting conditions and a 5 hour RF regime in the long-term study had indications that the locomotor activity rhythm was split into two separate components. Rhythm splitting has been reported for a variety of mammals under many different conditions (Albers et al., 1983; Aschoff et al., 1973; Jilge, 1980; Johnson et al., 1983; Pittendrigh, 1960; Pittendrigh and Daan, 1976c) although it was first described as a response to constant lighting (LL) conditions (Pittendrigh, 1960). In this long-term study, rhythm splitting occurred during DD conditions with one of the observed locomotor activity components entrained by the cyclic presentation of food. This locomotor activity component was referred to as anticipatory activity (AA) when observed and reported by Bolles in 1968. The second, freerunning component of locomotor activity seen in this long-term study did not appear to be affected by the cyclic food presentation. Other research has shown that increasing the strength of the feeding cue as a zeitgeber only affects the AA component, and not the freerunning component of locomotor activity (Boulos and Terman, 1980; Meyer-Lohmann, 1955). The present study found that the freerunning component of locomotor activity was not entrainable by restricted feeding regimes of

3, 5, and 7 hour length. Relative coordination was apparent in at least one long-term record (Appendix A; animal number 4), but this has been attributed to changes in coupling strength between the two oscillators in other studies (Moore-Ede et al., 1982; Pittendrigh and Daan, 1986c). Similar split locomotor activity patterns have been described when either a restricted food or a restricted water regime are used (Bolles, 1968; Bolles and Duncan, 1969).

All of the patterns seen in the locomotor activity records from the long-term study can best be explained by invoking a two-oscillator model for the circadian time-keeping mechanism. Proposed in different forms by many different authors, the two-oscillator model is rapidly gaining support in the circadian literature (Albers et al., Bolles and Moot, 1973; Boulos et al., 1980; Boulos and Terman, 1979; Coleman et al., 1982; Daan and Pittendrigh, 1976; Honma et al., 1983; Meyer-Lohmann, 1955; Moore-Ede et al., 1982; Pittendrigh, 1960; Pittendrigh and Daan, 1976c; Wiedemann, 1984). The anticipatory activity (AA) component has been shown to be endogenously driven by an oscillator other than the suprachiasmatic nucleus (SCN), since it can only be entrained in animals with lesions of the SCN by feeding cycles approximating 24 hours in length (Phillips and Milkulka, 1979; Stephan et al., 1979). It has also been shown that control of many rhythms normally driven by the SCN can be shifted to another driving oscillator when a restricted feeding regime that conflicts with the animal's subjective rest period is employed before the SCN is lesioned (Krieger et al., 1977). The restricted feeding regimes in this study did not shift SCN-mediated control of rhythmicity in the freerunning locomotor activity component, as they were timed to fall in the active period of the animals' cycle. Locomotor activity cyclicity in constant conditions normally is lost when lesions are placed in the SCN (Stephan et al., 1979).

In the two-oscillator model, the SCN only responds to cyclic photic temporal cues. A secondary self-sustaining oscillator responds to temporal feeding and drinking cues and normally is coupled to the SCN. In the absence of severe stress on the system, the SCN and the secondary oscillator work in concert to drive the overt rhythm of locomotor activity (Moore, 1983). Minor perturbations to the system thus produce results which appear to be indicative of a single-oscillator. However, when lesions are placed in the SCN, the locomotor activity becomes arrhythmic in constant conditions (Elliot, 1976; Kawamura and Ibuka, 1978; Stephan and Zucker, 1972; Stetson and

Watson-Whitmyre, 1976). Locomotor activity in the form of anticipatory activity (AA) can be recovered in animals with lesions of the SCN when a restricted feeding regime is employed (Krieger et al., 1977). This occurs because the AA locomotor component is driven mainly by the secondary oscillator. When coupling between the two oscillators is stressed by placing animals in a restricted feeding regime with constant lighting conditions, the two locomotor activity components are driven independently by their separate oscillators as each responds to its own temporal cues. With no photic temporal cues for entrainment, the SCN oscillates at its inherent frequency ($\tau$) and drives the freerunning locomotor activity component. The secondary oscillator, on the other hand, responds to the cyclic presentation of food and drives the anticipatory activity component (Stephan et al., 1979). As the two oscillators change their phase-relationship to one-another, coupling strength varies. This occurred with animals in the long-term portion of this study (Appendix A; numbers 4, 9, 10, 11, 14, 15, 16, 17, and 20). When the two oscillators approach a phase-angle difference of zero (as is seen when the freerunning component enters the restricted feeding window) the coupling strength is greatest, and the locomotor rhythm as a whole is most pronounced (see Appendix A; number 4). This explains why locomotor activity increases so dramatically within the feeding window when this phase relationship is reached. As the freerunning component moves out of phase with the anticipatory activity component, the overt expression of locomotor activity is reduced in strength. When the restricted feeding regime is removed, the two locomotor activity components respond according to their coupling strength. If they are 180° out of phase with respect to one-another (see Appendix A; number 15), the two components may both freerun until the anticipatory activity (AA) dampens out due to the lack of feeding cues. If they are in the process of reducing their relative phase-angle to one-another when the RF regime is removed, they will be drawn together as a function of their relative position and strengths (Appendix A; numbers 9 and 10). The phase-angle between the two oscillators is at a minimum when the locomotor activity components that they drive coincide in real time. At this point, the coupling strength between the oscillators may be so great that the two locomotor activity components can switch their relative positions in the subsequent freerunning locomotor rhythm (Appendix A; number 12). If one accepts a reasonable amount of inter-animal variation in the relative oscillator strengths between the two

locomotor activity components (as shown by Pittendrigh and Daan, 1976a), all of the patterns observed in the long and short-term studies presented in this report can be explained by this model.

The physiological mechanism by which the circadian system resets its timing with only daily feeding cues needs to be examined further. The neural structures driving the circadian plasma glucose and insulin rhythms have been implicated also in the control of food intake (Gagliardino et al., 1984; Idahl and Martin, 1971; Martin et al., 1974; Szabo and Szabo, 1975). The possibility that rising plasma glucose concentrations might act as a temporal cue for the circadian system was examined in this study. Unfortunately, the experimental protocol did not provide a strong enough feeding zeitgeber to produce a statistically significant blood glucose peak. Given the fact that the lateral hypothalamic nuclei (LHN) can indirectly detect changes in blood glucose and are directly innervated with the SCN (Gagliardino et al., 1984), it is important that feeding induced plasma glucose changes be investigated further. This study demonstrated that a short feeding window (1 hour) can produce a blood glucose peak. Therefore, future studies would need to provide a stronger feeding zeitgeber by either reducing the feeding time or restricting the amount of food presented.

This study had no selection for running ability in its experimental protocol, and yet almost all of the locomotor records showed indications of rhythm splitting. Interactions between the two locomotor activity components in most of the records can not be explained by a single-oscillator driving mechanism. Thus, the generality that two coupled oscillators can drive many of the overt rhythms seen in mammals is given further support as a result of these data. Unfortunately, however, the proposed two-oscillator model can only be tested in a purely qualitative manner with the type of data presented in this study. In order to be more effective, the model must have the ability to quantify precisely the expected response of an animal's circadian system to a given set of experimental conditions. This is impossible at the present time given the limitations involved in studying the actual clock mechanism at the biochemical/neural level. Until a method can be found to directly assess the instantaneous state of the internal circadian oscillators and their relationship to the overt measurable rhythms which they drive, as much information as possible will have to be gleaned from observational studies such as this one.

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# Appendix A. Locomotor Activity And Weight Records: Long-Term Study

NOTE:

Figures in this appendix show changes in locomotor activity and body mass for individual mice over time. The left side of the abcissa for each figure is marked in time units (hours) with the original light/dark cycle indicated. The right side of the abcissa is marked in weight units (grams). The ordinate is also marked in time units (days) and is valid for both parts of the abcissa. In order to provide the best visual display possible for this long-term study, the locomotor records for each animal are a triple-plot, with time proceeding in hours from left-to-right, and in days from top-to-bottom. Prior to the recording of locomotor rhythms, animals were placed in an LD 16:8 (lights on at 0700 and off at 2300) and given hard chow *ad libitum*. At day one of the experiment, animals were switched to a DL 12:12 (lights on at 1900 and off at 0700). The first small arrow at day 50 on the ordinate denotes where the light/dark cycle was switched to total darkness. The second arrow at day 99 indicates where animals were switched to their experimental diets. The solid vertical lines in the figures denote the temporal feeding window. Restricted feeding regimes were either seven hours in length (food from 0730 to 1430) or five hours in length (0830 to 1330). White blocks in the locomotor records indicate a loss of data. The experiment ended on day 228.

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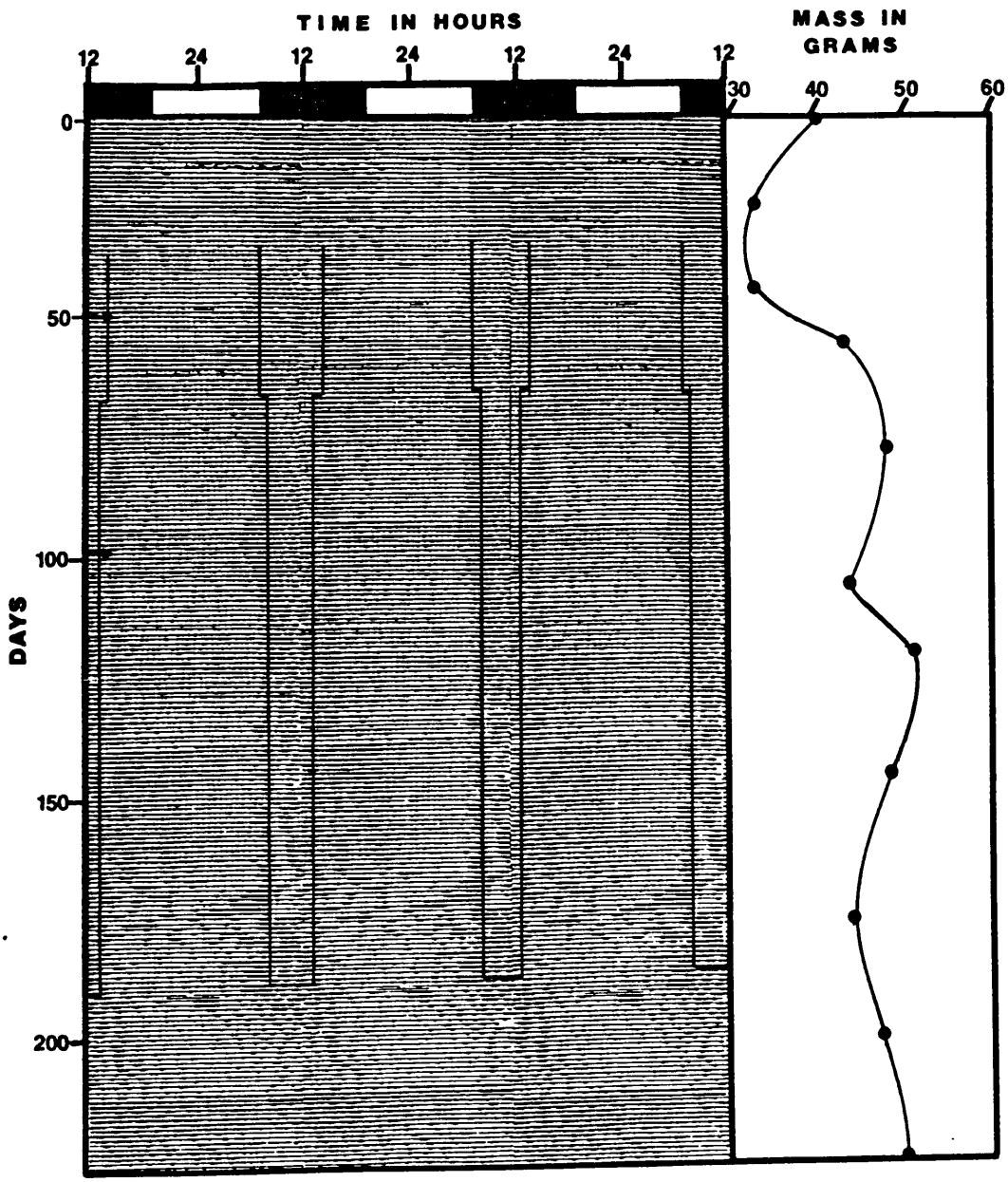
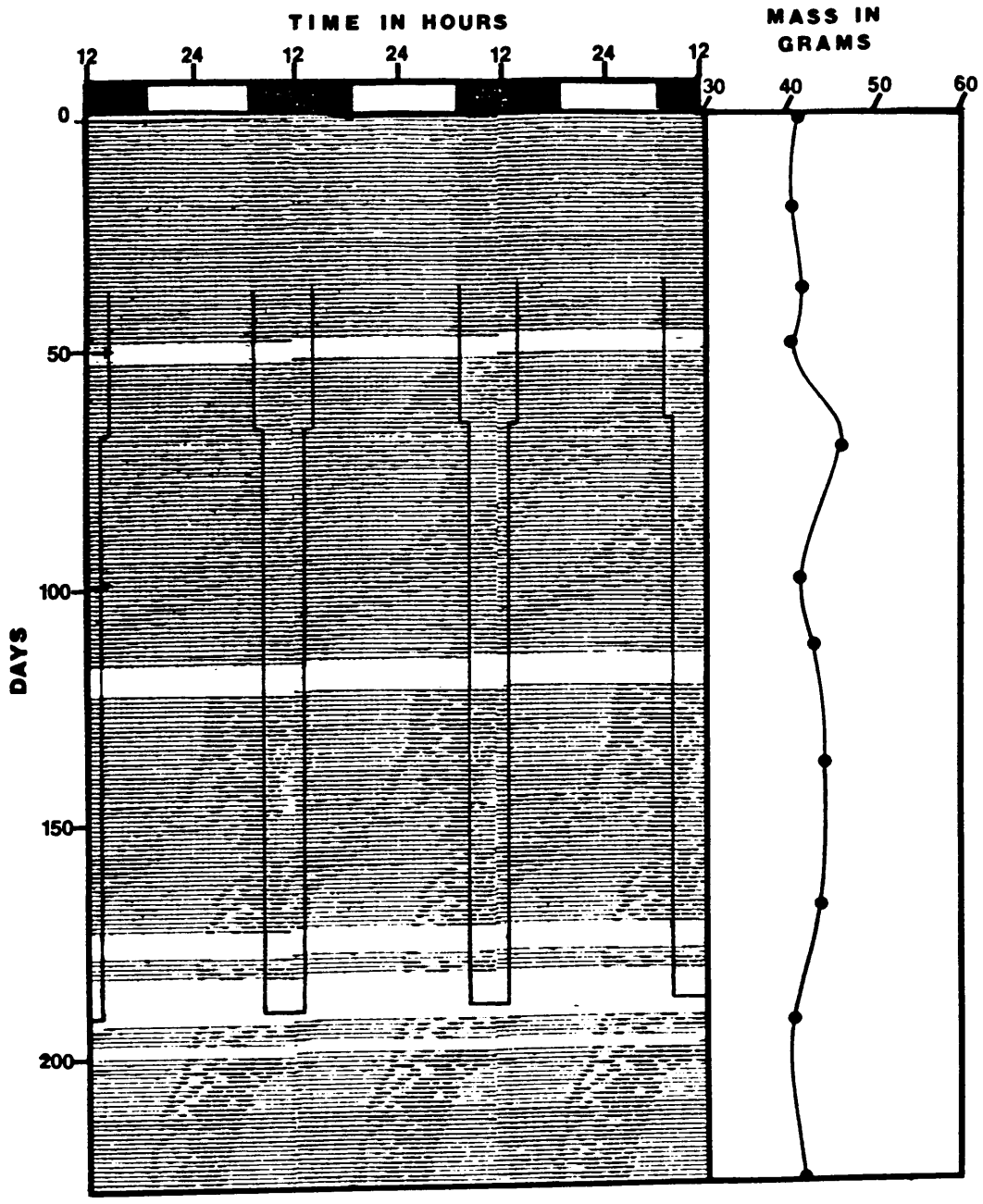


*Animal Number 4 (Chow-glucose experimental diet)*



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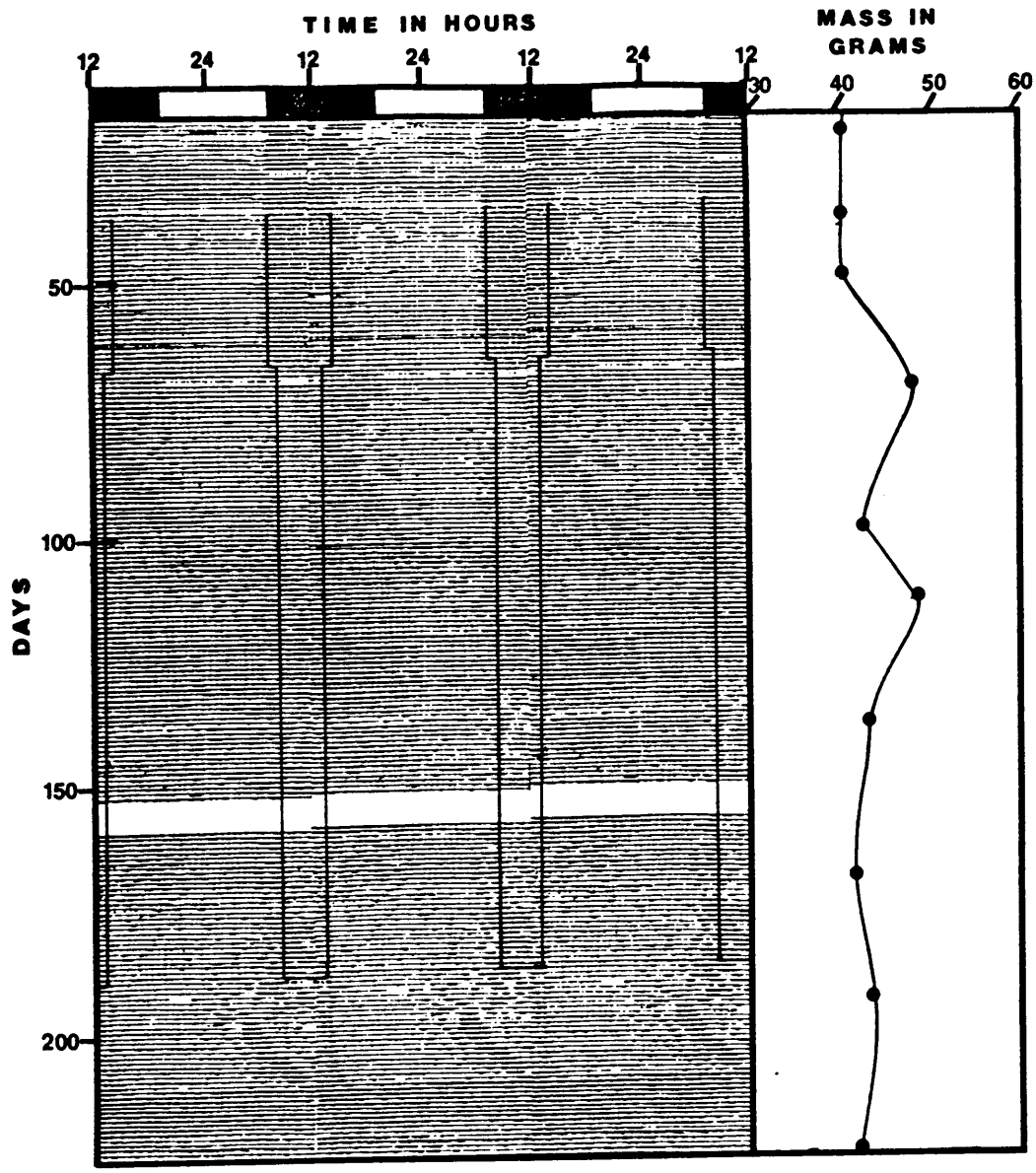

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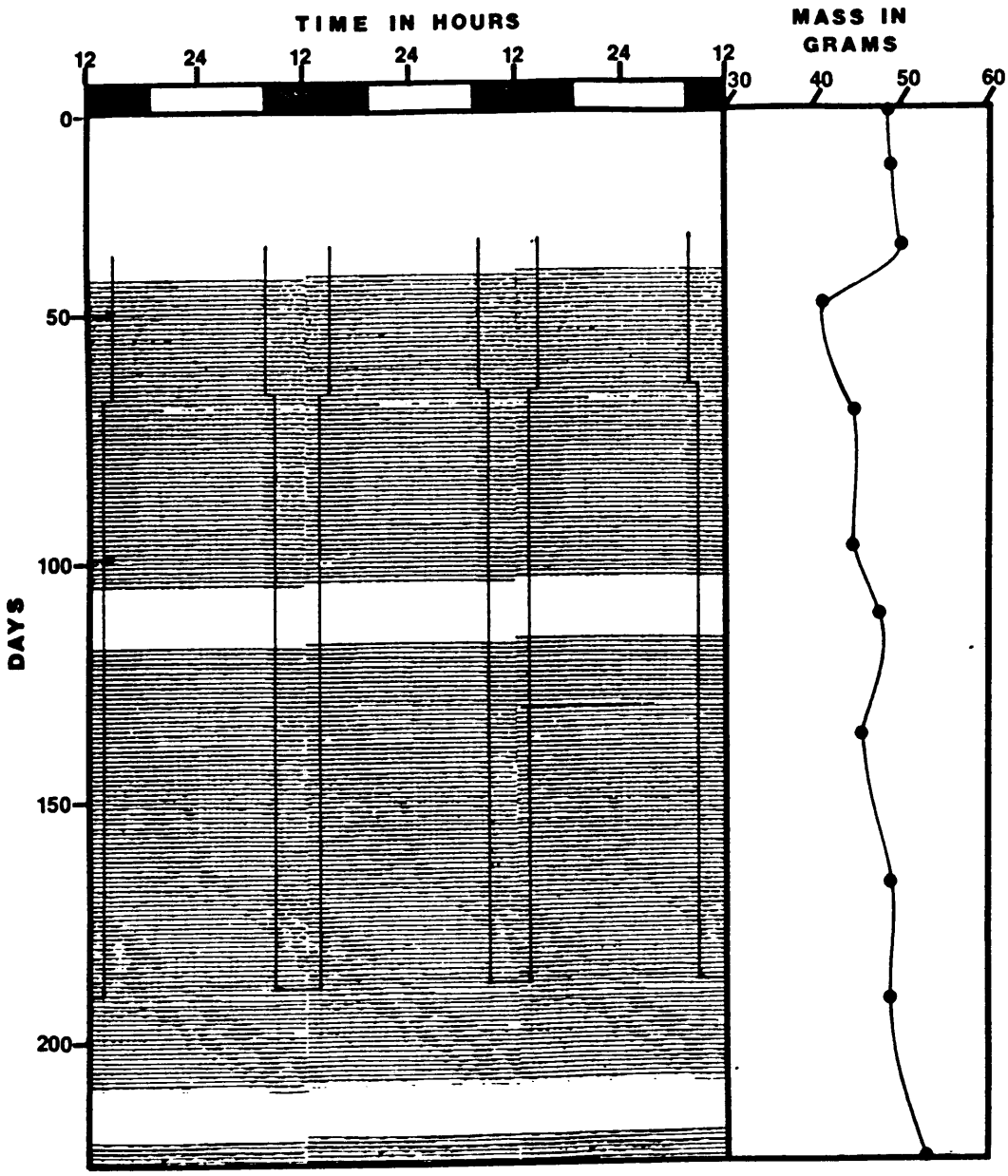

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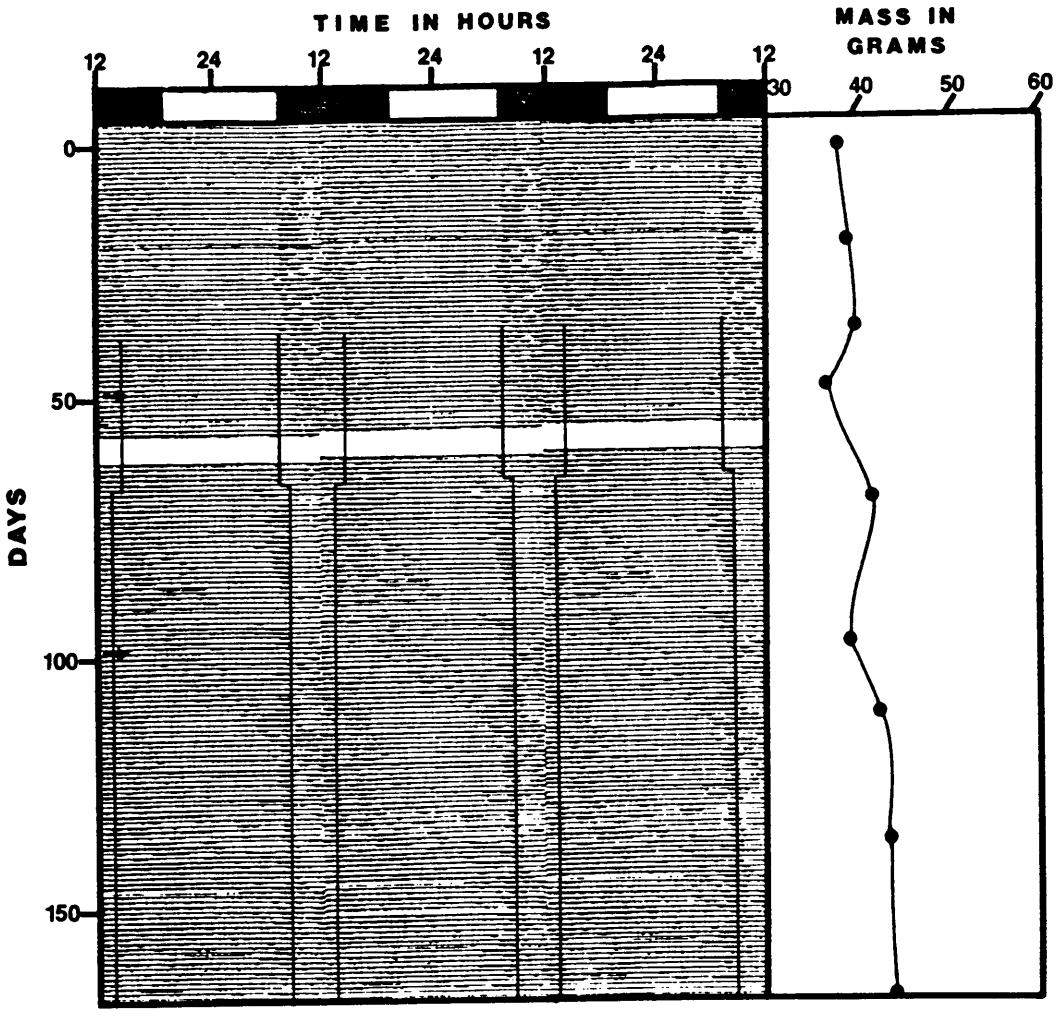

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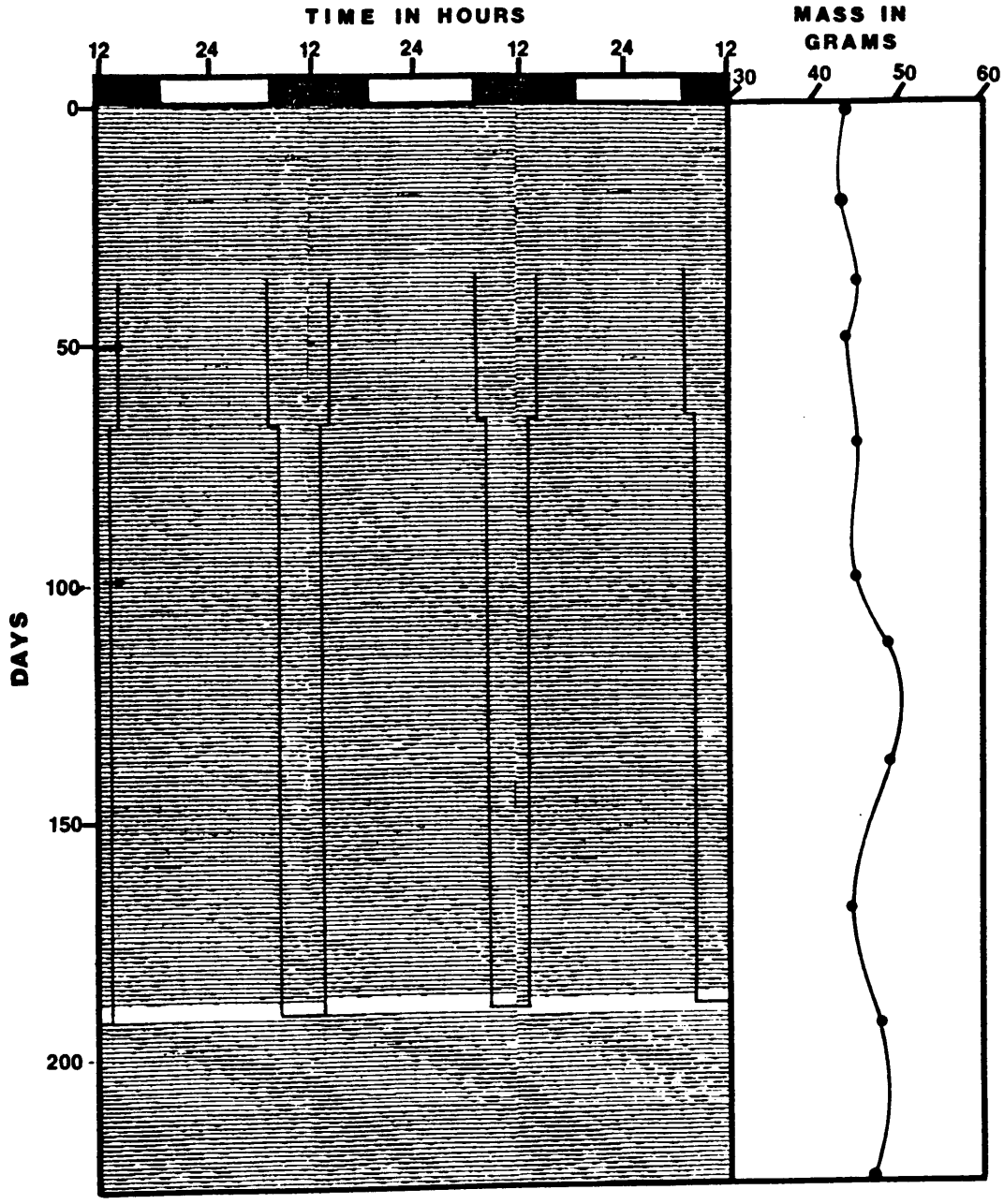
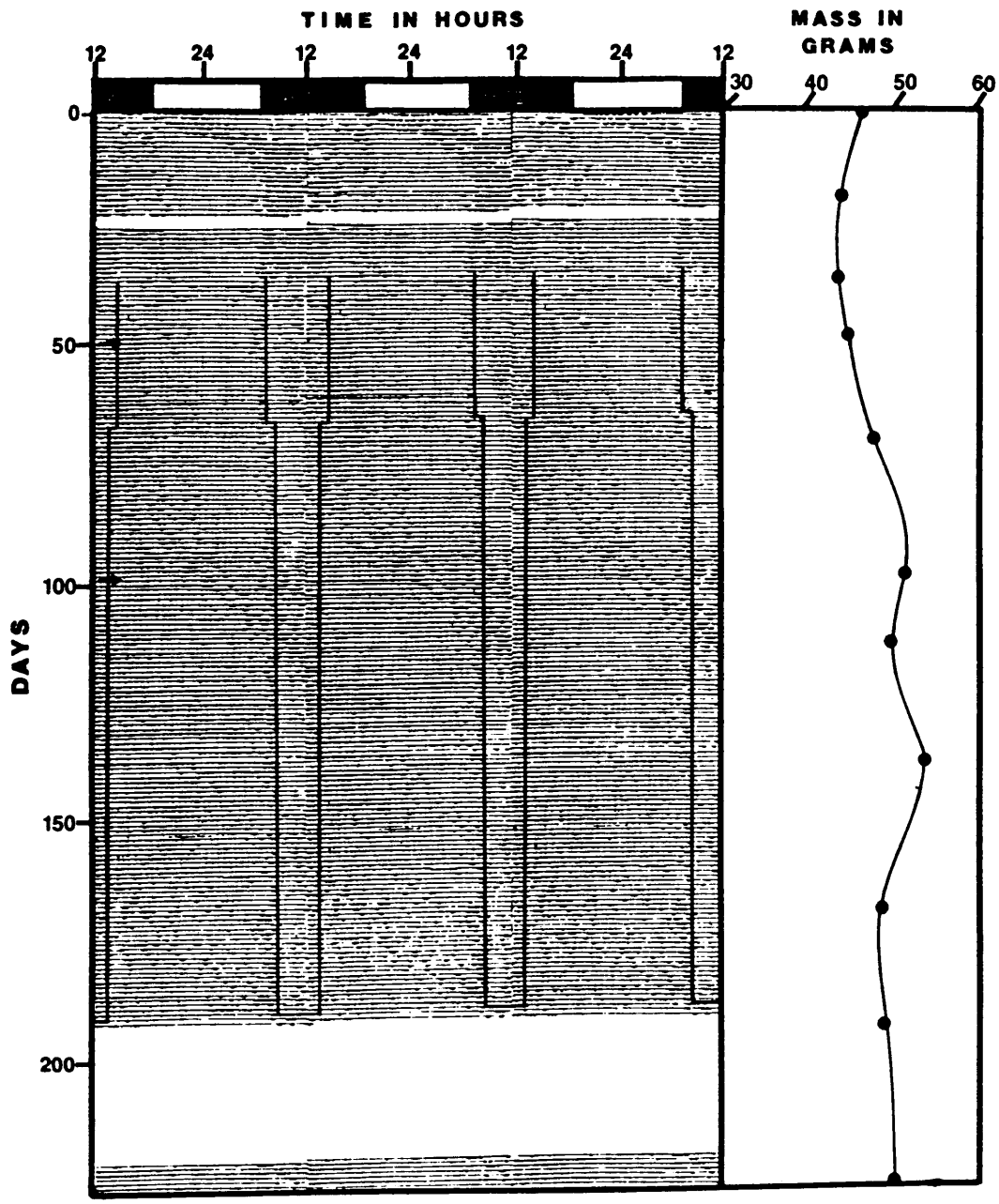


*Animal Number 17 (Chow-glucose experimental diet)*



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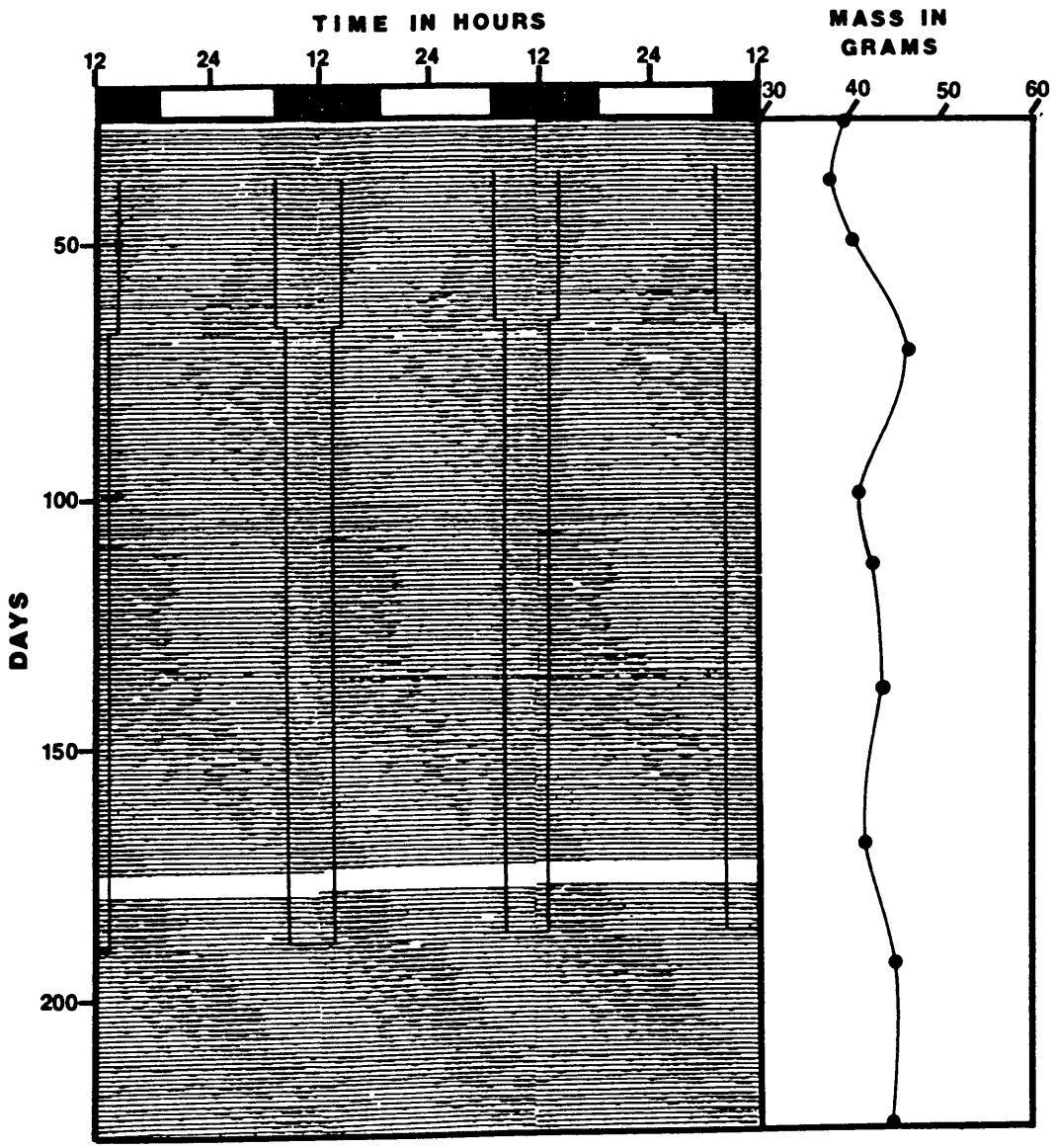
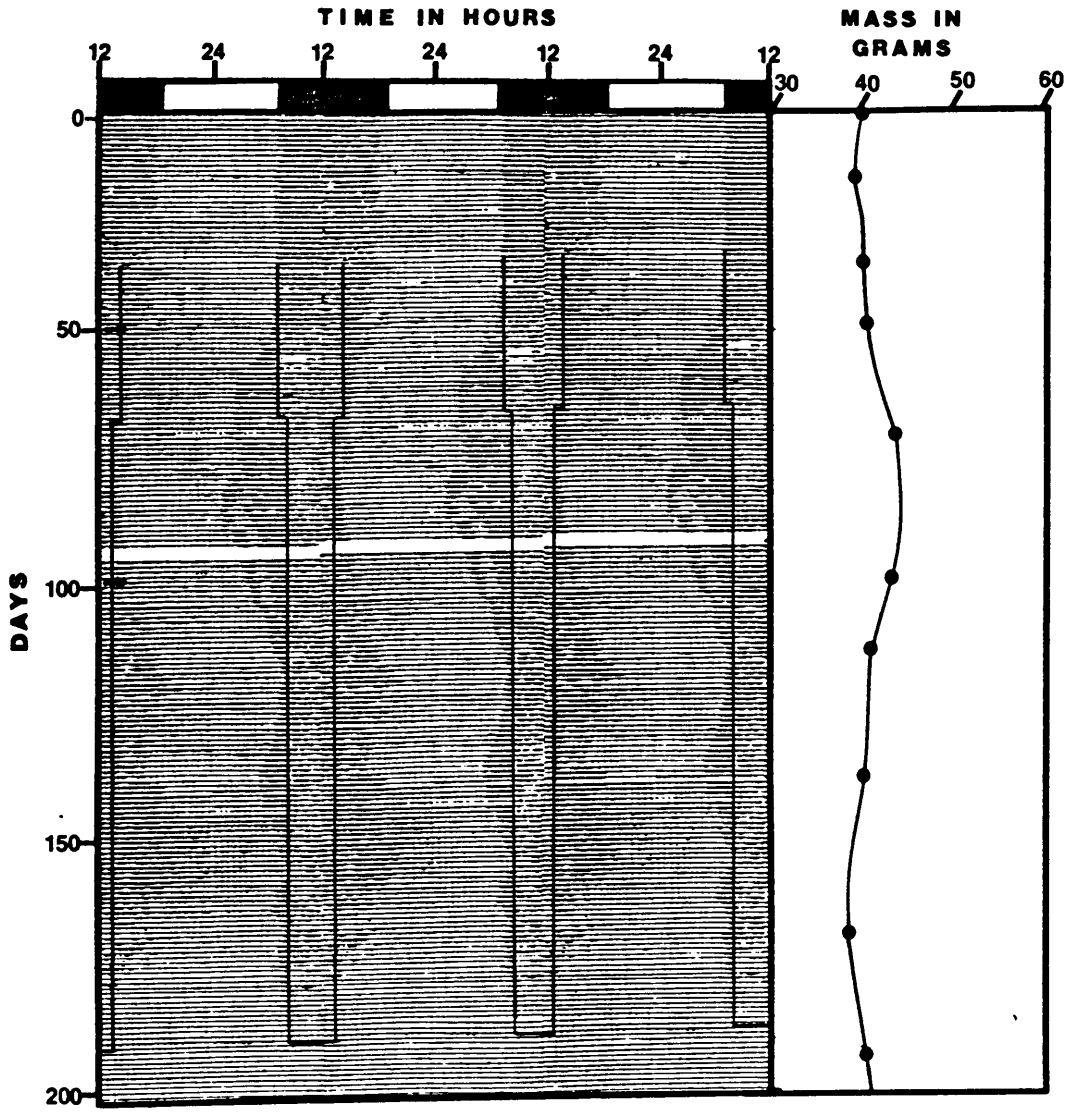


*Animal Number 5 (Chow-starch experimental diet)*



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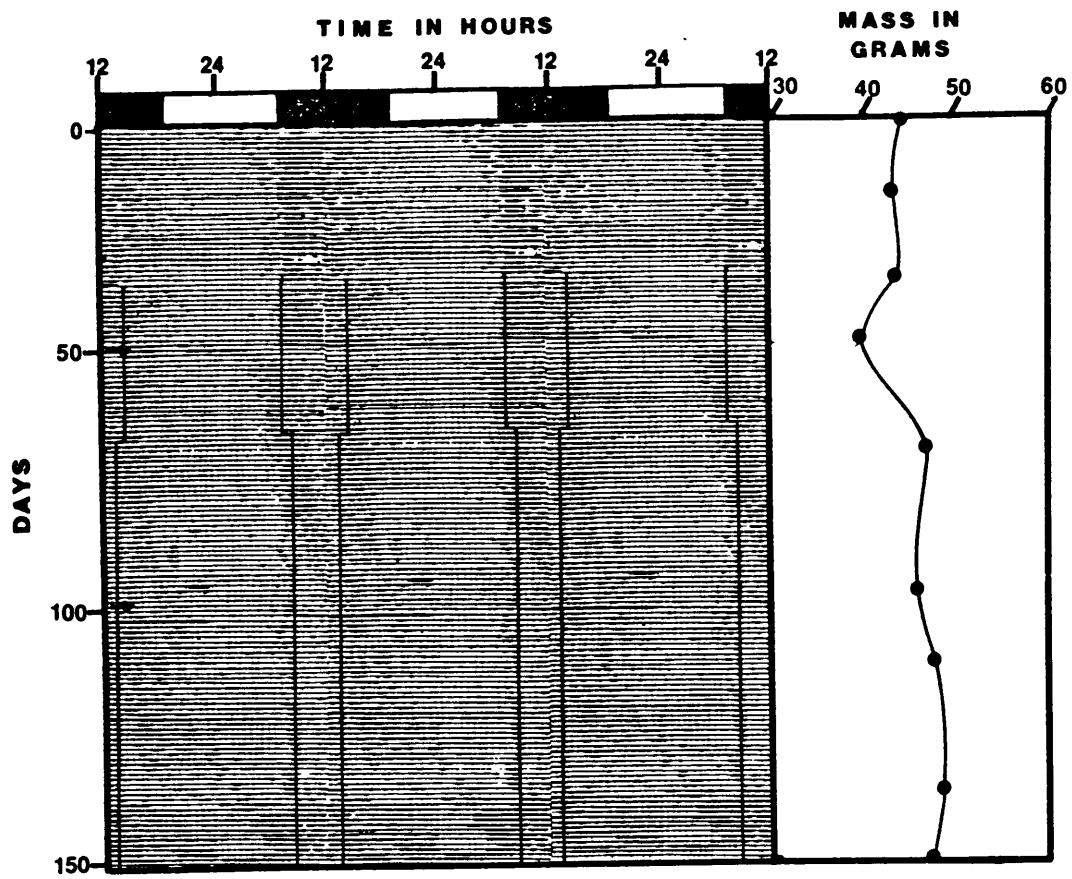

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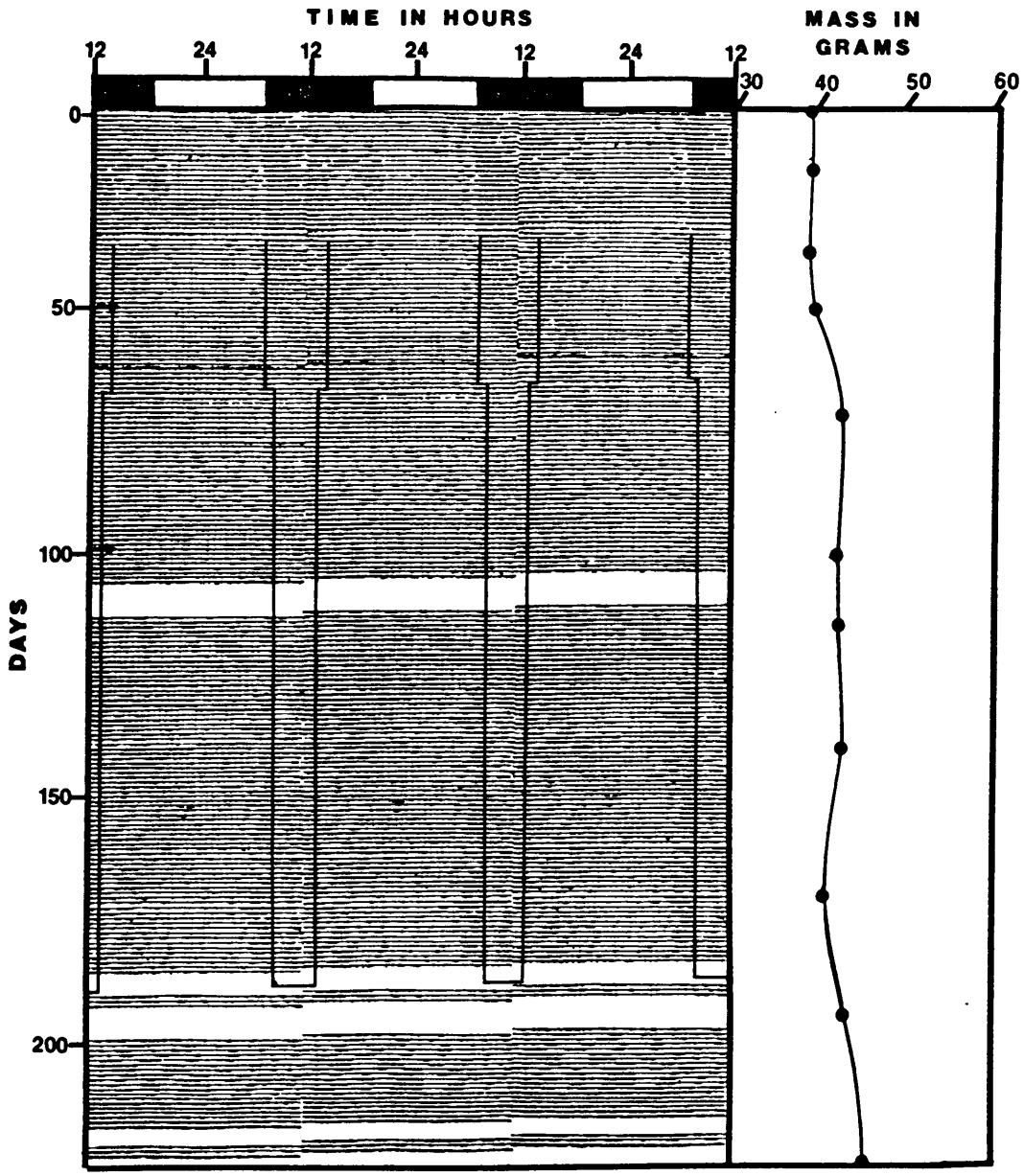

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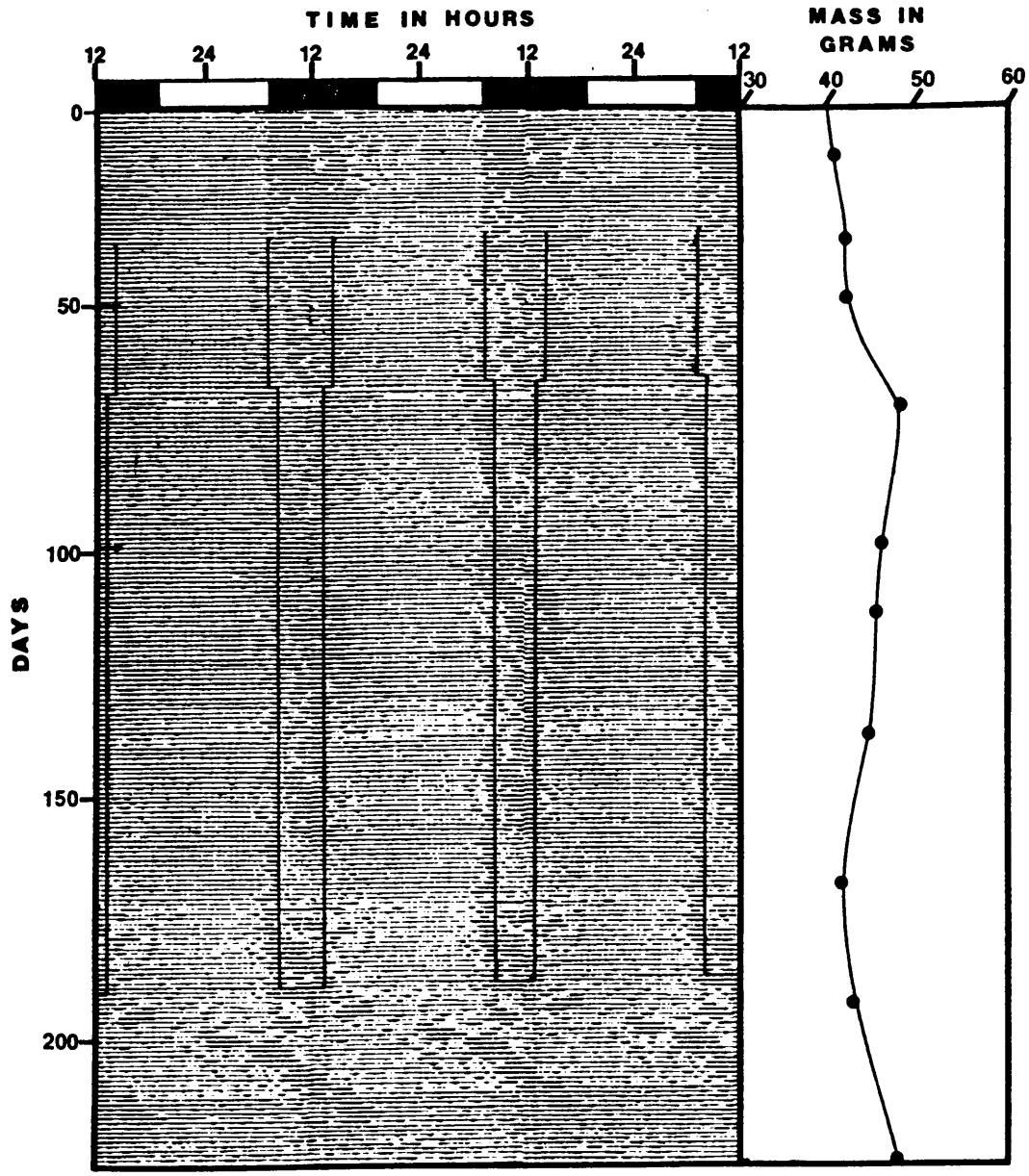

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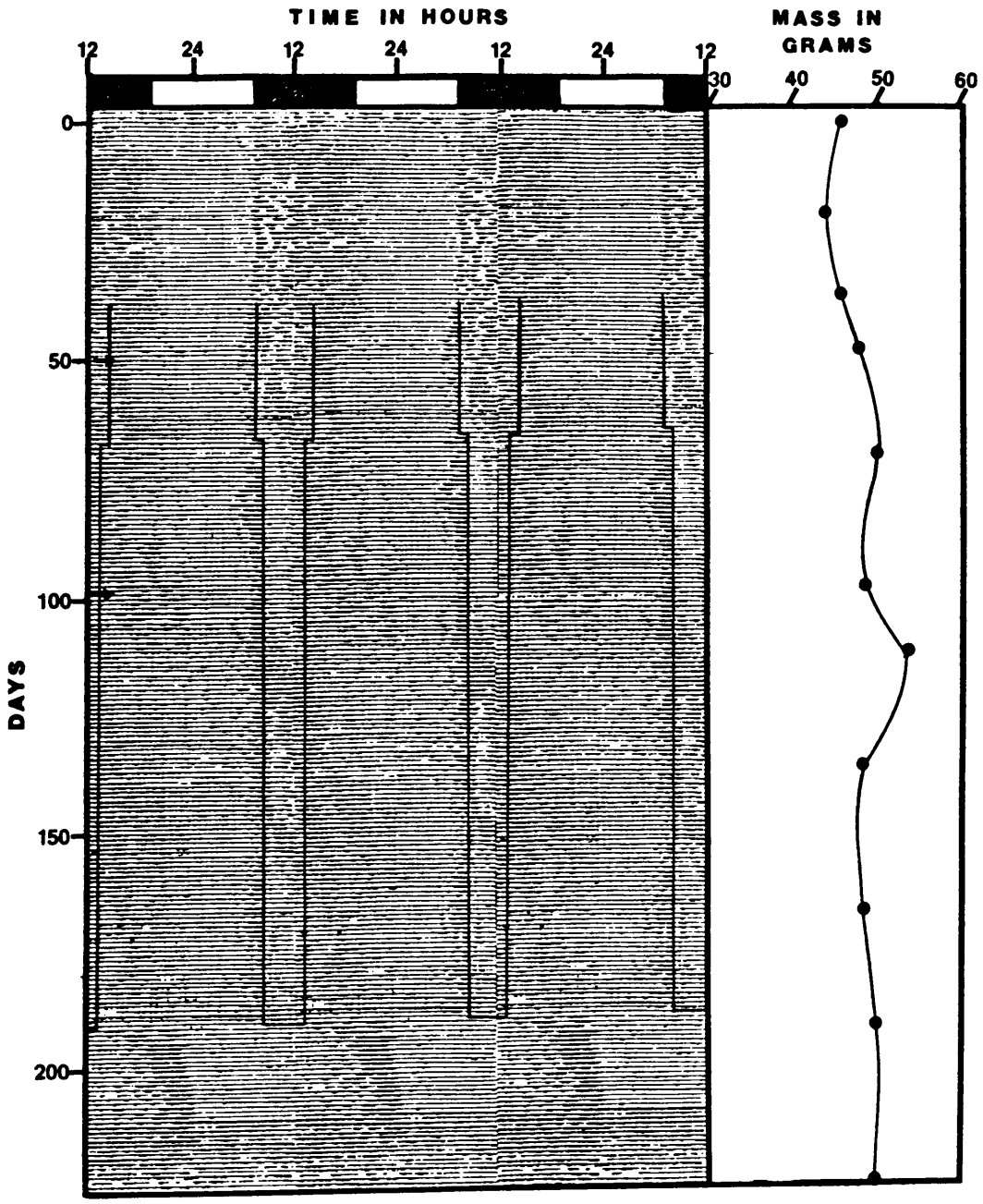
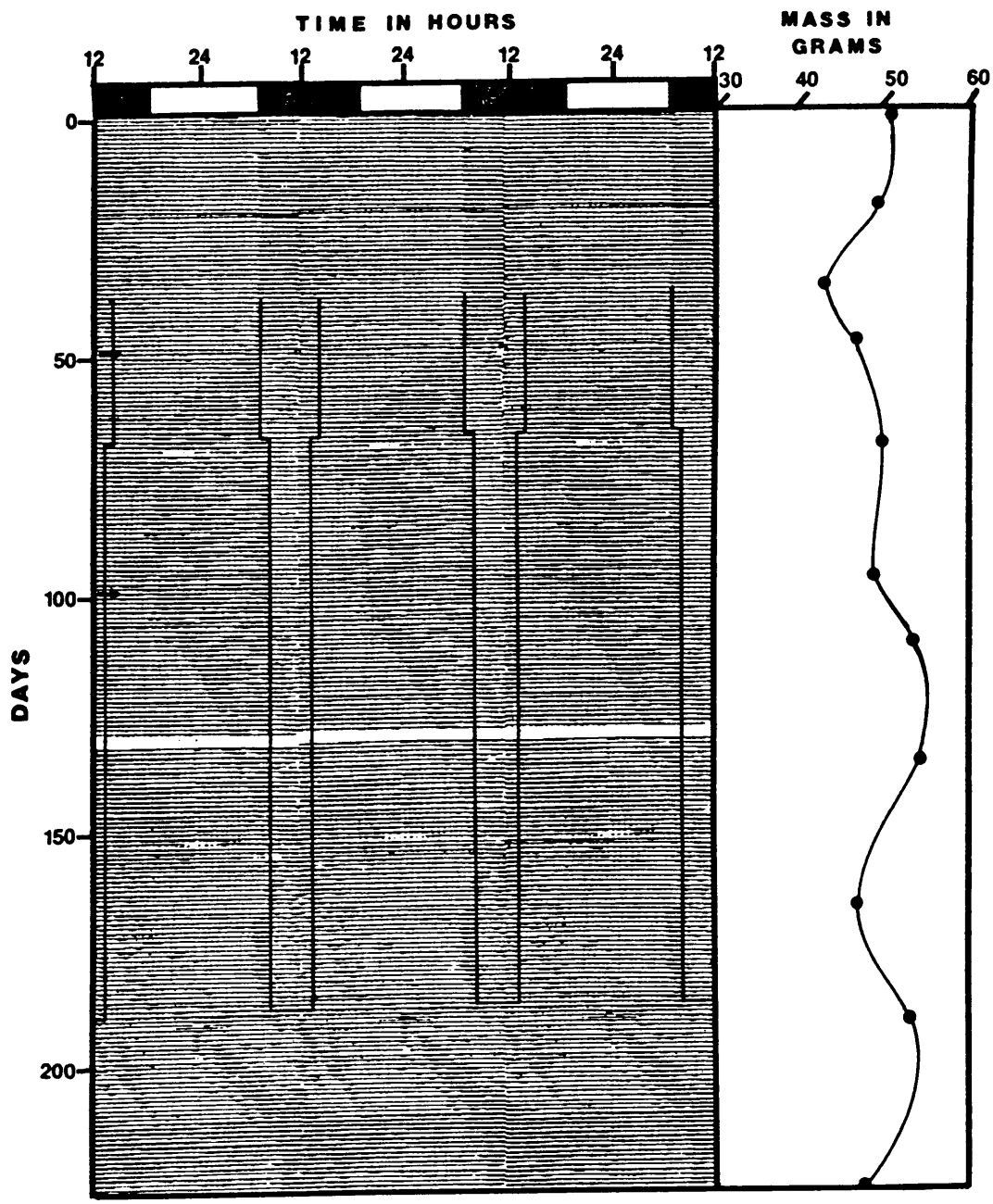
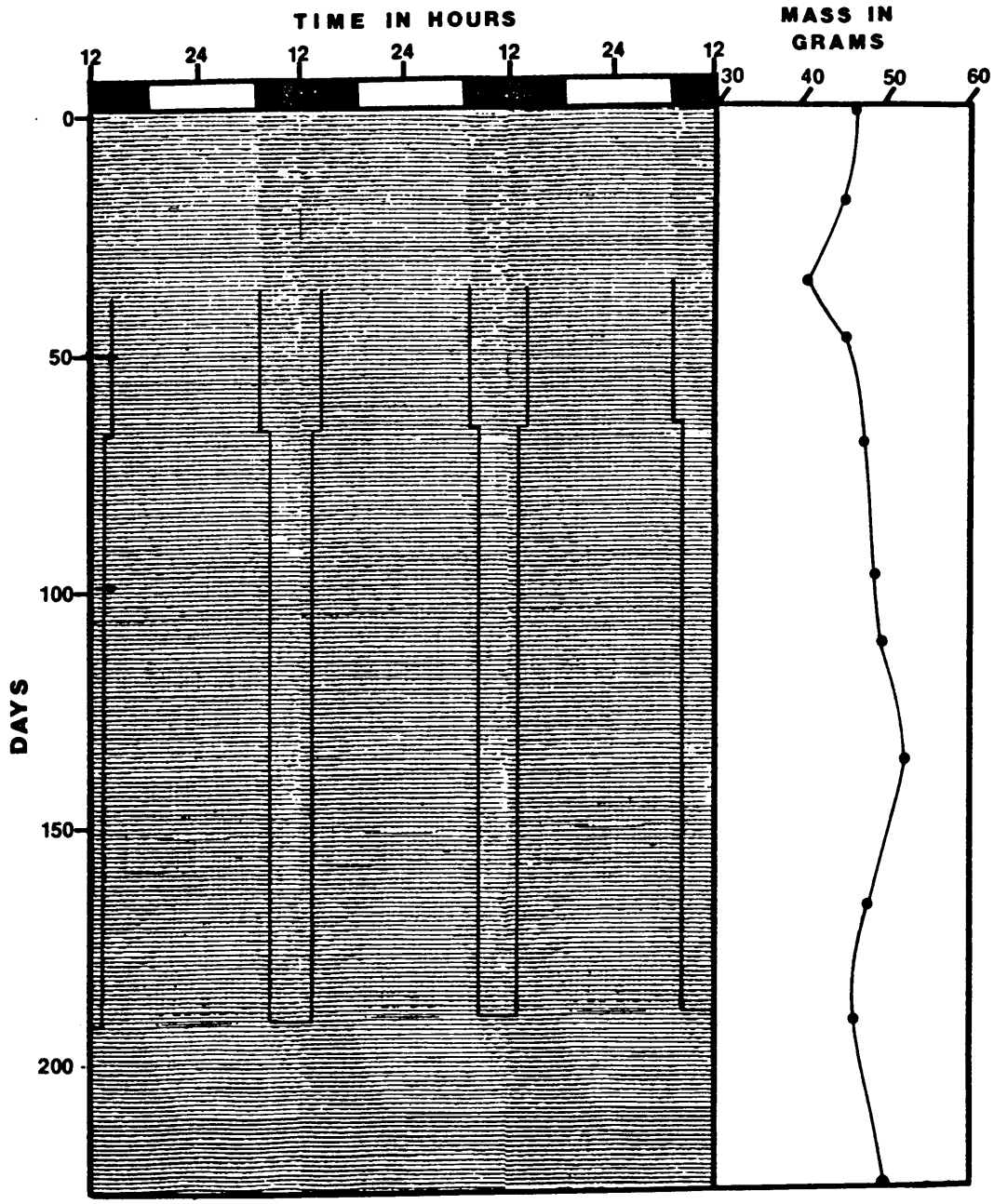


*Animal Number 15 (Chow-starch experimental diet)*



*Animal Number 19 (Chow-starch experimental diet)*

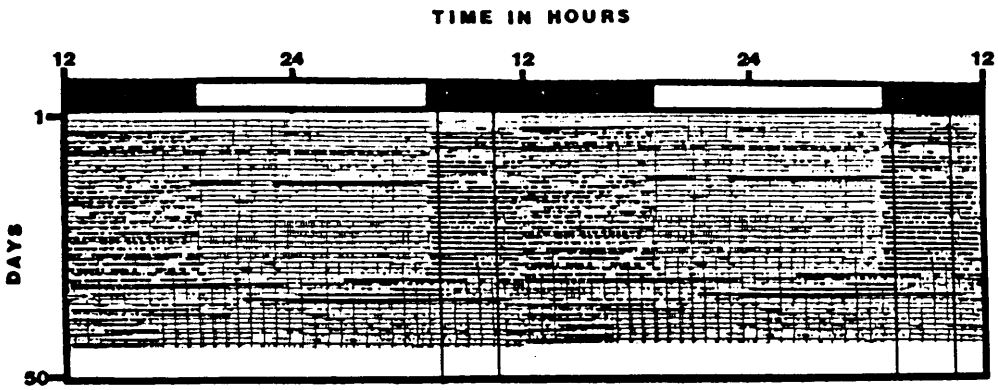


# Appendix B. Locomotor Activity And Weight Records: Short-Term Study

NOTE:

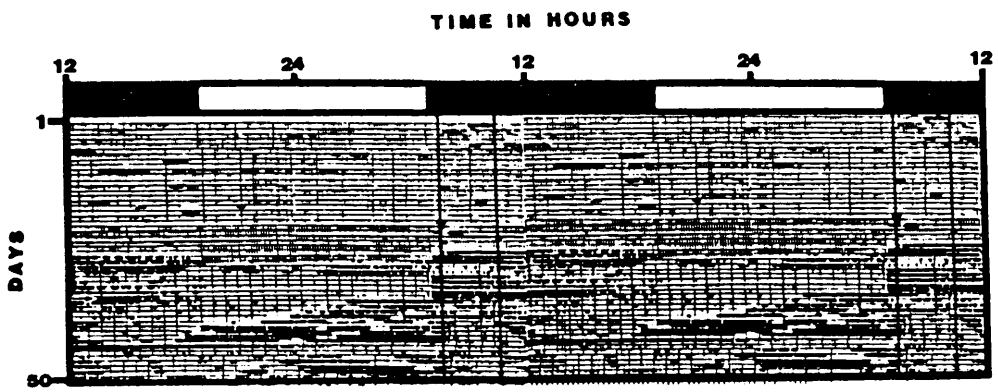
Figures in this appendix show the changes in locomotor activity and body mass for individual mice over time. The abcissa for each figure is marked in time units (hours) with the original light/dark cycle indicated. The ordinate is marked in time units (days). In order to provide the best visual display for this short-term study, locomotor records for each animal are a double-plot, with time proceeding in hours from left-to-right, and in days from top-to-bottom. Prior to the recording of locomotor rhythms, animals were placed on a DL 12:12 (lights on at 1900 and off at 0700) and given hard chow *ad libitum*. At the beginning of the records, animals were still in the same lighting cycle, but were only given powdered chow from 0730 to 1230 (five-hour restriction) or from 0730 to 1030 (three-hour restriction). The temporal feeding window is indicated by the solid vertical lines. The small arrow at day 35 on the ordinate denotes the point at which the light/dark cycle was switched to total darkness. The weight changes over time are depicted below each figure, with the day value relative to the beginning of the experiment. White blocks in the locomotor records indicate a loss of data. The experiment ended on day 50.

*Animal Number 23 (3 hour restricted feeding regime)*



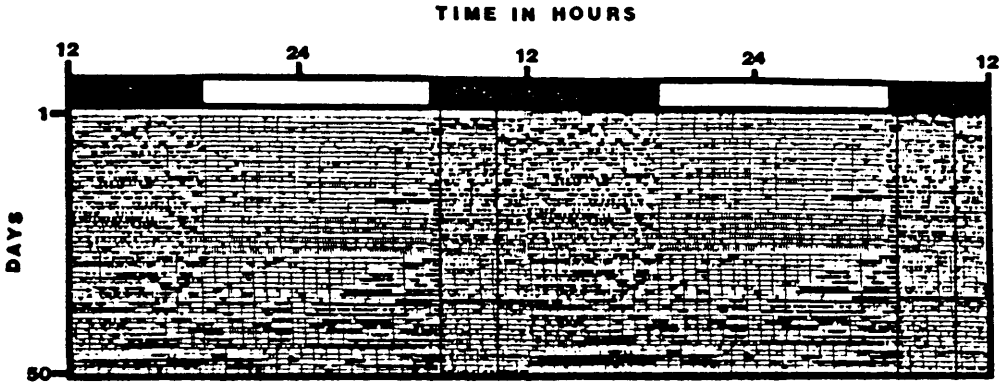

| Mass Changes: | Day | Mass(gms) |
|---|---|---|
| | -8 | 14.2 |
| | 22 | 23.2 |
| | 50 | 26.1 |

*Animal Number 28 (3 hour restricted feeding regime)*



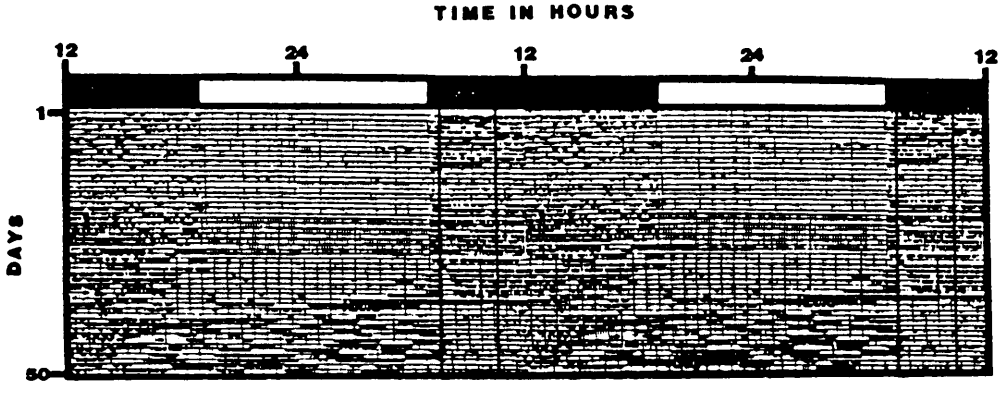

| Mass Changes: | Day | Mass(gms) |
|---|---|---|
| | -8 | 13.2 |
| | 22 | 25.4 |
| | 50 | 25.4 |

*Animal Number 32 (3 hour restricted feeding regime)*



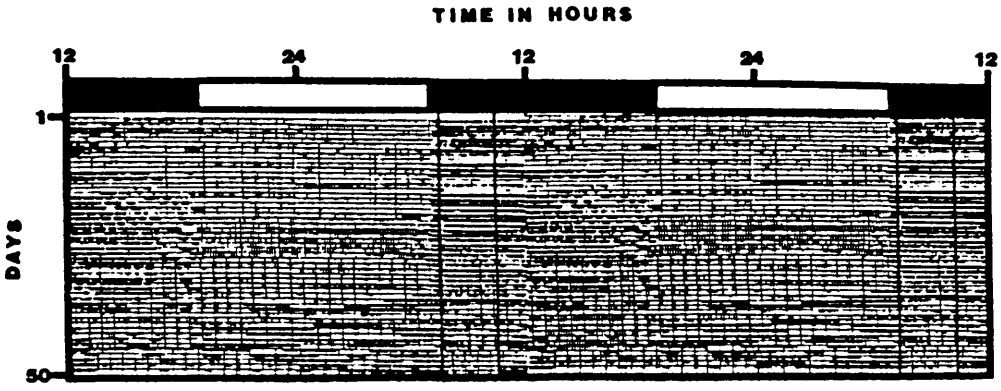

| Mass Changes: | Day | Mass(gms) |
|---|---|---|
| | -8 | 13.9 |
| | 22 | 21.8 |
| | 50 | 21.5 |

*Animal Number 33 (3 hour restricted feeding regime)*



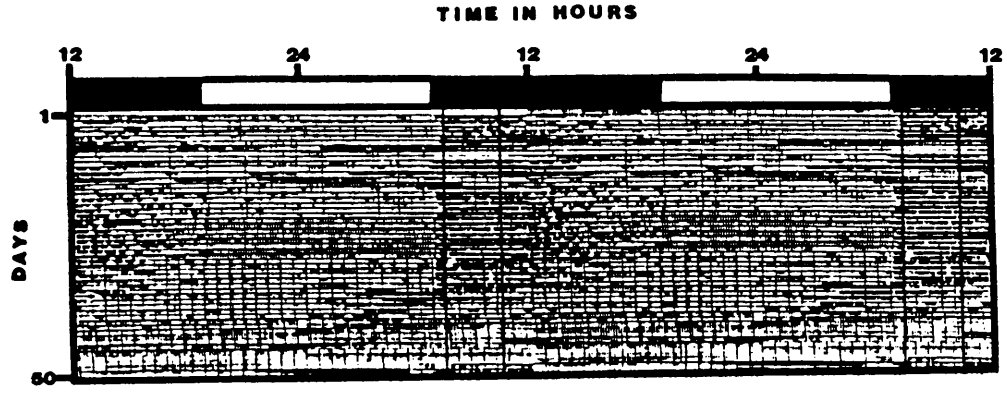

| Mass Changes: | Day | Mass(gms) |
|---|---|---|
| | -8 | 13.7 |
| | 22 | 21.1 |
| | 50 | 18.7 |

*Animal Number 35 (3 hour restricted feeding regime)*



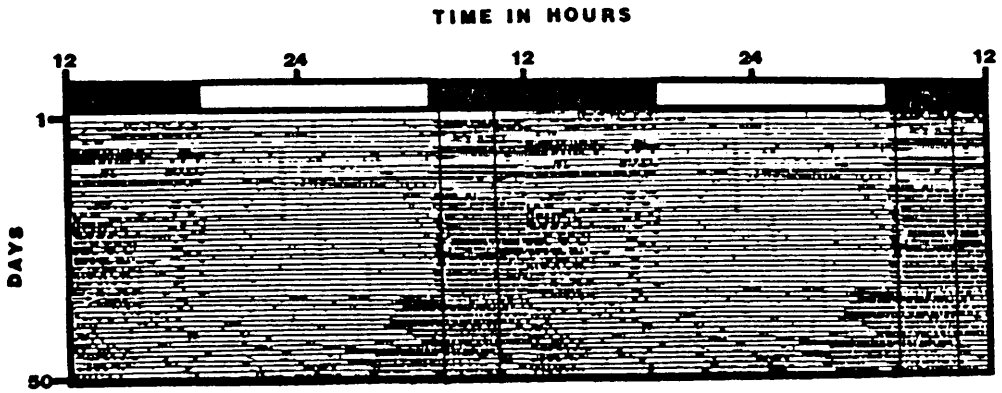

| Mass Changes: | Day | Mass(gms) |
|---|---|---|
| | -8 | 13.2 |
| | 22 | 19.4 |
| | 50 | 21.9 |

*Animal Number 37 (3 hour restricted feeding regime)*



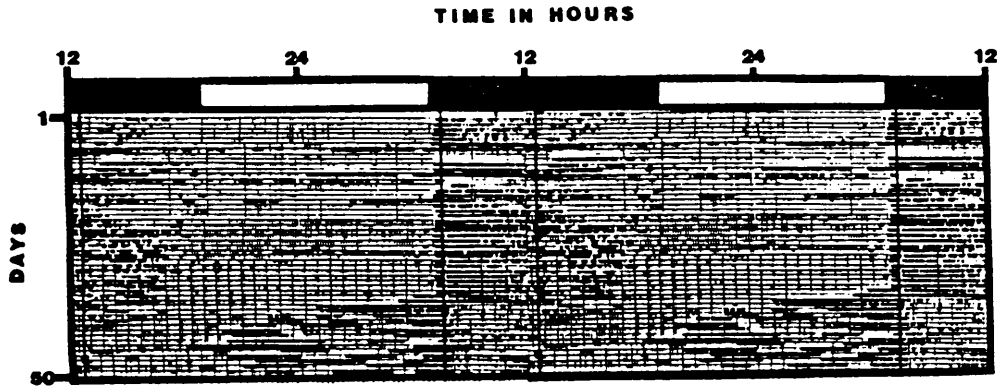

| Mass Changes: | Day | Mass(gms) |
|---|---|---|
| | -8 | 14.3 |
| | 22 | 23.2 |
| | 50 | 25.0 |

*Animal Number 40 (3 hour restricted feeding regime)*



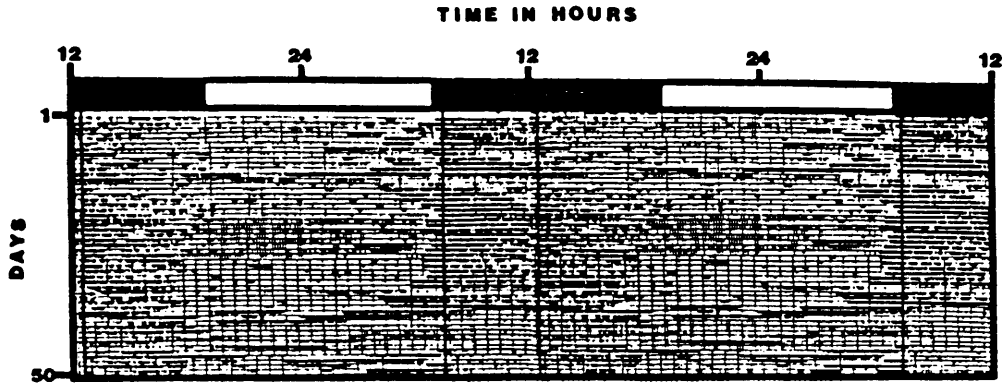

| Mass Changes: | Day | Mass(gms) |
|---|---|---|
| | -8 | 14.0 |
| | 22 | 20.4 |
| | 50 | 25.7 |

*Animal Number 22 (5 hour restricted feeding regime)*



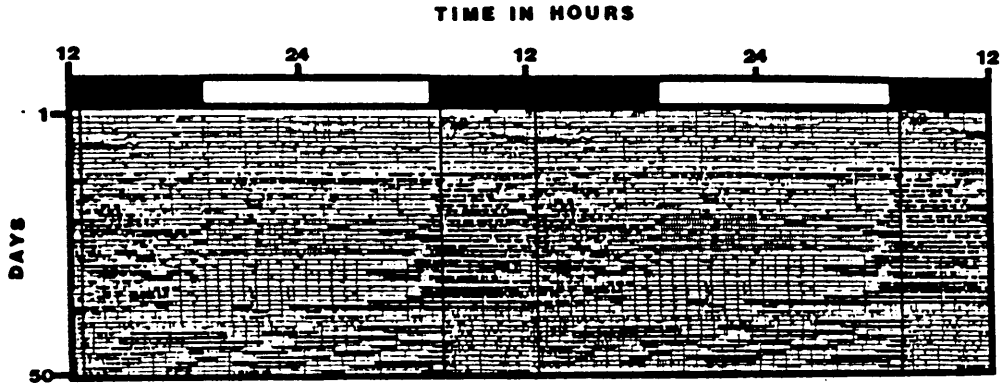

| Mass Changes: | Day | Mass(gms) |
|---|---|---|
| | -8 | 14.2 |
| | 22 | 23.7 |
| | 50 | 21.9 |

*Animal Number 25 (5 hour restricted feeding regime)*



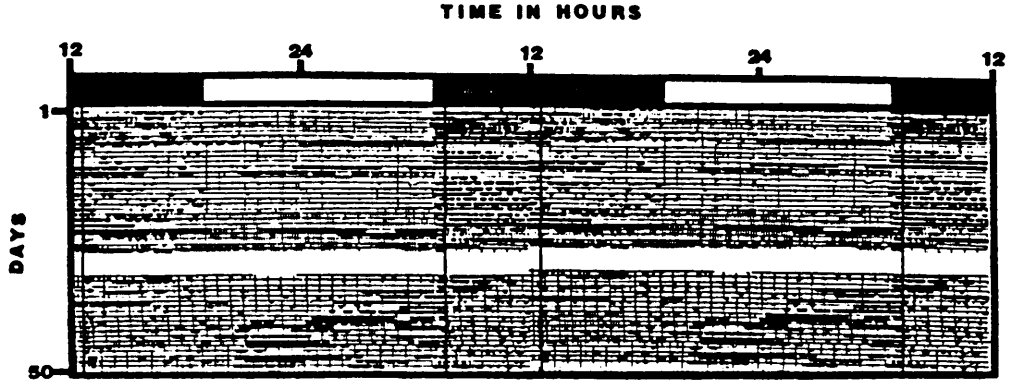

| Mass Changes: | Day | Mass(gms) |
|---|---|---|
| | -8 | 13.6 |
| | 22 | 23.8 |
| | 50 | 20.3 |

*Animal Number 29 (5 hour restricted feeding regime)*



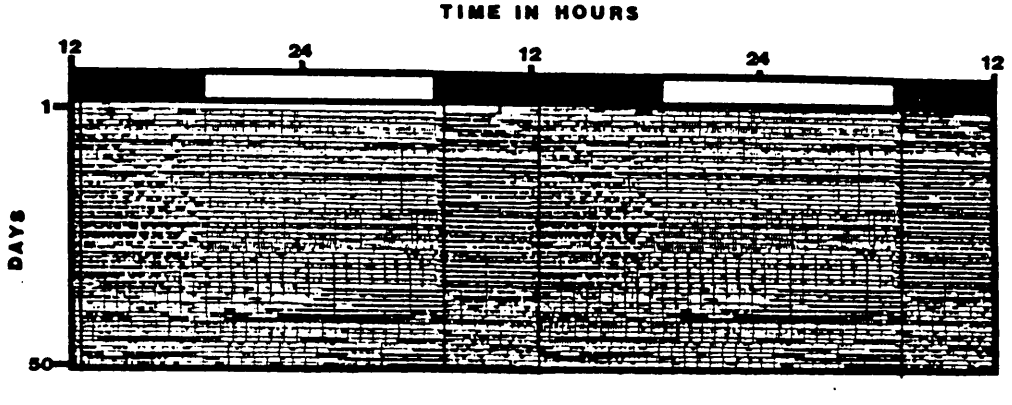

| Mass Changes: | Day | Mass(gms) |
|---|---|---|
| | -8 | 14.2 |
| | 22 | 21.5 |
| | 50 | 22.5 |

*Animal Number 31 (5 hour restricted feeding regime)*



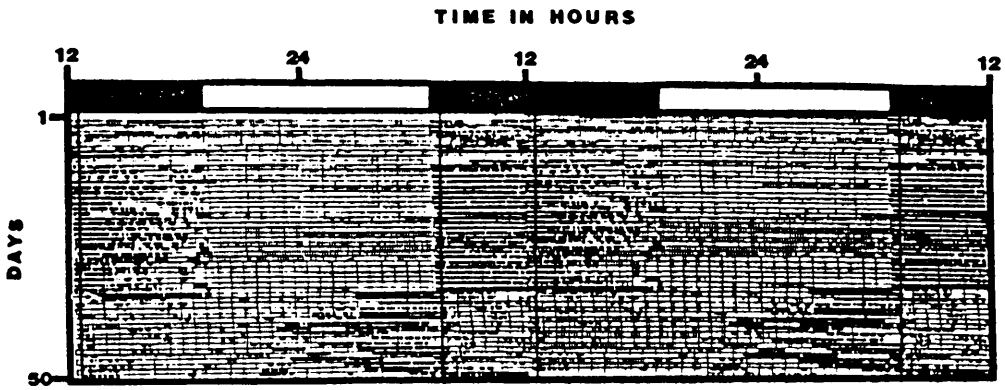

| Mass Changes: | Day | Mass(gms) |
|---|---|---|
| | -8 | 15.1 |
| | 22 | 22.4 |
| | 50 | 27.3 |

*Animal Number 34 (5 hour restricted feeding regime)*



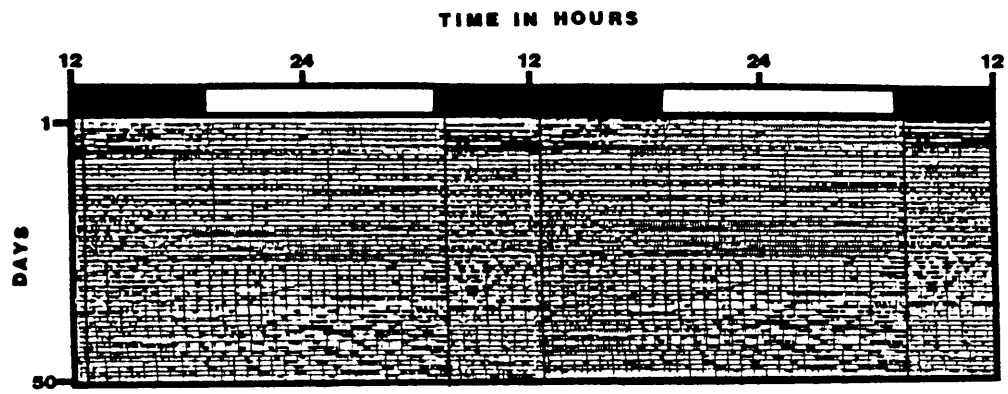

| Mass Changes: | Day | Mass(gms) |
|---|---|---|
| | -8 | 14.8 |
| | 22 | 20.9 |
| | 50 | 23.1 |

*Animal Number 36 (5 hour restricted feeding regime)*



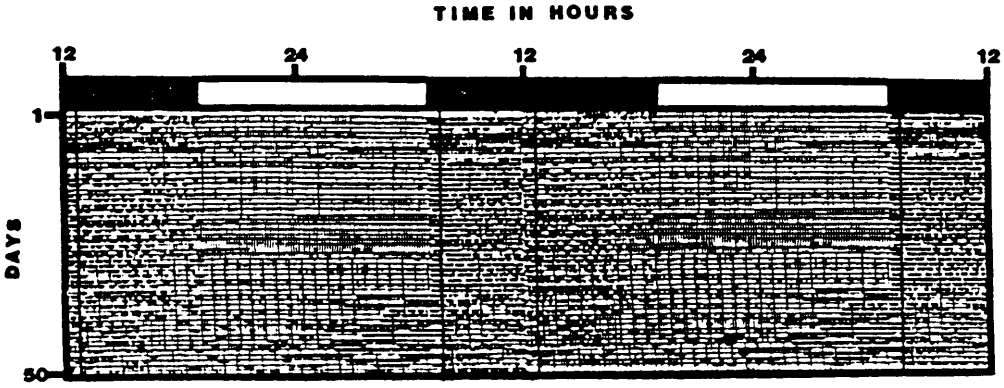

| Mass Changes: | Day | Mass(gms) |
|---|---|---|
| | -8 | 14.7 |
| | 22 | 22.0 |
| | 50 | 28.0 |

*Animal Number 38 (5 hour restricted feeding regime)*




| Mass Changes: | Day | Mass(gms) |
|---|---|---|
| | -8 | 14.1 |
| | 22 | 20.6 |
| | 50 | 22.9 |

*Animal Number 39 (5 hour restricted feeding regime)*




| Mass Changes: | Day | Mass(gms) |
|---|---|---|
| | -8 | 15.3 |
| | 22 | 23.2 |
| | 50 | 27.5 |

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