THE REGULATION OF THE TIMING OF MELATONIN SECRETION IN THE SHEEP

A thesis submitted by;

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SUMMARY

As with many animal species originating within the higher latitudes the sheep uses seasonal changes in daylength to adjust its breeding pattern to optimise the survival of its young (Yeates 1949). It is now recognised that the pineal gland plays a central role in the endocrine mechanisms which provide the sheep with hormonal signals concerning the changing parameters of the external photoperiod (Karsch et al 1986, Kennaway et al 1982).

This thesis addresses the nature of the central mechanisms involved in the regulation of the circadian pattern of secretion of the pineal hormone melatonin in the highly seasonal Suffolk breed of sheep. Insight was gained into these mechanisms by closely monitoring the changes in the pattern of melatonin secretion in sheep held in darkness to remove inhibitory photic input following their adaption to specified photoperiods. Through using the timing of the onset and offset of melatonin as phase reference points it was found that comparisons could be made to other circadian mechanisms using established chronobiological principles.

The experimental work is presented in four sections. The first section presents two preliminary experiments initially designed to examine the effect of pulses of light administered during the dark phase.

In the first of these experiments ewes were allowed a period of six weeks to acclimatise to a defined photoperiod and the pattern of melatonin secretion was then determined both under the prevailing photoperiod and during a period of extended darkness. A one hour light pulse was then introduced into the latter part of the dark phase (Experimental

10L:10D:1L:3D versus Control 10L:14D) resulting in a reduction of the period of melatonin secretion and accompanying endocrine responses mimicking those normally found in ewes exposed to long days. The second experiment examined the effects of the application of a light pulse in the first half of the dark phase. In this experiment two groups (1 and 2) each of four rams, were transferred from the prevailing photoperiod (October) to ultra short days (L:D,6.5:17.5) where they were maintained for four weeks. One group of rams (Group 2) was then transferred for a period of six weeks to a lighting regime where the dark phase was interrupted by a half hour light pulse (6L:7.5D:.5L:10D) while the other group remained on L:D,6.5:17.5 as controls. It was found that the rams in the control group as a result of treatment, completely lost their ability to generate a pattern of melatonin secretion. By contrast rams in group 2 exhibited an abnormal rhythm with three of the four animals showing unusually high levels of plasma melatonin. The data clearly indicated that these manipulations of the lighting regime had in some manner disrupted the pineal pacemaker so that it was no longer able to generate a normal rhythm in melatonin secretion. In some animals the period of melatonin secretion even extended into the light phase which contrasted with the finding in other rams (n=6) monitored under natural photoperiod which displayed a typical pattern of melatonin secretion with an immediate curtailment of secretion at dawn.

The results of these studies highlighted deficiencies in our understanding of the control of pineal function in the sheep and stimulated my interest in further studies of these

mechanisms. The data gained in the initial experiments indicated that under appropriate conditions the pattern of plasma melatonin could provide insight into the functioning of the pacemaker centres controlling pineal function especially if the function of the centres could be perturbed by judicious modification of the lighting regime. Experiments described in the subsequent section of the thesis aimed to extend the initial observation and establish baseline measurements on the pattern of melatonin secretion in ewes which were conditioned to a set photoperiod then subjected to darkness so that the functioning of the putative biological clock was free of photic input.

In the first experiment ewes were entrained to either short (L:D, 10:14) or long days (L:D, 14:10) for four weeks and their melatonin profiles then determined under the prevailing photoperiodic regime and under extended darkness. Under the ambient photoperiods, a normal pattern of melatonin secretion was observed with the onset of melatonin secretion occurring soon after lights off and terminating immediately the lights were turned on. However, under extended darkness, the onset of melatonin secretion occurred earlier and the offset approximately three hours later for both photoperiods, indicating that, contrary to expectation, the duration of melatonin secretion under extended darkness was longer for ewes adapted to short days than those adapted to long days.

A subsequent experiment using the same paradigm examined the effect of delaying dusk, i.e., the time of lights off, by four hours. It was found that under extended darkness ewes experiencing delayed dusk showed a delay in the offset of melatonin secretion by more than 4.5 hours indicating that

the offset of melatonin secretion was affected by the time at which dusk occurred.

To further characterise the interaction of the onset and offset of secretion, subsequent experiments examined the pattern of melatonin secretion in ewes held under continuous darkness for several days, following a period of entrainment to specific photoperiods.

In ewes entrained to short days (L:D,10:14) the episodic output of melatonin was sustained in continuous darkness, with the offset of melatonin secretion occurring as abruptly as that usually associated with the beginning of the light phase. However, the time of offset occurred later each day, suggesting regulation by a pacemaker centre with a period greater than 24 hours.

Subsequent studies on the onset of melatonin secretion, reported in the next section of the thesis, yielded conflicting results. Two experiments were carried out, one during autumn and one in winter. In both experiments, ewes were entrained to a short day photoperiod, (L:D,10:14) for four weeks before monitoring the onset of melatonin secretion in continuous darkness but the results differed markedly. In autumn individual ewes showed considerable variation in the timing of the onset of melatonin secretion, with the onset in some ewes advancing whilst in others it delayed whereas in a similar experiment carried out in winter, the onset of melatonin secretion in all animals appeared to delay.

To resolve these apparently contradictory results the experiment was repeated in early summer and both the onset and offset of melatonin secretion monitored under continuous darkness in a the same experiment. It was found that during initial cycles in continuous darkness, the offset of

melatonin secretion was delayed while onset was advanced. However, after the third cycle there appeared to be a change in the functioning of the control centres as offset continued to delay whereas onset ceased advancing and began to delay.

The thesis concludes with a general discussion attempting to integrate the various observations made with current hypotheses on the nature of pacemaker centres.

Accepting that the melatonin onset and offset reflects the activity of pacemaker centres controlling pineal function, the results obtained so far are best interpreted as adding support to the view that control of the pineal pacemaker may be understood as being determined by two interacting pacemakers as in the model described by Pittendrigh and Daan (1976). However, it is conceded that data acquired so far does not refute a simple single pacemaker model. The challenge for future research aimed at developing our understanding of the pacemaker mechanisms determining regulation of melatonin secretion will be to design experiments which will allow differentiation between these competing hypotheses.

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DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University, and to the best of the author,s knowledge and belief, the thesis contains no material previously published or written by any other person except where due reference is made in the text.

Permission is given to any person to photocopy or borrow this thesis.

COLIN R. EARL

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Introduction

Animals occupying niches other than in equatorial regions are dependent on a capacity to monitor changes in photoperiod in order to anticipate and adapt to seasonal cycles. How this is done is only partly understood. There is compelling evidence to indicate that the pineal organ is centrally involved in the process of photoperiodic time measurement. This evidence is derived from studies which demonstrate that in all species so far investigated, pinealectomy, that is removal or ablation of the pineal, renders the individual incapable of responding to changes in photoperiod (Thorpe and Herbert 1976, Hoffman and Reiter 1965, Bittman et al 1983). Following the discovery and isolation of the pineal hormone melatonin by Lerner and his associates, (1958,1959,1960) the effects of melatonin on many photoperiodic species were investigated and compelling evidence acquired to show that the pattern of melatonin secretion was critically involved both in the mechanism through which the pineal plays a central role in photoperiodic time measurement and seasonality of reproductive activity (Reiter 1974, Turek and Campbell 1979, Cardinali 1981, Vaughn 1981).

Many hypotheses have been formulated attempting to explain how the pineal participates in photoperiodic time measurement. All now include reference to the pineal and its involvement in the circadian pattern of melatonin secretion (Rollag et al 1978, Carter and Goldman 1983). Although pineal rhythms are generated by an endogenous circadian pacemaker little is known about the nature of this pacemaker (Klein and Weller 1970), Ralph et al 1971, Yellon et al 1982). This thesis was directed at improving our knowledge of the nature

of the pineal pacemaker in the sheep to provide a better basis for understanding the interrelationship between endogenous biological rhythms and seasonality in breeding activity exhibited by this species.

Studies with laboratory rodents have indicated that the pineal pacemaker is sensitive to light acting via the eyes resulting in melatonin secretion being supressed in the presence of light. Consequently the unrestrained behaviour of any pacemaker controlling melatonin secretion can only be monitored in the absence of light. (Reppert et al 1979, Lewy et al 1980). To date most of the studies of melatonin secretion in the sheep have been made under circumstances where light could influence pacemaker function. Studies reported in this thesis attempt to rectify this deficiency and gain insight of the functioning of the pineal pacemaker unconstrained by light. This has been addressed by monitoring the patterns of melatonin secretion generated in sheep during exposure to defined photoperiods and then again when they are held under extended or continuous darkness.

Introduction to the Literature Review

The literature review includes studies from the fields of pineal physiology, circadian physiology and photoperiodic time measurement. Each of these areas now embrace a vast literature and for the purposes of this thesis only those studies which are closely related to the main theme of the thesis have been cited.

The review is firstly concerned with research identifying the role of the pineal hormone melatonin in the photoperiodic time measuring system. Much of the data stems from experiments with rodents where it is found that melatonin production is principally controlled by the level of one enzyme, N-acetyltransferase which is in turn regulated by a circadian pattern of B-adrenergic stimulation. The effects of light and pharmacological treatments on the level of N-acetyltransferase activity in these species and hence on the pattern of melatonin production are reviewed.

The review then concentrates specifically on the research data obtained in studies in the sheep, the species of central interest to this thesis. Firstly an overview is provided of data relating the annual cycle of reproduction and photoperiod. The effects of melatonin administration in intact and pinealectomised sheep are then reviewed followed by what is known of the pattern of melatonin secretion observed under various photoperiods yielding data

which suggests that these patterns are a consequence of the activity of circadian central mechanisms.

Theories of photoperiodic time measurement developed prior to the discovery of pineal involvement in this process are then presented followed by a review of two hypotheses which have emerged to explain the role of the pineal in this process.

The complex nature of biological pacemakers is then discussed together with some of the more recent models that have been proposed to provide a basis for explanation of their function. The review concludes with a discussion of experiments which have attempted to probe the nature of the suprachiasmatic nucleus, a centre which is believed to play a central role in regulating circadian rhythmicity and a consideration of the two major hypotheses which attempt to explain the nature of the pineal pacemaker.

LITERATURE REVIEW

1.1 The Pineal and the neuroendocrine gonadal axis

1.1.1 Introduction

Having reviewed the pineal literature up to 1954 the only consistent association that Kitay and Altschule (1954) could find between the functioning of the pineal gland and other biological functions was with reproduction. An important element in their argument was the apparant relationship that existed between pineal tumours and sexual malfunction. This clinical link between the pineal gland and the neuroendocrine gonadal axis provided an important impetus to the development of research into the links between pineal function and reproductive activity.

1.1.2 Pinealectomy

The classical first step in investigation of glandular function, that of ablation, showed that pinealectomy could indeed influence gonadal activity in mammals (Reiter 1974, 1977, Minneman and Wurtman 1975) but as shown in subsequent studies any influence of the pineal was not direct but through its central role in the photoperiodic time measuring system (Hoffman 1981, Herbert 1981). Turek and Cambell (1979) highlighted the importance of the pineal in photoperiodic time measuring system with the following statement. "The importance of this gland in the photoperiodic response in mammals is underscored by our failure to find in the

literature a single demonstration that the removal of the pineal gland did not alter how an animal responded to a subsequent photoperiodic change."

1.1.3 Melatonin Injections

The discovery of the pineal hormone melatonin and its subsequent availability led to a plethora of experiments in a wide variety of species investigating the effects of exogenous melatonin on the neuroendocrine gonadal axis. An important advance in these experiments was achieved when it was recognised that the effectiveness of melatonin injections varied with the time of administration (Stetson et al 1983, Tamarkin et al 1976). This helped resolve many apparant contradictions developing in the literature and controversy as to whether melatonin was acting in a pro or antigonadotrophic manner. For example daily injections given in the late afternoon to animals cued to breed by long days antagonised the stimulatory effect of long photoperiod (Sisk and Turek 1982) whereas when melatonin was administered in the late afternoon to animals cued to breed on short days, it elicited a progonadotrophic response, overcoming the inhibitory effect of long days (Kennaway et al 1982). Melatonin is therefore capable of eliciting both antigonadotrophic and progonadotrophic responses. It has also been reported that melatonin injections given in the morning will overcome the antigonadotrophic effect of afternoon injections in long day breeders (Chen et al 1980).

Whilst the discovery of the importance of time of administration of melatonin provided an insight into the manner in which melatonin was involved in the photoperiodic

time measuring system, much of the early literature must be interpreted with caution as it is now known that at some of the dose rates used plasma melatonin would have been elevated in small laboatory animals for the full 24 hour period in many of the experiments and many of the effects attributed to timed injections may in fact be caused by a continuously high level of melatonin (Brown et al 1985).

1.1.4 Melatonin Implants

Continuous administration of melatonin through implants given to long day breeding rodents such as the hamster generally act in an antigonadotrophic manner and induce testicular regression (Turek et al 1975, Turek et al 1976). Testicular recrudescence on transfer to long days is similarly suppressed by melatonin implantation (Turek et al 1976). Thus in these experiments the continuous release of melatonin gave a similiar response as exposure to short days. In these cases melatonin was acting in an antigonadotrophic manner. However melatonin implants can also have a progonadotrophic effect on long day breeding animals. If hamsters receive implants before being placed in a nonstimulatory photoperiod the antigonadotrophic effect of short days is blocked (Turek and Losee 1978, Reiter et al 1975). Furthermore the implantation of hamsters on short days hastens testicular recrudescence. Thus in rodents, "When photoperiod stimulates neuroendocrine gonadal activity melatonin is inhibitory and when the photoperid inhibits reproductive activity, melatonin is stimulatory" (Turek and Losee 1978).

1.1.5 Melatonin Infusions in Hamsters

To further investigate these effects programmed infusions of melatonin have been used by Goldman and his associates to monitor the effect of various patterns of melatonin infusion on the reproductive performance on pinealectomised and intact juvenile Djungarian hamsters (Carter and Goldman 1983, Goldman et al 1979). In this manner it was possible to investigate the effect of time and duration of administration as well as the amplitude and total amount of melatonin on the neuroendocrine gonadal axis. Importantly it was found that infusions of greater than 8 hrs always induce testicular regression and infusions of 4-6 hours or less were ineffective in inducing testicular regression in pinealectomised hamsters. This finding suggests that it is the duration of melatonin secretion which is the important feature of the melatonin signal rather than the time of day at which melatonin levels are elevated.

1.2 The Secretion of Melatonin by the Pineal Gland

1.2.1 Introduction

Hypotheses on pineal participation in photoperiodic time measurement now centre on the nature of the signal provided by the circadian pattern of melatonin secretion. However while numerous studies on the effects of melatonin on the neuroendocrine axis have been conducted, the manner by which the pineal rhythm in melatonin production is generated has received little attention. A brief overview of the available

literature is provided in the following section.

1.2.2 Melatonin biosynthesis

The biosynthetic pathway of melatonin production was originally determined by Lerner et al (1958, 1959 & 1960) and has been reviewed on many occasions (Balemans 1978, Lewy 1983, Wurtman et al 1964). Briefly the essential amino-acid tryptophan is taken up into pineal parenchymal cells (Minneman and Wurtman 1976, Wurtman and Moskowitz 1977) and converted to 5-hydroxytryptophan (5 H.T.P.), through the action of tryptophan hydroxylase (Sitoram and Lees 1978, Jequier et al 1969), before decarboxylation to serotonin through the action of the enzyme L- aromatic amino acid decarboxylate (Snijder and Axelrod 1964). Conversion of serotonin to melatonin proceeds by a two step process catalysed by two pineal specific enzymes. The first N-acetyltransferase (N.A.T.) converts serotonin to N- acetyl serotonin (N.A.S.), (Klein 1978,).
N-acetylserotonin is then converted to melatonin by a second pineal specific enzyme, hydroxy-indole-o-methyl transferace (H.I.O.M.T.) which transfers a methyl group from S adenosyl methionine to the 5 hydroxyl of N.A.S. to form melatonin (Axelrod and Weissbach 1961).

1.2.3 Rate Limiting Steps in the Secretion of Melatonin

It is now generally accepted that melatonin secretion is principally regulated by the activity of one enzyme, N.A.T.. Support for this view has been provided by studies which show

that conditions which led to an increase in N.A.T. resulted in a fall in serotonin concentration, its substrate and an increase in its product N.A.S. (Brownstein et al 1973). Conversely conditions which decreaseed N.A.T. activity were associated with an increase in serotonin and a decrease in N.A.S. (Deguchi and Axelrod 1972, Klein and Weller 1972). Further support was provided by studies of pineal organ cultures which demonstrated that the stimulation of melatonin secretion by the addition of norepinephrine was associated with parallel increases in N.A.T. synthesis (Klein 1978, Oleshansky and Neff 1978).

1.2.4 B-adrenergic Stimulation and the Level of N.A.T. Activity.

The complex biochemical processes involved in the regulation of N.A.T activity have been reviewed by Oleshansky and Neff (1978) and Lewy (1983). A brief summary of experiments which indite B-adrenergic input as a major factor in the regulation of pineal melatonin content is included below.

Initial studies in this area were carried out by Brownstein, Holz and Axelrod (1973) and Brownstein and Axelrod (1974). In these studies the effect of a beta blocking agent, l-propanolol and an alpha blocking agent, phentolamine on the rise in N.A.S. activity were investigated. In rats it was found that N.A.S. production was stimulated by the administration of l-isoproterenol, and the rise was reversed by l-propanolol whereas phentolamine had no effect. Further, it was shown in organ culture experiments that there

was a rise in N.A.T. activity associated with the addition of the B-adrenergic agonist norepinephrine to pineal gland tissue (Klein et al 1970) which could also be stopped by the application of B-adrenergic blocking agents (Brownstein, Saavedra and Axelrod 1973). Thus it was concluded that the level of N.A.T. activity was controlled by the stimulation of a beta adrenergic receptor.

The activity of N.A.T. is mediated by cyclic AMP as first indicated by Strada and coworkers (1971) who found that the addition of the neurotransmitter norepinephrine (N.E.) caused a six fold increase in the concentration of adenosine 3-5-monophosphate (cyclic 3-5-AMP) which preceded the elevation in N.A.T. activity and the increase in the formation of melatonin.

Evidence that light influences the level of pineal activity through its effect on the level of B-adrenergic stimulation is provided by studies which showed that the fall in the level of N.A.T. activity associated with lights on was accompanied by a reduction in the turnover of N.E. in pinealocytes (Oleshansky and Neff 1978, Brownstein and Axelrod 1974).

Collectively these findings support the generally accepted hypothesis that the production of melatonin is under B-adrenergic control.

1.2.5 Circadian Control of Pineal Activity

The hypothesis that pineal activity is controlled by an endogenous circadian pacemaker was developed as the result of a number of studies. Firstly it was found that there was a

circadian rhythm in N.A.T. (Klein and Weller 1971) along with a circadian rhythm in its substrate and its product (Klein and Weller 1970, Quay 1963, Snyder et al 1965, Klein and Weller 1972).

Secondly, the circadian rhythm in pineal melatonin was observed to persist in rats maintained in continuous darkness (Ralph et al 1971). In the same experiment the authors also established not only that the rhythm persisted, but that it was phase locked to the activity rhythm. Subsequent experiments showed similar phase linkages in the Syrian and Djungarian hamsters (Tamarkin et al 1980, Yellon et al 1982).

Thirdly, a circadian rhythm in the turnover of N.E. in sympathetic nerves innervating the pineal has also been observed (Brownstein and Axelrod 1974). This rhythm persisted in blinded animals but was suppressed by light which led the authors to suggest that the daily rhythm in the stimulation of B-adrenergic receptors of pineal cells by N.E. is probably responsible for the circadian cycle in pineal indole metabolism.

1.3 Light and pineal activity

1.3.1 The action of light on the rhythm of pineal Activity

Interruption of the dark (night) period of the daily cycle affects the behaviour of a number of circadian rhythms including the rhythm of pineal activity. The action of light on pineal rhythms however is mediated via the eyes and the central nervous system resulting in a reduction in the turnover of N.E., a sharp decline in N.A.T activity and cessation of melatonin production (Deguchi and Axelrod 1972, Klein and Weller 1972, Moore and Klein 1974).

The study of the pineal rhythm is difficult because of the extreme sensitivity of this rhythm to the effects of light. Even a brief exposure of animals to light can alter the phase of the rhythm which it is hoped to monitor. Zatz (1979) has attempted to overcome this problem by applying a light or pharmacological treatment to some animals and blinding all animals so that the differences in the timing of the onset and cessation of N.A.T. activity between treated and untreated animals could be monitored without interference. Using this drastic approach it was shown that pulses of light around the time of lights off delayed the rhythm in N.A.T. activity whereas pulses of light during the last few hours of darkness had little effect.

A similar appraoch was used by Illnerova and Vanecek (1979, 1982, 1985) in their exhaustive studies of the effect of light pulses on the rhythm of N.A.T. activity in rats. However they avoided blinding, by placing the rats in complete darkness to prevent further effects of light on the pineal pacemaker. This important series of experiments provides us with the most detailed studies of the behaviour of the pineal pacemaker in any species and formed the basis of the approach used in this thesis. To monitor the changes in the rhythm of N-acetyltransferase activity they used the rise and the fall in N.A.T. activity as two phase reference points. An important feature of their experiments was that the rats were maintained in darkness until the rats were sacrificed to assess the level of pineal N.A.T activity. Phase shifts in the rhythm of N.A.T. activity were calculated by comparison of the timing of the rises and falls in pineal N.A.T. activity between pulsed and control animals.

Using this approach it was found that when a one minute pulse of light was delivered early in the night, N.A.T. levels quickly dropped to a low level and then rose after a lag period. This lag period was found to vary in a manner dependent on the time of night the pulse was applied. In animals on L:D,12:12 maintained with lights off from 1800h to 0600h, the rise was delayed for 1.6h after a pulse at 2000h, 2.5h after a pulse at 2100h, 4h after a pulse at 2200h and 4.8 hrs after a pulse at 2300h. These delays occurred even though N.A.T. activity could quickly be restimulated under these circumstances by pharmacological B-adrenergic stimulation. Pulses of light at 2400h resulted in a decline in N.A.T. activity in all animals, followed by a subsequent rise in about half of the animals. Pulses at 0100h, 0200h and 0300h caused declines in N.A.T. activity none of which were followed by later rises in N.A.T. activity.

The authors interpreted this data as indicating that the light pulses caused instantaneous shifts of the endogenous pacemaker controlling the rise in the level of N.A.T.. Measurements made one day after administration of the pulse to animals entrained to L:D,12:12, L:D,18:6, and L:D,6:18, showed delays in the rise of N.A.T. activity for all photoperiods whereas the decline in N.A.T. activity showed both advances and delays. These results were used to construct phase response curves. On the basis of the differences in these phase response curves Illnerova and Vanecek (1982) have suggested that the pacemaker controlling pineal activity is comprised of two interacting oscillators.

1.3.2 Pharmacological investigations of the rhythm of N.A.T. activity

The same authors, Illnerova and Vanecek (1982), also investigated the timing of the rise in N.A.T. activity in animals exposed to different photoperiods. They found that the lag time between the time of lights off and the rise in N.A.T. activity increased as animals were placed in shorter photoperiods. They examined whether these differences could be attributed to an effect of different photoperiods on the internal mechanisms regulating pineal function by conducting several pharmacological experiments.

In these they found that the timing of the increase in the level of N.A.T. activity in response to injections of the B-adrenergic agonist isoproterenol given at the time of lights off was similar in rats exposed to different photoperiods indicating that there was no difference in the ability of animals to respond to B adrenergic stimulation. On the basis of their data the authors concluded that the differences in the lag times before the rise in N.A.T. activity between animals on different photoperiods was therefore not due to a difference in sensitivity of receptors for B-adrenergic agonists.

To test whether N.E. was being released but in insufficient quantities to stimulate the pineal, animals were treated with the antidepressant imipramine which potentiates N.A.T. induction with low levels of catecholamines. Again differences in time of the increase in N.A.T. activity between photoperiods were observed. The authors concluded from these findings that the differences in the time of the rise of N.A.T. activity under different photoperiods was due to an interaction between the photoperiod to which the animals are exposed and the central mechanisms driving the

N.A.T. rhythm.

These findings were interpreted to support their view that the time of the rise and fall of pineal activity as assessed by changes in the level of N.A.T. activity or melatonin concentrations under extended or continous darkness reflects the behaviour of the pineal pacemaker centres. The action of light on the level of N.A.T. activity has also been investigated by Romero and Axelrod (1974) and Deguchi and Axelrod (1972). These researchers showed that the application of cycloheximide, a blocker of translation at night when N.A.T. levels were high only caused a slow decline in the level of N.A.T. activity in contrast to the the abrupt decline in N.A.T. activity caused by light at night. Brownstein interpreted these differences as indicating that the effect of light must result from disaggregation, inactivation or proteolytic degradation of N.A.T.. Further investigations using actinomycin D, a blocker of transcription, suggest that the rise in N.A.T. activity associated with lights off also involves transcription as well as translation (Brownstein 1977).

When the level of N.A.T. activity was lowered by turning on the lights at night and N.A.T. activity reinduced by the application of B-adrenergic stimulation, the rise in the level of activity is more rapid than that normally observed, suggesting that that this may occur because adequate levels of messenger R.N.A are already be present in the gland and the normal lag time during which messenger R.N.A. is synthesised may not be required (Strada et al 1971).

Together with the other data described in this section these results indicate that light not only determines

whether an elevated level of N.A.T. activity and hence melatonin production can occur but also determines the time during the daily cycle when this can occur.

1.3.3 Pathways involved in the neural control of pineal rhythms.

The effect of the light dark cycle on the circadian rhythm of pineal activity is so dramatic that it was originally thought that pineal activity was a passive response to darkness. It has since been proposed that light has its effect on the pineal gland via a pathway involving the retina, the retinohypothalmic tract, the suprachiasmatic nucleus, and the superior cervical ganglia. Comprehensive reviews of the neural pathways involved in the regulation of pineal rhythms have been undertaken by Kappers (1965), Ueck (1979) and Klein (1985).

Evidence that the R.H.T. (retino-hypothalmic tract) which leaves the optic nerves at the level of the optic chiasm and terminates in the S.C.N. is involved in mediating the effects of light on pineal activity has been provided by Moore and co-workers who traced this pathway by monitoring the movement of radioactive amino acids injected into the eye (Moore and Lenn 1972, Moore 1973). Studies by Zucker et al (1976) and Rusak (1979) have shown that lesions which did not impinge directly on the R.H.T could also effect certain aspects of circadian activity rhythms. These findings suggest that pathways other than the R.H.T. may be involved in the entrainment of circadian rhythms.

1.4 Pineal involvement in seasonality in the sheep

1.4.1 The annual cycle of reproduction in the sheep

Under constant daylength the sheep goes through cycles of reproductive competence and incompetence with a period of approximately one year (Howles et al 1982, Ducker et al 1973) suggesting control by some endogenous mechanism. Support for this view is provided by the finding that the transition from the non breeding to breeding state takes place in the same time interval whether animals are held on long days or experience naturally shortening daylength (Robinson et al 1985). Similarly the reverse transition is not delayed by continuation of short day treatment (Robinson and Karsch 1984). This ability of animals to undergo changes in reproductive status contrary to that expected due to the prevailing photoperiod has been termed photorefractoriness and is attributed to the existence of an endogenous mechanism which controls the annual cycle of reproduction in the sheep.

The reproductive cycle in the sheep can however be experimentally manipulated by appropriate changes in photoperiod. D,Occhio et al (1984) have shown that the annual cycle of reproduction can be compressed into 24 weeks by alternating short and long photoperiods for 12 weeks at a time. If the pineal is removed however the cycle of reproduction can no longer be driven by changes in photoperiod (Bittman et al 1983).

1.4.2 Pinealectomy and melatonin infusion experiments in sheep.

The initial experiments investigating the effects of pinealectomy on seasonal breeding in the sheep failed to provide the expected evidence that the pineal was playing a major role in the regulation of seasonal breeding in this species (Roche et al 1970, Seamark et al 1981, Kennaway et al 1983, Barrel and Lapwood 1979). This was surprising since oral administration and injections of melatonin had both been shown to influence the timing of breeding activity in the sheep (Kennaway et al 1982, Nett and Niswender 1982).

The dilemma was resolved when it was realised whilst the annual rhythm of reproductive competence in the sheep is not altered by pinealectomy and that pinealectomised sheep cannot respond to a change in photoperiod (Kennaway et al 1982 and Bittman et al (1983). Bittman et al (1983) for example, pinealectomised two groups of sheep and subjected them to alternating photoperiods of L:D 18:6 and L:D 8:16. One group which was left intact was readily manipulated by the changes in photoperiod whereas pinealectomised animals were not. Although pinealectomy altered the animals ability to respond to changes in photoperiod, these animals still showed seasonal changes in reproduction which coincided with that of pineal intact animals housed outdoors. Subsequently it was shown by Yellon et al (1985) that the appropriate infusions of melatonin into pinealectomised animals could drive the neuroendocrine axis in the same manner as changes in photoperiod. This was most powerfully demonstrated in an experiment where an infusion of the short day pattern of melatonin elicited a short day response even though the

pinealectomised animals were exposed to long days (Yellon et al 1985).

These findings suggest that the endogenous mechanism controlling the annual cycle of reproduction survives pinealectomy but requires the presence of an intact pineal to be successfully driven by changes in photoperiod.

1.4.3 Melatonin Secretion in Sheep

The complex mechanisms controlling melatonin secretion have principally been studied in rodents and while direct experimental evidence is lacking it is likely that they are similar in the sheep.

In natural lighting conditions the sheep exhibits a steep rise in melatonin levels following lights off and an equally steep fall following lights on so that melatonin secretion is usually continuous during the dark phase (Kennaway et al 1983, Rollag and Niswender 1976). During the night the secretion of melatonin is highly pulsatile with no consistent peak making it difficult to identify a single time as the point of maximum concentration (Rollag and Niswender 1976).

It has been suggested that under short days the pattern of melatonin secretion exhibits two peaks while under long days only one is present (Arendt et al 1981). However in more detailed studies of the melatonin profiles of groups or of individual sheep there is little evidence to support this view (Kennaway et al 1983) and given the natural pulsatility of melatonin secretion and the infrequent sampling period (3 hours) used in Arendt et al (1981) study it is not surprising that a number of peaks were found.

When the melatonin profiles of Corriedale ewes were monitored during different seasons of the year it was found that the period for which melatonin concentrations were elevated accurately reflected the the length of the night throughout the year (Rollag and Niswender 1978). Studies using merino cross ewes under controlled photoperiods also show a similiar association between the time their melatonin is elevated and the duration of the dark phase (Kennaway et al 1983). There is one study using Soay sheep where little difference was found between the duration of melatonin secretion under short and long days (Lincoln et al 1982).

The melatonin profiles of Suffolk Greyface cross sheep exposed to short (L:D,8:16) or skeleton long (7L:10D:1L:6D) were investigated by Brinklow et al (1984). They found no difference between the mean melatonin levels between the two photoperiods supporting the findings of Linclon et al (1982) who found no difference in melatonin levels between sheep held on L:D,8:16 and L:D,16:8. They also found that the levels of melatonin secretion after the light pulse were substantially reduced.

The effect of a light pulse in the latter part of the night (7L:9D:1L:7D) on melatonin profiles, prolactin levels and ovulatory activity has also been investigated in lle-de France ewes by Ravault and Thimonier (1988). Their results indicated that the light pulse given 16 to 17 hours after the onset of the main light phase led the animals to interpret this photoperiod as long days. The light pulse induced a decrease in melatonin levels in all animals followed by a subsequent increase in 6 of the 8 ewes studied. It is of interest that the 6 ewes which had a total duration of melatonin secretion of approximately 15 hours still

interpreted the photoperiod as long days. The authors suggest that this finding supports the hypothesis that periods of melatonin secretion throughout the day are more important than the total duration of melatonin secretion in determining photoperiodic resoponses. The duration of the longer period of melatonin secretion may still however play an important role in conveying the photoperiodic message.

In a further experiment Ravault and Thimonier (1988) investigated the effect of a 4 hour light pulse coupled with either 8, 20, 32 or 44 hours of darkness in what is known as a resonance experiment (see 3.1.3). Under these lighting regimes all groups showed a 24 hour rhythm in melatonin secretion supporting the hypothesis that melatonin secretion in sheep is controlled by a circadian pacemaker. Prolactin levels under 4L:8D, and 4L:32D indicated that these two photoperiods were interpreted as long days whereas 4L:20D and 4L:44D, the two photoperiods which were multiples of 24 hours were interpreted as short days.

1.4.4 A Circadian rhythm of melatonin secretion in the sheep

The persistence of a rhythm in melatonin secretion in sheep held in darkness to reduce photic input was first imputed by Rollag and Niswender (1976). More detailed studies of the rhythm in melatonin secretion under continuous darkness have been reported by Lincoln et al (1985). In one study animals previously entrained to L:D,16:8 were placed in continuous darkness for 10 days and plasma melatonin concentrations monitored on days 1-3 and on day 10. Using the onset of melatonin secretion as a phase reference point and

deriving the period of the rhythm from the time between the onset of successive peaks it was found that the the period of the rhythm on days 1-3 was 24.3 ± 1.2h and 23.0 ± 0.4 hours from days 3 to 10. It was also reported that the duration of the melatonin peak on day 1 was not significantly different from that under constant darkness on day 10.

Further evidence of the circadian nature of the melatonin rhythm was provided by Almeida and Lincoln (1982). In this study the authors housed rams on either L:D,8:40, L:D,8:28 and L:D,16:8. Clearly defined melatonin rhythms were observed under all photoperiods except L:D,8:28. Under L:D.8:40 animals showed a circadian pattern of melatonin secretion demonstrating the circadian nature of the pacemaker controlling the rhythm. Under L:D,8:28 melatonin concentrations appeared to be continuously high.

In a further experiment rams previously maintained on L:D 16:8 were moved from a 25h (L:D,16:9) light dark cycle to a 23h (L:D,16:7) light dark cycle. This was achieved by varying the length of the dark period from 9 to 7 hours. The results of these studies showed that the pacemaking system controlling melatonin secretion in the sheep could readily adapt to 23 or 25 hour cycles. Under both of these photoperiods the period of melatonin secretion coincided with the dark period.

Karsch et (1986) found that intact Suffolk ewes maintained on short days became refractory even though there melatonin profiles reflected that of short days. Pinealectomised (Px) ewes receiving a short day infusion pattern also exhibited photorefractoriness (Karsch et al 1986). In the Soay ram however the phenomenon of photorefractoriness has been associated with a disruption of

the melatonin rhythm (Almeida and Lincoln 1984). In their study the previously observed coupling between the melatonin rhythm and the light-dark cycle was weakened or lost after 21 weeks in constant photoperiod.

Robinson (1985) has demonstrated that the interpretation of a particular photoperiod may depend on the animals recent photoperiodic history. In an experiment in which ewes were transferred to L:D,13:11 after exposure to either short days (L:D,10:14) or long days (L:D,16:8), animals previously exposed to long days interpreted L:D,13:10 as short days and those previously exposed short days interpreted L:D,13:10 as long days. It is of interest that both groups of animals displayed similar melatonin profiles under L:D,13:10 in this experiment providing us with the only example in any species where animals with the same melatonin profile interpret a photoperiod differently. The daylength sheep experience will determine the duration of melatonin secretion they exhibit in most cases but how this duration in interpreted may depend on what stage of its annual reproductive that animal is at. Further studies in this area may help elucidate the role of the duration of melatonin secretion in conveying the photoperiodic message.

1.5 Photoperiodic time measurement

1.5.1 Introduction

Previous research into P.T.M. has demonstrated that the circadian system is fundamentally involved in the process by which animals measure the length of day. Evidence supporting this hypothesis is supplied by experiments known as " T " experiments and resonance light cycle experiments.

1.5.2 T Experiments

A "T " experiment is a series of experiments in which the period of the entraining signal is varied through a range extending from several hours less to several hours greater than 24 hours and the effect on a rhythm such as the activity rhythm and on the reproductive axis noted. In these experiments the rhythmic activity indicates how the animals have responded in order to remain entrained to each light cycle. If enough experiments are conducted it is possible to alter the relationship between the light pulse and the activity rhythm and observe at what stages of the activity rhythm the light pulse causes the reproductive axis to be stimulated. In this manner the sensitive phases of the activity rhythm to the effects of light pulses have been mapped.

An example of a T experiment has been provided by Elliott (1976). In this experiment he found that a 1 hr pulse of light delivered every 24 hours caused testicular regression and a 1 hr pulse every 23.34 hrs completely blocked testicular regression. To explain this phenomenon, reference has to be made to the process by which an

animal must arrange its circadian cycle so that the pulse occurs at that time which gives the appropriate advance or delay necessary for entrainment. In the example provided by Elliott, a T of 23.34 results in a pulse occuring at the end of the activity bout. With a T of 24.67 activity occurs immediately after the pulse. Under a T of 24 the pulse occurs some time after the end of activity. These findings demonstrate that in achieving stable entrainment under different photoperods the relationship between the light pulse and the phase of the activity rhythm is systematically varied. The finding that pulses occurring at slightly different periods to 24 hours could have such markedly different effects on the reproductive system highlighted the importance of 24 hour cycles in photoperiodic time measurement. A similiar experiment conducted with weanling male deer mice, (Peromyscus maniculatus), produced the same result (Underwood et al 1985). These experiments demonstrate that pulses of light at different times of the activity rhythm have different effects on P.T.M.. These results have been interpreted to support the hypothesis that a circadian rhythm of sensitivity to light is involved in photoperiodic time measurement.

1.5.3 Resonance light cycle experiments

In resonance light cycle experiments a constant duration of light is coupled with varying periods of darkness and the effect on the reproductive axis monitored. The intent of these experiments is to determine the importance of the duration of the light pulse and the circadian time of its

occurrence on the photoperiodic time measuring system. An example of such an experiment has been provided by Elliott et al (1972). In this experiment a 6 hour light pulse was coupled with dark periods of 18 hrs, 30 hrs, 42 hrs and 54 hrs. Those light cycles which were a multiple of 24 hrs were all interpreted as short days whereas those which were not were interpreted as long days. In the hamsters exposed to 24 hr cycles the active phase of the animals was never coincident with the light phase. This may explain why these photoperiods were interpreted as short days as the sensitive period to light is thought to occur during the active phase.

Results of experiments such as this clearly indicate that it is not the duration of light or darkness which determines the response but that P.T.M. involves a response to light which varies on a circadian basis.

1.5.4 The Bunning hypothesis

One of the first attempts to explain how the circadian system is involved in P.T.M is attributed to Bunning (1960). In this hypothesis Bunning has proposed that the animal posseses a circadian rhythm of sensitivity to the effects of light. If light is coincident with the sensitive portion then a different response is predicted than when light is not coincident with this phase.

In this model light plays a dual role. Firstly it entrains the rhythm of sensitivity and secondly its coincidence or noncoincidence with the sensitive portion of the circadian cycle determines the response.

1.5.5 Internal and External Coincidence Models and the Resonance model

Pittendrigh has referred to the Bunning hypothesis as an external coincidence model because it relies on the coincidence of an external stimulus, light, with an an endogenous sensitive portion of the circadian cycle (Pittendrigh 1972). Pittendrigh and Minis (1964) have concluded that " the only safe generalisation that can be made is that the amount of induction is a function of the steady entrained state of the circadian organisation: in some steady states, induction is maximal ; in others it occurs to a limited extent ; in still others no induction occurs." As Pittendrigh points out, this generalisation still accomadates the external coincidence model since it is only in some entrained steady state that the postulated photoinductible phase of the circadian cycle will normally coincide with light. This generalisation also accomadates many other interpretations one of which has been termed the "internal coincidence model." In this model it is recognised that multicellular organisms contain a population of circadian oscillations whose mutual phase relationships may have an effect on physiological function. This model suggests that a change in photoperiod may change the entrained steady state of the multioscillator system so that under some photoperiods constituent oscillators would be phased in a manner which resulted in induction whereas under other photoperiods this would not occur.

A third possible way in which P.T.M. may be achieved has also been discussed by Pittendrigh. In this theory the

physiological response of an animal is thought to depend on the degree of resonance an animal is experiencing with its entraining cycle. An example of how the degree of resonance can effect the physiological state of an animal has been provided by Went (1960) who showed that growth rate and total growth were maximal when organisms were driven with an external cycle close to that of their endogenous period and impaired when this period was altered from this period. Pittendrigh and Minis (1964) also found that the longevity of Drosophila melanogaster was maximal under 24 hour cycles and lowered if they were driven by cycles of 21 or 27 hrs.

Pittendrigh makes the point that nowhere in the current discussion on photoperiodism has this concept received much recognition.

1.6 Models of Pineal involvement in P.T.M.

1.6.1 Rollag's model

On the basis of their studies with sheep, Rollag and Niswender (1978) have proposed an external coincidence model in which light both determines when melatonin will be produced and also entrains a rhythm of sensitivity to the presence of melatonin. If there is coincidence between the period of production of melatonin and the sensitive period to the presence of melatonin then a short daylength response is predicted. If however melatonin is not present during the melatonin sensitive period then a long daylength response is predicted. Furthermore it is suggested that the relationship between the rhythm of sensitivity and the period of melatonin

production will be systematically altered as daylength changes throughout the year. Thus on short days the period of melatonin secretion may cover the sensitive portion of the cycle causing a short day response, whereas under long days the short duration of melatonin may not, resulting in a long day response. The effect of melatonin injections prior to dusk can easily be explained using this hypothesis since these may cover the sensitive period which was not previously covered by endogenously produced melatonin on that photoperiod.

1.6.2 Carter and Goldman's model.

The results obtained by researchers in Goldman's laboratory using hamsters have been summarised above in section (1.1.5.). On the basis of these results they have questioned the hypothesis put forward by Rollag and Niswender (1978). They suggest that the results of their infusion experiments show that it does not matter at what time of the daily cycle the melatonin is present as long as the period for which it is elevated exceeds a certain critical duration (Carter and Goldman 1983, Goldman et al 1984).

They propose that the photoperiodic responses of this species are dependent on the duration of melatonin to which the animal is exposed and that short day responses are elicited when the pulse duration exceeds a threshold duration and long day responses when the duration is less than this critical duration. In this model the duration of melatonin secretion is determined by an interaction between the

photoperiod to which an animal is exposed and the circadian oscillators which control the rhythm of melatonin secretion.

In both of these hypotheses the circadian secretion of melatonin plays a central role yet very little is known about the way in which the light dark cycle and the mechanisms controlling melatonin secretion interact to produce a particular melatonin signal.

1.7 Biological Pacemakers

1.7.1 Introduction

Using surgical and electrolytic lesioning techniques it has been possible to determine putative physiological mechanisms by which higher organisms process information from the external environment. The linkages between these mechanisms and the biochemical and neural pathways involved in the regulation of melatonin secretion have been reviewed in a previous section. Little however is known about the nature of the pacemaker which is responsible for the generation of the melatonin rhythm.

In this section some of the findings and problems confronting researchers studying the nature of biological pacemakers are provided and have been discussed so that the the problems involved in trying to improve our understanding of the nature of the pineal pacemaker can be appreciated.

Reference to some existing models has been made to illustrate certain points.

1.7.2 Terminology

The literature on circadian physiology continually refers to pacemakers, oscillators and biological clocks, terms which are poorly defined in the literature. Pittendrigh however provided us with the following definition of the term pacemaker. " It is an undamped self sustaining oscillation in the control of activity and rest whose formal properties have functional meaning; they are clocklike" (Pittendrigh and Daan

1976). This implies that the terms biological clock and pacemaker are interchangable. The term oscillator differs in that the rhythm which it controls need not behave in a clocklike manner. There are however examples in the literature where the term oscillator has been used to describe rhythms which do show clocklike behaviour. An example of this is seen in the the two oscillator model described by Illnerova and Vanecek (1982) which will be discussed in detail in a later section. The important point about these definitions is that they tell us nothing about the nature of the mechanisms which generate rhythmicity, they merely describe the type of activity observed.

1.7.3 Pacemaker Complexity

Circadian physiologists investigate how neural tissue is organised to generate circadian signals, how these circadian signals are coupled to the circadian rhythms which they drive and how environmental agents such as the light dark cycle entrain or synchronise circadian pacemakers. To answer these questions detailed studies of the behaviour of circadian rhythms under a range of photoperiodic treatments have been made. Circadian physiologists who attempt to gain an understanding of the nature of biological pacemakers by observing overt rhythmicity however face a fundamental problem which is succintly summarised in the following way by Pittendrigh and Daan (1976). "Our point is simple, we cannot assume that functional singleness implies anatomical or structural singleness." In this statement Pittendrigh and Daan stress the point that although a rhythm may appear to

behave as if controlled by a simple pacemaking system there is no means of determining whether the neural output which controls the rhythm is from a simple pacemaker or is a simple output from a very complex pacemaking system. The possible complexity of pacemaking systems is demonstrated by models described by Moore-Ede et al (1976). Very simply their models suggest that there may be one, two or more central pacemakers and that these may act independently or interact with each other. These pacemakers then may control rhythmicity in a network of cellular systems which themselves may or may not be capable of sustaining oscillations in the absence of any outside input. To add further to the complexity, these cellular systems may also interact with each other.

A simple example of the problems this may provide for the circadian physiologist is found in the work of Earnest and Turek (1982). This study presents the activity rhythm of a hamster maintained on continous light for 174 days. For the first 92 days the hamster showed a single rhythm suggesting that the rhythm was controlled by a single pacemaker. After this period the rhythm was observed to split into two distinct components which each free-ran with the same period but with a period different from that previously observed for the single rhythm. There are numerous examples of similar behaviour in a number of species which has led Pittendrigh (1960) to state "There are now many cases where a free-running system gives evidence of comprising more than one component with different characteristic frequencies. Such examples are provided by the study of body temperature rhythms in hibernating bats and the effects of constant light on rhythms in mice (Mrosvosky and Hallonquist 1986,

Pittendrigh 1960).

The possible complexity of circadian pacemakers would seemingly make it impossible to gain any understanding of their nature by merely observing overt rhythmicity. This means that even when there is an enormous amount of data available as there is for behavioural rhythms there is a problem of determining which particular model provides the best explanation of events. This difficulty arises in part because even a simple pacemaker can be responsible for quite complex behaviour as is demonstrated by reference to a single pacemaker model defined by Wever (1965).

1.7.4 Wever's Single Pacemaker Model

Wever (1965) suggested a second order differential equation could be used to explain the behaviour of circadian rhythms. The oscillation defined by this equation obeys simple mathematical laws and is self sustaining. The organism is assumed to be active when the oscillation is above a certain threshold and at rest when below this threshold. Therefore the ratio of duration of activity to rest is determined by the level of the threshold. Under constant conditions the rhythm stabilises into a sine-wave with the times of onset of activity determined by the point of intersection of these two functions. Wever also explains the behaviour of this oscillation under the conditions of entrainment to different photoperiods and different light intensities. The results show the various shapes the oscillation would have under these various conditions. These changes would result in major differences between the

relationship between the lights off and on and the onset and offset of activity due to the strength of the entraining agent and the length of the photoperiod even with a nonvarying threshold.

Consideration of Wever's model makes two important points. Firstly it raises the important question about the suitability of onset and offset as markers for the phase of pacemakers since they can both be influenced by the level of the threshold.

Secondly it highlights the fact that even using simple mathematical models can produce a range of possible outcomes in rhythmic behaviour.

Wever has also stated that a model based on a third order differential equation could provide more variations including activity patterns with two peaks. Where Wever (1965) has started with a mathematical model and shown that it could with variations explain many of the observations made on the behaviour of circadian rhythms, other researchers have addressed the problem of determining the nature of circadian pacemakers in another manner. This approach is best demonstrated by reference to a two pacemaker model proposed by Pittendrigh and Daan (1976).

1.7.5 Multioscillator models for the control of circadian rhythms.

Aschoff (1960) was one of the first to observe that the activity rhythms of many species appeared to be made up of two components, one occuring around the beginning of the active phase and another around the end of the active phase.

These and other observations have led to the development of hypotheses which suggest that circadian rhythms may be controlled by pacemaking centres which contain more than one pacemaker.

The most compelling evidence to support this hypothesis is provided by those examples where various components of a circadian rhythm free-run with different periods when held under constant photoperiodic conditions. This phenomenon, termed splitting, has been demonstated in the golden hamster (Pittendrigh 1974), the tree shrew (Hoffman 1971), lizards (Underwood 1977) birds (Gwinner 1974). Pittendrigh and Daan (1976) also claim that the phenomenon of splitting and refusion are not readily explained by any pacemaker model based on a single oscillator but can be explained by reference to a system which involves two mutually coupled oscillators.

They have also suggested that models should be judged by two criteria. Firstly by their ability to explain the facts and secondly by their usefulness in advancing the analytical process. Their model which is successful in satisfying both these criteria is described in detail below.

1.7.6 Pittendrigh and Daans Two Pacemaker Model

In this model one oscillator which they have termed M is thought to control the morning component of activity and the other E is thought to control the evening component. The intrinsic periods of E and M, that is their periods when they are not influencing each other have been labelled tE and tM respectively. The model rests on four fundamental

propositions. The first of these states that the intrinsic periods of E and M react differently to light intensity with tE being a positive function of light intensity and tM negative. The second proposition is that when E and M are in the coupled state the period of the compound pacemaker will differ from tE or tM. The third proposition is that the relative influence of each pacemaker on the other will depend on the distance between E and M. The final proposition is that under certain circumstances two distinct coupling modes are possible. These modes correspond to the split and unsplit states observed in many activity rhythms. In this model any factors which can influence tE, tM or the interaction between E and M can have significant effects on the period a rhythm displays and can also cause it to alter its coupling mode and cause it to split or if already split, to refuse.

Pittendrigh and Daan (1976) have suggested that this model could provide a basis for some of the more unusual features of circadian rhythms, such as the circadian rule. This rule which was originally described by Aschoff specifies that for an activity rhythm, ratio of activity to rest and total amount of activity increases with increasing light intensity in diurnal animals and decreases in nocturnal animals (Aschoff 1960). Pittendrigh and Daan (1976) suggest that changes in light intensity would alter tE and tM in an opposite manner, either forcing E and M closer together or further apart, resulting in a change in the manner in which E and M interact. In their model, this change in the interaction component would ultimately result in a change in the period of the rhythm thereby explaining the effects of a change in light intensity on the period of rhythmic activity.

They contend that this model is also able to explain the feature of circadian rhythms termed the "after effects of photoperiod " an example of which is provided by experiments conducted with the white footed deermouse (Pittendrigh and Daan 1976). In this species the period of the freerunning activity rhythm is longer after exposure to a long photoperiod than a short photoperiod. Pittendrigh and Daan (1976) explain these observations by suggesting that exposure to photoperiods of different length will influence the manner in which E and M interact and on release into LL or DD differences will persist, at least initially and result in the observed after effects.

The model proposed by Pittendrigh and Daan (1976) has been developed and tested using computer modelling by Daan and Berde (1978). The model developed by Daan and Berde (1978) makes no assumption about the amplitude of the oscillation but is only concerned with the onset and offset of activity. In this model E and M can be assigned different periods and the degree of coupling can be programmed into the model. When different conditions were programmed into the model the results were very similar to that observed experimentally. Most convincingly the conditions which resulted in splitting in the golden hamster under laboratory conditions also produced splits when programmed into the model. Similarly, after effects of exposure to different photoperiods were also observed using this model.

The two pacemaker model as refined by Daan and Berde (1978) thus a provides a basis for a more plausible explanation for some of the more unusual behaviour of circadian rhythms.

The above discussion makes the point that pacemaking systems which operate to generate circadian rhythmicity may be extremely complex or relatively simple. If they are of a very complex nature then our chances of gaining an understanding of their nature by studying overt rhythmicity are probably small. If however they are of a simpler nature then the chances are much better. The finding that the simple two pacemaker model of Daan and Berde (1978) can explain not only the the normal behaviour but the unusual behaviour of circadian rhythms is encouraging.

1.8 The Role of the Suprachiasmatic Nucleus in Mammals

1.8.1 Introduction

The physiological mechanisms through which the suprachiasmatic nucleus (S.C.N), the putative site of the biological clock behaves to generate circadian rhythmicity is a subject of active research. Evidence identifying the S.C.N. as the location of the pacemaking centre has been demonstrated by Moore (1979) who demonstrated that bilateral lesions of the S.C.N. abolish the circadian rhythm in N.A.T in the pineal. Previously Nishino et al (1976) had linked the S.C.N. with the pineal thtrough their finding that light excites certain groups of neurons in the S.C.N which exert an inhibitory action on the cervical sympathetic nerves resulting in a reduction in N.E. release by nerve fibres innervating the pineal. These and other findings have shown that the S.C.N are as important in the generation of circadian rhythms in pineal function as they are for behavioural rhythms. This assumption is further supported by those studies which show that activity rhythms and pineal rhythms appear to be linked (Yellon et al 1982, Tamarkin et al 1980).

Anatomically the S.C.N. is a complex nucleus comprised of dense accumulations of small neurons lying dorsal to the optic chiasm and lateral to the third ventricle (Gurdjian 1927, Guldner 1976). Present data indicates that the bilaterally paired nuclei have at least two subdivisions (Soroniew and Weindl 1982). These consist of a small rostral component and a larger caudal component which contains a

dorsomedial and a ventrolateral division. Several detailed reviews of the structure, connections and cell types present in the S.C.N. have been published (Guldner 1976, Soroniew and Weindl 1982, Groos 1981).

1.8.2 Circadian Rhythmicity Within the S.C.N.

Evidence supporting the hypothesis that the S.C.N. is fundamentally involved in the generation of circadian rhythms is provided by two main areas of research. The first area is those experiments which demonstrate that the S.C.N. is a unique area of the brain in which endogenous rhythmicity exclusively occurs. This is best demonstrated by studies conducted by Inouye and Kawamura (1979) who showed that a rhythm in neural multiple unit activity persists in blinded animals in sections of the hypothalamus containing the S.C.N. which have been neurally isolated from the rest of the brain. Rhythmicity within these sections has been shown to be dependent on afferent inputs from elsewhere in the brain. Additional compelling support is also provided by studies which showed that a circadian rhythm of glucose utilisation existed in the S.C.N. but not elsewhere in the brain (Schwartz et al 1980).

Evidence that the S.C.N. plays an important role in the regulation of circadian rhythms is provided by studies which show that electrical stimulation applied directly to this area give similar results as exposure to light with stimulation early in the active phase causing delays and stimulation late in the active phase, advances (Rusak and Groos 1982).

The results of the experiments cited above clearly reveal the S.C.N is a unique area of the brain which can generate rhythmic activity independently of neural input from other centres.

1.8.3 Lesions of the S.C.N.

The second area of research which supports the hypothesis that the S.C.N. are involved in the generation of circadian rhythms is provided by studies of the effects of lesions of the S.C.N. on circadian rhythms. Initial studies by Richter (1967) reported that hypothalmic lesions eliminated rhythms in eating, drinking and activity in rats. Moore and Eichler (1972) and Stephan and Zucker (1972) subsequently found that total destruction of the bilaterally paired S.C.N. resulted in the abolition of the circadian rhythms of drinking behaviour, locomotor activity and adrenal corticosterone secretion in rats. Furthermore S.C.N. lesions disrupt other events known to have a circadian component such as those events which involve photoperiodic time measurement (Rusak and Morin 1976, Turek et al 1980, Turek and Pickard 1981).

1.8.4 The nature of pacemakers in the S.C.N.

Whilst recognising that circadian pacemakers are present in the region of the S.C.N. and also that they control circadian rhythms in activity behaviour, hormonal levels and also the rhythm in pineal activity the results of several studies suggest that other areas outside of the S.C.N may also play an important role in the generation of circadian

rhythms. In studies conducted by Rusak (1977a, 1979), hamsters with lesions to the S.C.N. generated rhythmic components which broke away from stable entrainment and sometimes free ran for several cycles and occasionally reentrained. When maintained under constant conditions the activity component often showed circadian rhythmicity for several cycles. This suggests that there must be some component of the pacemaking system which resides outside of the S.C.N. which survives lesioning. This finding is also supported by experimental work conducted with the Squirrel monkey. In the Squirrel monkey maintained in constant light S.C.N. lesions disrupted the rhythm of drinking behaviour but did not alter the rhythm of core body temperature (Fuller et al 1981). These findings suggested that the pacemaker controlling the rhythm in core body temperature may reside outside of the S.C.N..

Rusak (1979) has also reported that some S.C.N. lesioned hamsters generated rhythmic components that were entrained for some periods and free-ran for other periods. Others continued to show circadian or ultradian periods of activity over many cycles. He hypothesised that circadian pacemakers whether inside or outside the S.C.N. were still operating in S.C.N. lesioned hamsters but that they were no longer interacting to form an organised output. Rusak has suggested that there may be a multitude of self sustaining oscillators and that the S.C.N. may contain a large proportion of the oscillator population. In this suggestion the S.C.N. may normally maintain synchrony among these oscillators but when the S.C.N. are lesioned the level of mutual coupling may be insufficient to keep all of the elements in phase. The

influences of these smaller sub populations of activity is assumed to account for the presence of components that transiently free-run synchronise and sometimes merge. A similar possibility has been expressed by Pittendrigh (1976) in the statement "The observed frequency of the individual must be a compromise of a spectrum of frequencies that would be individually manifest if the constituent oscillations could escape entrainment from the rest of the system and freerun." The coupling mechanisms that bring about the complex of mutual entrainments must involve discontinuities making a range of system frequencies realizable.

Other research has shown that unilateral lesions induce changes in the period of activity rhythms but the rhythm is still maintained (Pickard 1981, Pickard and Turek 1983). In hamsters displaying split activity rhythm, unilateral lesions abolish one of the rhythmic bouts of activity.

On the basis of these findings Turek (1985) has concluded that each S.C.N. has the capacity to maintain circadian rhythmicity and that under normal conditions there is interaction between the bilaterally paired nuclei in the generation of the circadian rhythm of activity.

The main object of lesion studies has been to determine the precise location of circadian pacemakers not only within the brain but also within the S.C.N.. However as Moore (1983) suggests the elimination of a circadian rhythm after the lesioning of an area does not prove that the endogenous circadian oscillator was located in that area, since the lesion may only have severed neural output from a pacemaker located elsewhere.

The results of such experiments have been reviewed by Turek

(1985) and he states that " the key question - how are circadian rhythms generated remains unanswered." He speculates that " the generation of circadian signals may depend on the interaction of a large number of neurons with a specific structure " or "alternatively ,circadian rhythmicity may be a property of individual cells within a pacemaker , and the interactions between the cells may serve only to achieve overall synchronisation." These conclusions demonstrate our lack of knowledge of the basic physiology responsible for the generation of circadian rhythms.

The section above demonstrates not only our lack of knowledge in this area but also that until we can directly investigate the biochemical processes which act to generate circadian rhythmicity, all of our hypotheses concerning the nature of these pacemakers will be of a speculative nature.

1.8.5 The effects of melatonin on the S.C.N.

Steps forward in our understanding of the biochemical processes which may be acting to control circadian rhythmicity have been made by researchers investigating the effects of melatonin on the S.C.N. and on free running circadian activity rhythms.

Redman, et al (1983) and Armstrong et al (1986) have shown that daily injections of melatonin (1mg/kg) entrain the free running circadian activity rhythms of adult male Long-Evans rats held in constant darkness such that entrainment occurs when the onset of activity coincides with the time of day at which melatonin is injected.

Armstrong et al (1987) have also shown that single injections of melatonin (50ug/kg) phase advance the activity of free-running rats when given 1-3 hours before activity onset. Cassone et al (1988) suggest that these findings are consistent with the hypothesis that melatonin entrains free-running rats by daily phase advances. Other lines of evidence that the S.C.N. are a site for the entraining effects of melatonin are provided by the finding that surgical ablation of the S.C.N. prevents entrainment to injections of melatonin (Cassone et al 1986) and the observation that the S.C.N. specifically binds radio labelled melatonin ligands (Cardinali et al 1979, Niles et al 1979, Vanecek et al 1987). Studies of the effects of exogenous melatonin on the uptake of 2-deoxy-glucose also indicate that the effects of melatonin also depend on the time of day of administration (Cassone et al 1988). At this stage however it is not clear whether the effects of exogenous melatonin described above reflect a role for endogenous melatonin produced by the pineal gland since pinealectomy has little or no effect on rat free-running locomotor rhythms (Cheung et al 1982, Quay 1968). Pinealectomy has been observed to alter the apparent rate at which animals re-entrain to phase shifts (Armstrong et al 1985, Finkelstein et al 1978) and this has been interpreted as indicating that pinealectomy may alter the coupling between endogenous oscillators (Armstrong et al 1985, Chesworth et al 1987). Alternatively it has been suggested that similar effects would be observed if pinealectomy increased the subjective sense of light intensity (Aschoff et al 1982).

It is known that the activity of the pineal gland is controlled by neural signals emanating from the S.C.N. The further findings that the resultant production of melatonin may alter the behaviour of the S.C.N. suggests a familiar feedback self regulating system employed by other biological systems. A clear understanding of the biochemical processes and the resultant physiological outcomes of this system would provide a significant step forward in our understanding of circadian control systems.

1.9 The pacemaker which controls the level of pineal activity

1.9.1 Introduction

In the pineal literature there are two models for the pineal pacemaker which are commonly referenced. They are the "clock-gate" model and the two oscillator model.

1.9.2 The "Clock-Gate " Model

Lewy (1983) has proposed a single oscillator model for the control of pineal activity which he has titled the "clock-gate "model. It was so termed because in this model the internal circadian pacemaker controlling neural input to the pineal acts as a clock regulating the time when melatonin production can occur and the light dark cycle acts as a gating mechanism only allowing melatonin production to occur when the animal is in darkness. In this model light acts to entrain the pacemaker controlling neural input to the pineal so that under normal photoperiods this occurs during the dark period of the daily cycle. If animals are subjected to a change in photoperiod the phase of the pacemaker is gradually adjusted so that neural stimulation again occurs during the dark period of the daily cycle.

A lag thus occurs between the onset of neural stimulation and the time when melatonin is actually produced. It is postulated that this time is required for the biochemical processes which occur between release of N.E. and production of melatonin to occur. Clock on represents the time when the pacemaker is capable of sending neural stimulation to the

pineal and the period during which the clock is on is assumed to be constant. The gate, is the period of time between dusk and dawn during which melatonin secretion can occur. Melatonin secretion commences when the clock is on, the gate is open and the lag time has passed. Another feature of this model which Lewy has included to explain observations on the timing of pineal activity in the rat is that the phase of clock on may be changing in relation to middark. This feature was included to explain the much greater delay between dusk and onset of pineal activity under short photoperiods compared with long photoperiods.

This model successfully explains many of the observations made of pineal activity. It however does not in this form allow for any differences in the duration of pineal activity under extended darkness an observation made in animals which have previously been entrained to photoperiods of different lengths (Illnerova and Vanecek 1982).

In discussing this point with Dr. Lewy he suggested that the time of clock-on might be determined by the pacemaker and that the time of clock-off may be determined by the depletion of enzyme. Dr. Lewy has put forward this hypothetical model as a valuable basis for discussing neural control of the melatonin rhythm. This model has similarities to Wever's, single pacemaker model but also takes into account the masking effect of light on the melatonin rhythm (Wever 1965). Also the termination of rhythmic activity in Wever's model is determined independently of the time of onset whereas in Lewy's model one may affect the other.

To use melatonin secretion as a marker for the endogenous pacemaker controlling pineal activity careful consideration

must be given to the masking effect of light. Lewy (1983) suggests that on the day of observation the gate must be maximally open. That is darkness must occur well before the expected time of clock on and continue until after the expected time of clock off

1.9.3 A Two Oscillator Model Proposed by Illnerova and Vanecek

Illnerova and Vanecek (1982) have used the two oscillator model proposed by Pittendrigh (1976) as the basis to construct a model for the control of pineal activity . In this model the evening oscillator E, which is entrained by dusk is thought to control the rising phase of melatonin secretion and the morning oscillator M, entrained by dawn is thought to control the falling phase of melatonin secretion. Evidence supporting the two oscillator model was provided by numerous experiments in which rats were given pulses of light at various times of the night. The details of the manner in which these experiments were conducted have already been described in section 1.5.1..

From the data gathered with animals entrained to L:D, 12:12, the authors were able to construct phase response curves for both the rising and falling phases of N.A.T. activity. These phase response curves differ in that the rising phase of N.A.T. activity has only phase delays whereas the morning rise has both advances and delays. Similar experiments were conducted with animals which had been entrained to L:D, 18:6 and L:D, 6:18 before the pulses were applied (Illnerova and Vanecek 1985). The phase response

curves obtained showed that the evening rise showed mostly delays and the morning fall showed both advances and delays. The magnitude of the shifts were however far greater for animals previously entrained to L:D, 6:18.

The findings that the phase response curves for E differed from that of M under the three photoperiods investigated has been used to support the hypothesis that the melatonin rhythm is controlled by a pacemaker comprised of two interacting oscillators.

They suggest that under L:D, 6:18 the distance between E and M may result in less interaction between them. The responses of E and M to pulses of light in this situation will be a closer reflection of phase shifts of E and M since the shift will be less affected by interactions between them. They suggest that as in the model of Pittendrigh and Daan (1976), E and M will interact with degree of the interaction being dependent on the distance between the two oscillators. It is postulated that when E and M are forced closer together as under long photoperiods the greater the interaction will be and that this will result in a greater decompression shown by E and M when they are placed in extended or continuous darkness.

On the basis of the effect of pulses of light on the onset and offset of N.A.T. activity under both short and long photoperiods Illnerova and Vanecek (1982) have drawn some conclusions about the manner in which E and M are entrained by the light dark cycle. Since pulses of light 1h after the onset of darkness causes no delays in the timing of the evening rise under L:D, 6:18 but do under L:D, 18:6 the authors suggest that the timing of the rise in N.A.T.

activity under L:D, 6:18 which occurs 6-7 hrs after lights off is determined by morning light only, whereas under L:D, 18:6 the rise may be influenced by both evening and morning light. Pulses of light 1h before lights on cause advances under both periods. As a result of the above finding the authors suggest that morning light be more important in the entrainment of this rhythm.

They suggest that the position of the morning fall is fixed by dawn and that the position of the evening rise is determined by the period of E and its interaction with M. The finding that there is no further decompression after 1 day in continuous darkness and a shift towards the morning hours supports this view.

It is suggested that under very long photoperiods pulses of light in the middle of the night might interfere with both oscillators causing small advances to the evening oscillator and delays to the morning oscillator. The finding that the evening rise and morning fall did not shift in a parallel manner after the light pulses however might also be explained by reference to a single oscillator model as described by Wever (1965) in which the time of the rise or fall do not move parallel because of differential changes to the threshold or mean value of the oscillation responsible for the rhythm. Thus a pulse of light during the latter part of the night may lower the threshold for the rise leading to a small advance and raise the threshold for the fall sufficiently to cause a large advance. It is also possible that the pulses may disturb the oscillation so that it goes through a number of transient cycles before attaining

stability. Under these circumstances the timing of the rises and falls may only be temporarily be disrupted and show parallel shifts after the rhythm has again stabilised.

1.10 Conclusions

Consideration of the research into the nature of the mechanisms controlling the timing of melatonin secretion highlights the problems encountered in trying to improve our understanding of this system.

The most detailed and encouraging work has been conducted by Illnerova and Vanecek who first demonstrated that through monitoring the onset and offset of pineal activity of rats held under extended or continuous darkness useful information could be obtained on the nature of mechanisms regulating pineal function. However in using small laboratory rodents they were forced to use different animals to determine the level of pineal activity at each time point. This technique is not well suited for longer term studies of pineal activity in continuous darkness because of possible differences in the periods of the pacemakers of individual animals. The sheep is a much better species for this purpose as it allows monitoring of pineal activity over longer periods in the same animal in continuous darkness.

A significant question on the choice of onset and offset of activity as markers for the behaviour of the underlying pacemaker controlling a rhythm has been raised by Wever (1965). In his hypothetical single pacemaker model changes in the level of the threshold can cause changes in the onset and offset of of activity even though no changes in the controlling oscillation had occurred. If his model is correct then onset and offset would be poor choices as markers in studies of the nature of the controlling oscillation. This itself highlights a major problem encountered in this thesis,

that is how do you determine the best marker for a rhythm before you have gained some understanding of the nature of the mechanisms controlling the rhythm. In this thesis their was little option but to choose onset and offset of melatonin secretion as markers since the pulsatile nature of melatonin secretion in the sheep shows no clear acrophase.

Compounding this problem is the question of interpretation raised by Pittendrigh and Daan (1976) and Moore-Ede at al (1976) in which they suggest that although a rhythm may appear to be controlled by a relatively simple pacemaking system there is no way of excluding the possibility that it may just be a simple output from a much more complex structure.

The problems raised are significant and there is no easy way in which they can be overcome. They will probably only be resolved by further study of the behaviour of the pineal over a range of photoperiodic treatments coupled with advancements in our understanding of the biochemical nature of the pacemaking centres responsible for the generation of rhythmic activity.

In the absence of any breakthrough at the level of the biochemical basis of pineal activity the determination of the true nature of the pineal pacemaker may take considerable time since the only method of advancement is to collect data on the behaviour of pineal rhythms under darkness to determine if this behaviour can be best explained by reference to a particular hypothetical model. This model can then be tested and amended in the light of future results.

This thesis is directed along these lines. Experiments have been conducted investigating the behaviour of the

melatonin rhythm under extended and continuous darkness after a range of photoperiodic treatments and the findings have been considered in the light of two currently existing models which purport to explain the function of the pineal pacemaker.

2.0 EXPERIMENTAL SECTION

2.1 Introduction

Although the melatonin signal plays a central role in photoperiodic time measurement our understanding of the nature of the pacemaker which controls the circadian pattern of melatonin secretion is poor.

As outlined in the literature review there is considerable conjecture about the nature of pacemakers which control circadian rhythms. Only two models have however been formally put forward describing the nature of the pineal pacemaker. These are the single pacemaker model described by Lewy (1983) and a two oscillator model proposed by Illnerova and Vanecek (1982).

The experimental work in this thesis was directed at gathering information on the pattern of melatonin secretion under a range of photoperiodic conditions which could be used to improve our understanding of the nature of the pineal pacemaker and to determine which of these models best describes the behaviour of the pineal pacemaker.

2.2 Materials and methods : General section

2.2.1 Animals

All animals used in the experiments conducted in this thesis were two year old Suffolk sheep. They were obtained from a local Suffolk stud from which they were culled for reasons not associated with their seasonality. When involved in an experiment each animal was maintained on a ration of 750 grams of commercial sheep pellets and 400 grams of alfalfa chaff per day.

2.2.2 Light rooms

Two rooms lit with fluorescent tubes which were of similar size and lighting intensity (400 lux) were used in the experiments. Each room had individual elevated pens and daily feeding and cleaning of the rooms occurred at 0800h.

2.2.3 Blood sampling

To facilitate collection of blood samples indwelling cannula (Medical grade polyethylene tubing I.D. 1.00mm O.D. 1.5mm) were placed in a jugular vein on the day prior to the beginning of sampling. Samples were collected into heparinised tubes and stored at 4 degrees centigrade until centrifugation after which plasma was collected and stored at -20 degrees centigrade until they were assayed.

A 40 watt red globe producing less than 2 lux remained on in the light control rooms at all times to facilitate the collection of blood

samples.

2.2.4 Melatonin assay

Melatonin concentrations in plasma were measured by R.I.A. using a slight modification of a validated assay (Kennaway et al 1982). In the assay used in this thesis, 500 ul of plasma was added to 500 ul of 0.5M borate buffer (pH 9.6) and extracted with 2.5 ml dichloromethane-hexane (1:1). The solvent phase was evaporated under a gentle stream of nitrogen gas and 600 ul of assay buffer (0.1 M sodium phosphate, 0.9% sodium chloride, 0.5% BSA, 0.05% bovine gamma globulin) was added together with 100 ul tritiated melatonin (SA,87.4 Ci/m mol;New England Nuclear, Boston, MA) and 100 ul antiserum. Separation of free and antiserum bound melatonin was achieved by addition of 100 ul 0.1% activated charcoal and centrifugation at 800 times g for 20 minutes. The antiserum (G280) bound 50% of the tritiated melatonin at a final dilution of 4.8×10^5. Cross reactivities at the 50% displacement level were: 6-hydroxymelatonin 0.02% ; 6-sulfatoxymelatonin,0.3% ; N-acetylserotonin, 0.3%. The present melatonin assay (D) was compared with the previous assay employing lipidex 5000 chromatography (C) and concentrations (fentomoles per ml) measured by the two assays were described by the regression equation C =.952 D +25.9. The intra and inter assay coefficients of variation were both less than 10% based on duplicate samples.

The sensitivity of the assay was calculated using techniques described by McIntosh and McIntosh 1980.

2.2.5 Prolactin Assay

Serum prolactin (PRL) concentrations were determined by double antibody Radioimmunoassay. Radioiodinated ovine PRL (NIADDK-oPRL-I-1) and standards (NIADDK-oPRL-16) or sample were mixed with antiserum (NIADDK-oPRL-1) at the same time. After 24-h incubation at 20-25C ovine anti-rabbit gamma globulin was added as second antibody and tubes allowed to stand at 20-25C for a further 24-h before separation of antiserum-bound and free PRL components. The intrassay coefficient of variation based on duplicate samples was less than 10%.

2.2.6 Phase reference points

To assess the duration of melatonin secretion and the times of onset and offset of melatonin secretion it was necessary to define phase reference points for onset and offset of secretion. The first time point at which the plasma melatonin concentration was 2 S.D. above assay sensitivity and stayed above this level for a further hour was taken as the phase reference for the onset of melatonin secretion. Similarly the phase reference point for the offset of melatonin secretion was taken as that time point at which the plasma level fell to within 2 S.D. of assay sensitivity and remained below this level for a further hour.

2.2.7 Statistical analysis

The data for the duration of melatonin secretion was analysed by two-way analysis of variance and Student's t test. Estimates of the period of onset and offset were calculated by regression analysis.

The magnitude of prolactin responses to thyrotrophin releasing hormone represent the highest concentration observed within 40 minutes of injection, minus the hormone concentration at the time of injection. Areas under the prolactin response profiles were determined by extrapolating the weights of standard graphing paper to arbitrary area units. The effects of light treatments on prolactin responses to thyrotrophin releasing hormone were analysed by t test.

3.0 EXPERIMENT 1

MELATONIN PROFILES AND ENDOCRINE RESPONSES OF EWES EXPOSED TO A PULSE OF LIGHT LATE IN THE DARK PHASE.

3.1 Introduction

Experiments with hamsters and sheep have shown that a pulse of light given during the night can alter an animals interpretation of the prevailing photoperiod (Hoffman et al 1981, Thimonier et al 1978). Furthermore, research with sheep, rats and hamsters has shown that a light pulse in the later part of the night shortens the the duration of pineal activity so that it resembles that seen under long days (Illnerova et al 1982, Brinklow et al 1984, Hoffman et al 1981). Little however is known of the response of the pineal pacemaker to such inputs.

To examine the effects of a short light pulse in the latter part of the night the melatonin profiles of control and pulsed ewes were monitored on the night when they received the pulse of light and on the following night under extended darkness.

To determine whether the light pulse had altered the animals interpretation of the photoperiod they were exposed to the animals prolactin responses to thyrotrophin releasing hormone were measured. The prolactin response has been shown by other researchers to be a useful indicator of the ewes interpretation of a particular photoperiod (Fitzgerald et al 1981, Howland et al 1983).

3.2 Materials and Methods

The experiment was conducted in autumn. Eight two year old Suffolk ewes previously held under natural lighting conditions were randomly allocated to two equal groups and

housed in individual pens in separate light control rooms. Ewes in both rooms were acclimatised to a lighting schedule of L:D,10: 14 for five weeks before the lighting in one room was changed to 10L:10D:1L:3D (Group 1) while the other group (Group 2) remained on L:D,10:14. Subjective dawn for both groups was kept constant at 0700h. After six weeks on these photoperiods blood samples were collected to characterise melatonin profiles. Half hourly samples were collected from 1500 to 1900h and from 0200h to 1830h. Additional samples were collected at 15 minute intervals between 0300h and 0400h in animals receiving the pulse during this period. To collect samples in continuous darkness the lights in both rooms were switched off at 1200h and samples taken at 30 min. intervals between 1400 and 1830h and from 0200h until 1100h.

The functioning of the pituitary gland was assessed by determining the prolactin response to a bolus intravenous injection of thyrotrophin releasing hormone (50ng/kg BW); Sigma (Chemical Company, St. Louis, Mo). Blood samples were taken at -40, -20, 0, 20, 40, 60, 80, 100 and 120 minutes relative to the time of the injection

3.3 Melatonin Results

The plasma melatonin profiles of the two groups of ewes both under entrainment and under extended darkness are shown in figures 1-4. Ewes exposed to L:D,10:14 (group 1) showed a significantly ($P<.01$) longer period of melatonin production (15.0 $\pm$ 0.4 mean $\pm$ SE; Fig 1) than ewes exposed to 10L:10D:1L:3D (group 2, 9.0 $\pm$ 0.4; Fig 2). The pulse of light between 0300h and 0400 shortened the period of

melatonin production in the latter group. In continuous darkness there was a significant ($P<.05$) lengthening of melatonin secretion in the pulsed group (9.0 $\pm$ 0.4 versus 13.2 $\pm$ 1.4 Fig 4) but no change in the control group (15.0 $\pm$ 0.4 versus 16.1 $\pm$ 0.5h Fig 3) resulting in both periods being of similar length

3.4 Prolactin responses

The magnitude of prolactin responses to an injection of thyrotrophin releasing hormone shown in figure 5 were significantly less ($P<.05$) in control ewes (478 $\pm$ 134 mg/ml) than in pulsed ewes (1578 $\pm$ 175 mg/ml). The area under the prolactin response curves was reduced in control ewes compared with pulsed ewes (140 $\pm$ 49 vs 501 $\pm$ 92 arbitrary area units $P<0.1$).

3.5 Discussion

Common explanations of light interruption experiments have until recently made reference to the Bunning hypothesis which proposes that the brain is sensitive to the effects of light during specific periods of the daily cycle (Bittman et al 1983). Responses to light interruptions were therefore postulated to be dependent on whether the light pulse was coincident with the light sensitive phase. With progress in our understanding of the role of the pineal in P.T.M. and in particular, new data which followed the availability of assays for melatonin, this concept was developed to include the idea that the sensitive phase is to the presence or absence of melatonin (Rollag et al 1978). Light pulses in this model are therefore considered to effect a shift in the location of melatonin sensitive phase in relation to the melatonin secretory profile. More recent findings have led to the development of a model which suggests that it may simply be the duration of melatonin secretion which conveys the photoperiodic message and that light pulses produce their effect by simply through shortening the duration of melatonin secretion (Carter and Goldman 1983). In the present study the duration of melatonin secretion was shortened by the light pulse occurring late in the dark phase. The finding that ewes recieving the pulse had serum PRL concentrations characteristic of sheep kept under long days clearly indicates that the ewes interpreted this pulse as a dawn signal (D,Occhio M.J., and C.R Earl unpublished).. Whilst these findings are consistent with the theory that P.T.M. essentially involves the duration of melatonin secretion,

they also allow the alternate hypothesis that the light pulse provides a new dawn signal, which changes the position of the melatonin sensitive phase so that it is no longer coincident with the shortened melatonin secretory period.

The rhythm of melatonin secretion persists in constant darkness presumably due to the continuation of signals emanating from pacemaking centres in the suprachiasmatic nucleus (Moore 1979). In the present studies the period of melatonin secretion in the pulsed ewes increased whereas the period of melatonin secretion in the control group was unchanged in extended darkness. This finding can be interpreted on the basis of a theoretical two-oscillator model for entrainment of circadian rhythms to the daily light dark cycle (Pittendrigh and Daan 1976). When the entraining signal, the light dark cycle was removed in the current experiment, the response of the two putative oscillators controlling pineal function reflects not only the behaviour of the individual oscillators but also the degree of interaction between them. Transfer of the pulsed ewes to extended darkness resulted in a relatively large increase in the duration of melatonin secretion in relation to the control group which could be explained in the two oscillator model as being due to the greater interaction between E and M brought about by changes in their relative positions. However since there was no difference between the duration of melatonin secretion between pulsed and control ewes under extended darkness it could be suggested that exposure to the light pulse even for 4 weeks had no effects on the underlying neural control of the melatonin rhythm as the duration of melatonin secretion was unchanged by the light interruption.

If endogenous circadian rhythms coupled to other endocrine functions also remained unaltered, it is possible that the rhythm in sensitivity to melatonin was unchanged and therefore the shortened duration of melatonin secretion in pulsed ewes was no longer coincident with the sensitive phase.

The data might be equally well interpreted by reference to a single oscillator model proposed by Lewy (personal communication). In his interpretation the light dark cycle entrains the onset of the rhythm of melatonin secretion and the duration of melatonin secretion is assumed to be fixed and dependent on the availability of substrate. The results of the current experiment with sheep are consistent with this hypothesis since the onset of melatonin secretion is closely associated with the time of lights off and the duration of secretion under extended darkness is the same under both photoperiods.

However, in the rat a pulse of light in the later part of the night shortens the duration of secretion under extended darkness which does not agree with the prediction from this model. This conflict can easily be overcome if one assumes that the pulse may alter the availability of substrate or enzyme in some way.

In conclusion, the present study provides additional evidence that the circadian rhythm of pineal gland activity is influenced by photoperiod. Furthermore, it supports the hypothesis that the pineal delivers a melatonin signal to the photoperiodic time measuring system which results in appropriate responses in other endocrine functions. The precise manner in which the pineal is controlled by photoperiod is not known but may involve two separate but

interacting oscillators.

FIGURE 1

The melatonin profiles of group 1 ewes (mean ± S.E.) under entrainment to the photoperiod L:D,10:14.

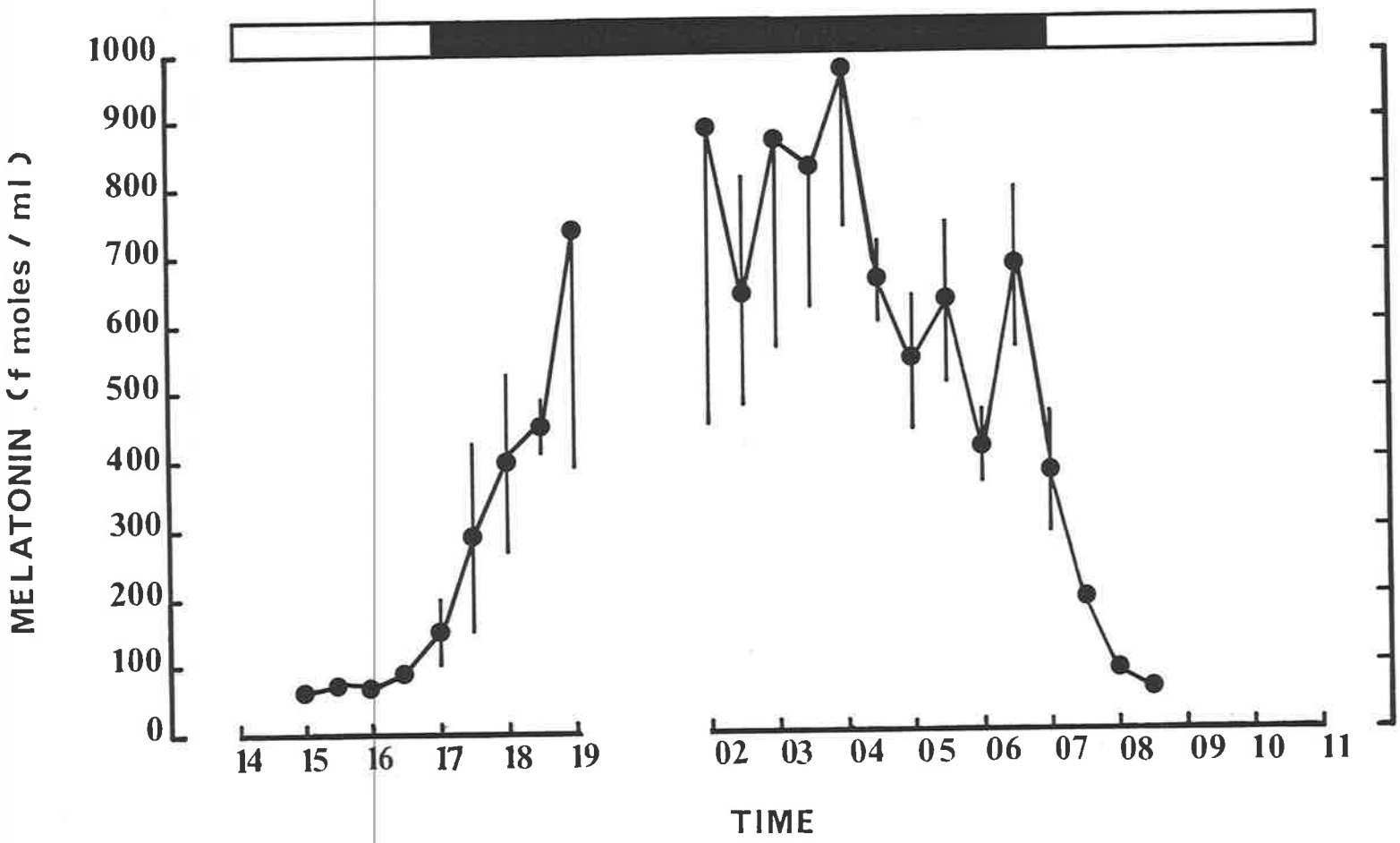

MELATONIN (f moles / ml)
1000
900
800
700
600
500
400
300
200
100
0
14
15
16
17
18
19
02
03
04
05
06
07
08
09
10
11
TIME

FIGURE 2

The melatonin profiles of group 2 ewes (mean ± S.E.) ewes under entrainment to the photoperiod 10L:10D:1L:3D.

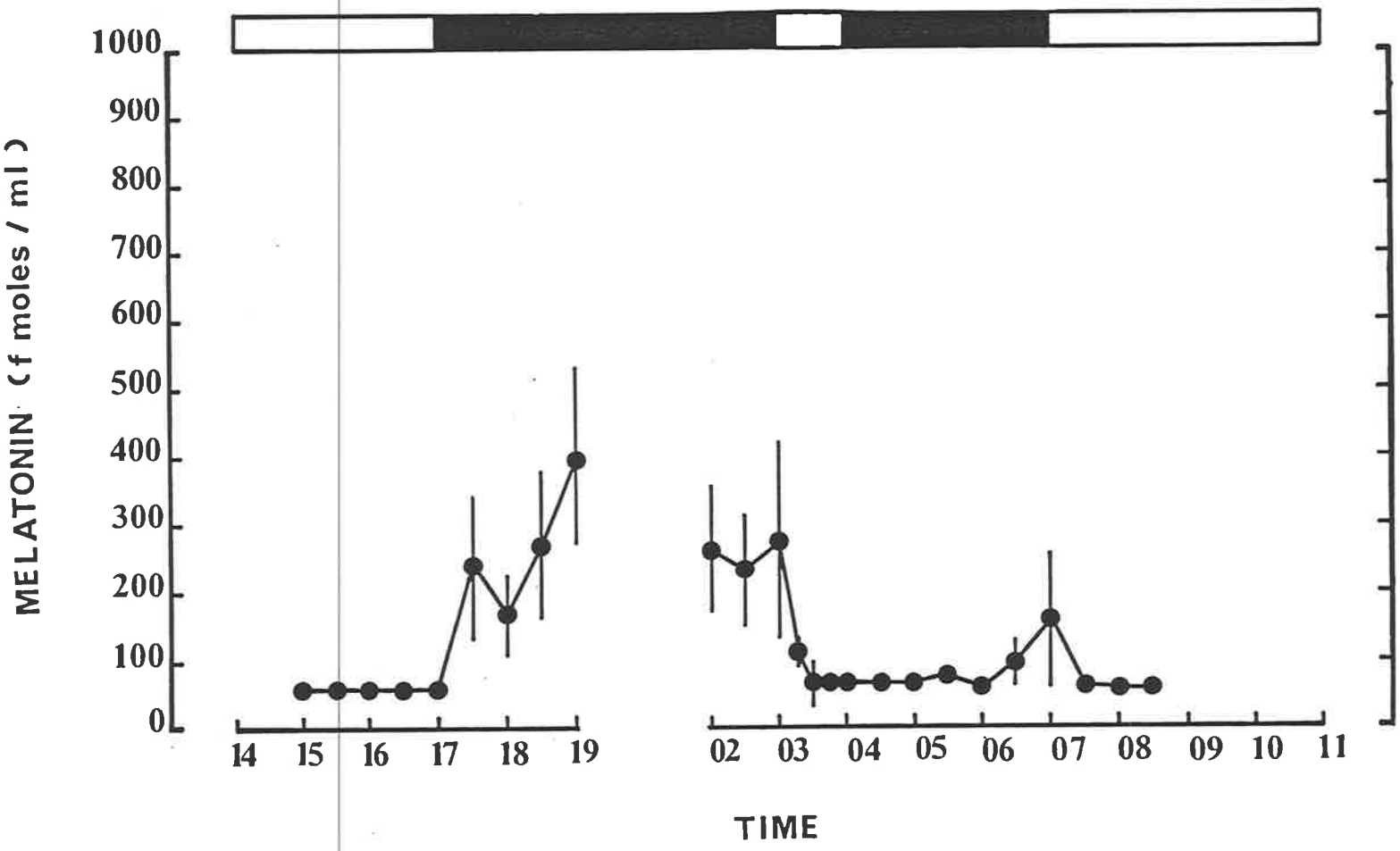

MELATONIN (f moles / ml)
1000
900
800
700
600
500
400
300
200
100
0
14
15
16
17
18
19
02
03
04
05
06
07
08
09
10
11
TIME

FIGURE 3

The melatonin profiles under extended darkness of group 1 ewes (mean $\pm$ S.E.) which were previously entrained to L:D,10:14.

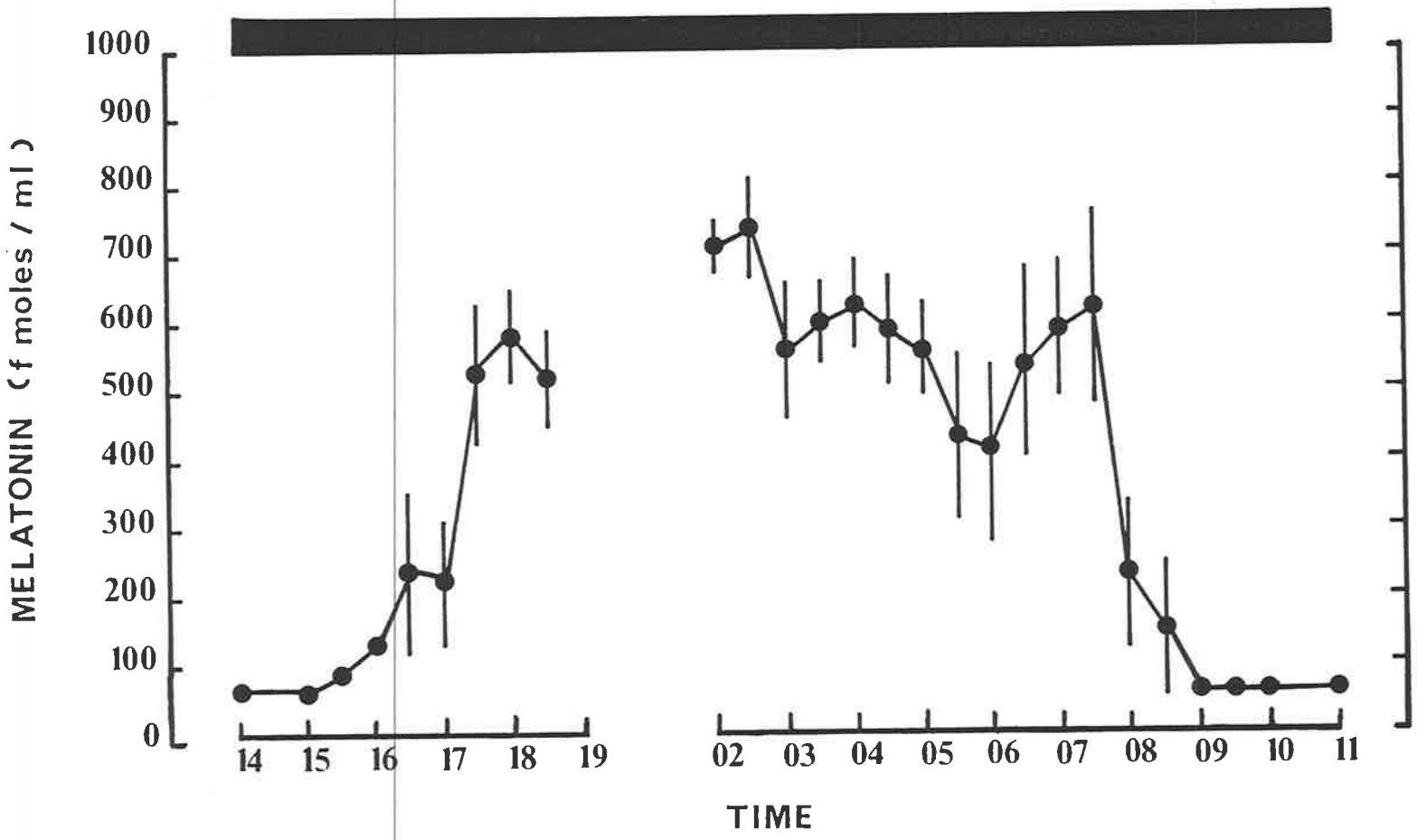

MELATONIN (f moles / ml)
1000
900
800
700
600
500
400
300
200
100
0
14
15
16
17
18
19
02
03
04
05
06
07
08
09
10
11
TIME

FIGURE 4

The melatonin profiles under extended darkness of group 2 ewes (mean ± S.E.) which were previously entrained to 10L:10D:1L:3D.

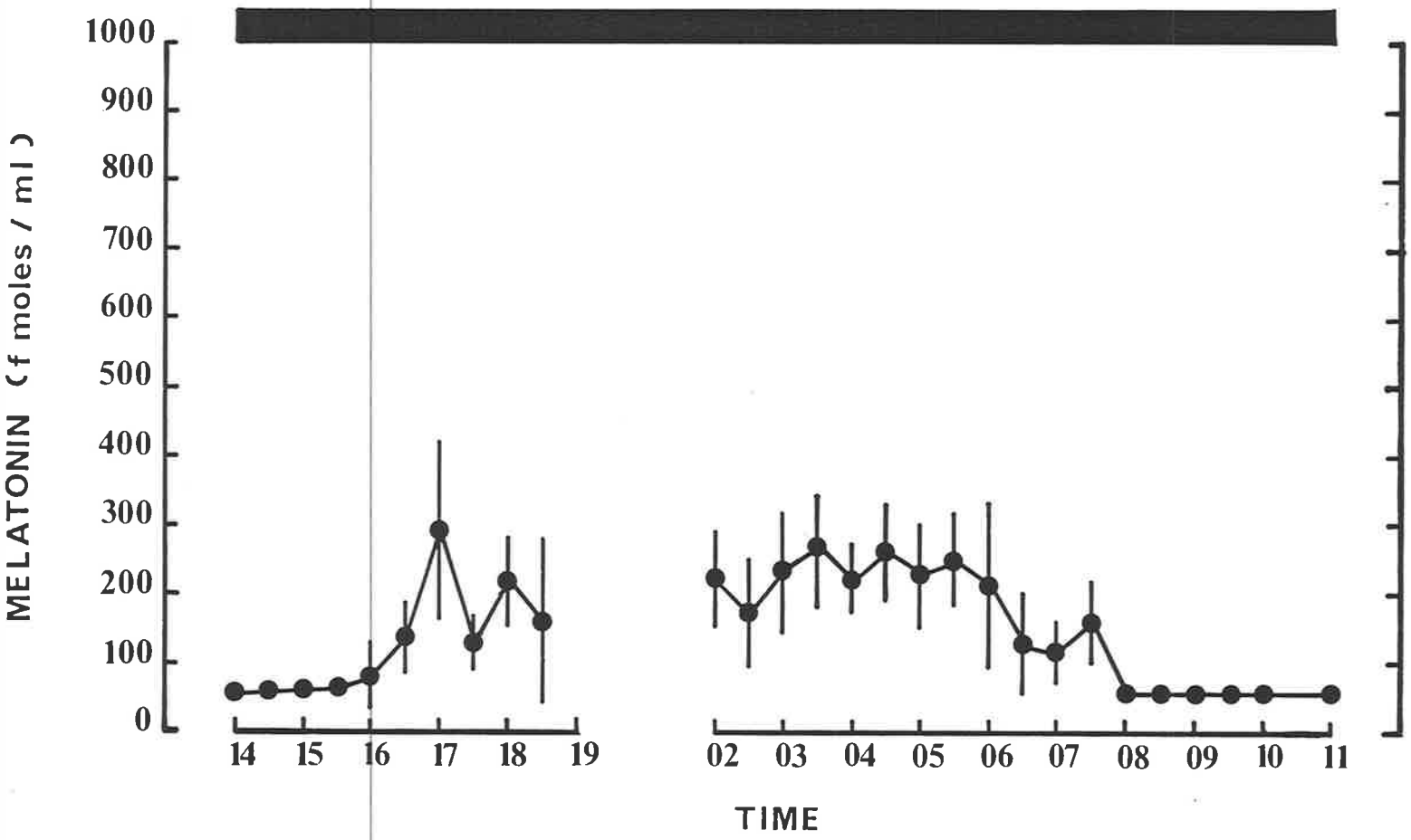
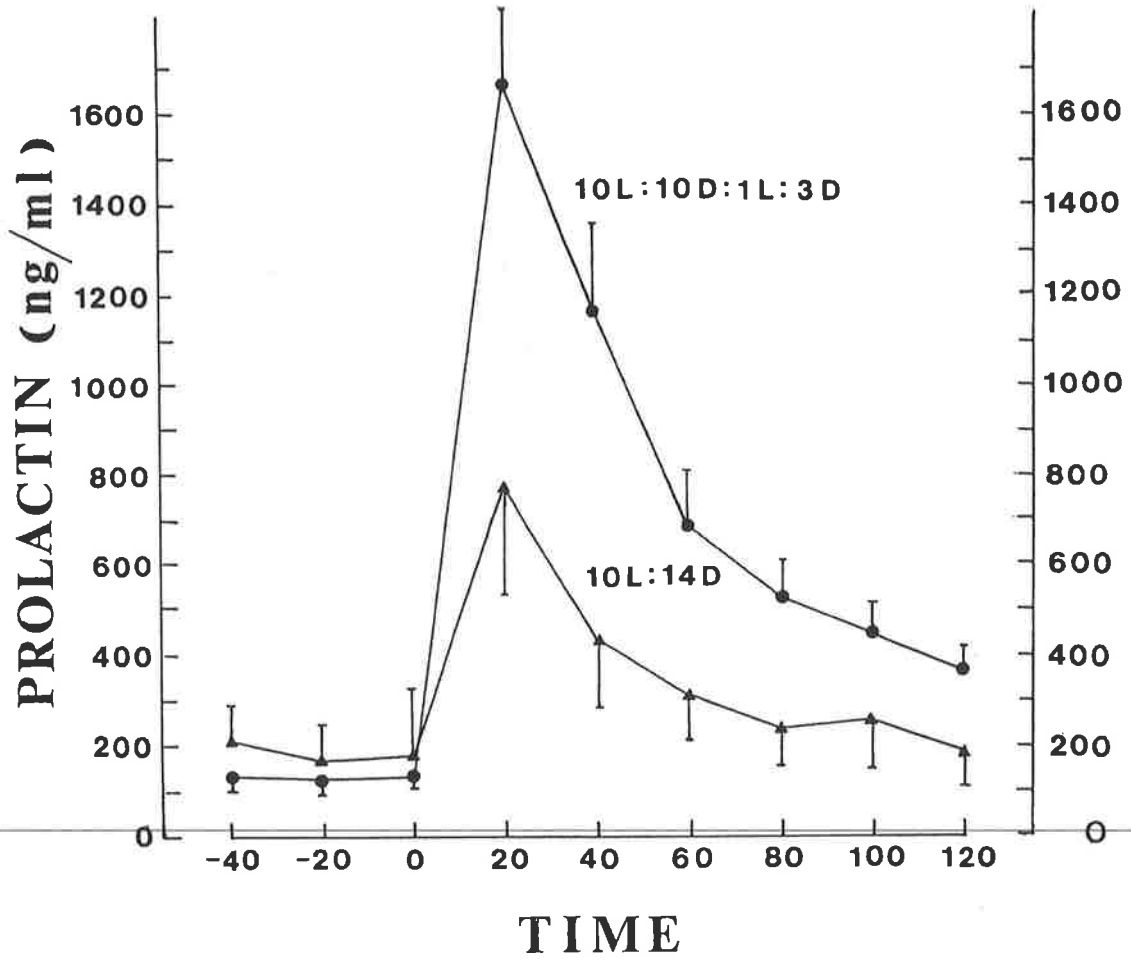

MELATONIN (f moles / ml)
1000
900
800
700
600
500
400
300
200
100
0
14
15
16
17
18
19
02
03
04
05
06
07
08
09
10
11
TIME

FIGURE 5

The prolactin responses of control and pulsed ewes (mean ± S.E.) in response to a T.R.H. injection (50 ng/kg).


PROLACTIN (ng/ml)
1600
1400
1200
1000
800
600
400
200
0
10L:10D:1L:3D
10L:14D
-40
-20
0
20
40
60
80
100
120
TIME

4.0 EXPERIMENT 2

THE EFFECT OF A LIGHT PULSE EARLY IN THE DARK PHASE ON THE PATTERN OF MELATONIN SECRETION IN THE RAM

4.1 INTRODUCTION

In the rat Illnerova and Vanecek (1982) found that a light pulse in the first half of the night caused a reduction in pineal N-acetyltransferase activity followed by a subsequent rise in activity after an intervening lag period. The length of the lag period was found to vary in length in a manner dependent on the time at which the pulse was administered. The second experiment was therefore designed to determine whether a light pulse in the early part of the night would have similar effects on the mechanisms controlling the rhythm of pineal activity in the sheep.

4.2 Materials and Methods

The experiment which was carried out in Spring involved fourteen two year old Suffolk rams which were randomly allocated to one of three treatment groups. Group 1, comprising 6 animals was housed in the animal house and exposed to natural photoperiod at all times. Groups 2 and 3 consisting of 4 rams each were exposed to L:D, 6.5:17.5 for four weeks to entrain the mechanisms regulating pineal function to this photoperiod. The photoperiod of group 3 animals was then changed to 6L:7.5D:.5L:10D while that of group 2 animals remained unchanged. The time of subjective dawn in both groups 2 and 3 was 0400H. After a further 4 weeks hourly blood samples were collected from animals from 0600h for 24hrs.

4.3 Results

All rams in group 1 housed on natural photoperiod showed a distinct circadian rhythm in melatonin secretion. The melatonin profile of this group of animals is displayed in figure 6. Animals in group 2 showed spikes of melatonin secretion at intermittent times throughout the sampling period. The individual profiles of each animal in this group are displayed in figures 7(a), (b), (c) and (d).

Animals in group 3 which were subjected to a pulse of light during the dark phase showed melatonin profiles of an abnormal nature. The melatonin profiles of this group are shown in figures 8 (a), (b), (c) and (d). Three of the four animals in this group showed much higher levels of melatonin than those observed in the controls. The melatonin profiles of both groups maintained under artificial photoperiod were unusual and so the profiles of all animals in each of these groups have been presented.

The melatonin profiles of Groups 2 and 3 did not lend themselves to routine analyses as the rams in Group 2 only secreted melatonin in intermittent spikes and the rams in Group 3 showed large variations in the times of onset and offset of melatonin secretion.

4.4 Discussion

The melatonin profiles of rams monitored under natural photoperiod (Group 1) exhibited a normal pattern of melatonin secretion, that is, the onset of melatonin secretion commenced at dusk and terminated at dawn. Rams in Group 2 which were suddenly shifted into a very short photoperiod (L:D, 6 1/2:17 1/2) from the natural photoperiod lost the ability to generate a rhythm in melatonin production. Previous research has shown that sheep are capable of producing a normal, nyctohymeral pattern of melatonin secretion under shorter photoperiods than that used in this experiment (Kennaway et al 1983, Bittman et al 1983). Thus the abnormal rhythm of melatonin secretion would appear to reflect a major perturbation in the internal mechanisms regulating the melatonin signal due to the special circumstances of the experiment.

A disorganisation of the pattern of melatonin secretion under artificial photoperiods has also been reported by Almeida and Lincoln (1982). In their study which was carried out in Soay rams the disorganisation occurred in animals which were exposed to constant short or long photoperiods for a prolonged period. The length of the photoperiod in this experiment was not changed but the animals still lost the ability to generate a normal pattern of melatonin secretion. The results of both experiments suggest a failure of the internal regulatory system to accomadate to the photoperiod to which the animals were exposed. Thus disorganisation in the mechanisms regulating pineal function can occur following an

abrupt change in photoperiod or prolonged exposure to a specific photoperiod. In contrast to the animals in Group 2 all animals in Group 3 secreted melatonin for extended periods. The insertion of the short light period restored the ability of these animals to generate a melatonin signal. The melatonin profiles of each animal are presented in figure 8 and are unusual in two aspects. Firstly the levels of melatonin concentration in 3 of the 4 animals are much higher than that of the animals exposed to natural daylength. Secondly the secretion of melatonin continues for some time after lights on.

Higher than normal levels of melatonin have been reported by Lincoln (1985) under three different photoperiodic conditions. These were, firstly, when animals were held on the photoperiod 8L : 28D, secondly, when animals were held on photoperiodic cycles of unusual period lengths and thirdly when animals were held in constant darkness. In the first two cases the animals were exposed to noncircadian cycles and this may have disturbed the manner in which the external photoperiod interacted with the endogenous circadian pacemaker controlling melatonin secretion. The third example suggests that continuous darkness may have some effect on the pacemaker so that it could longer generate a stable rhythm. These examples suggest that under certain photoperiodic conditions the mechanism responsible for generating the melatonin rhythm no longer operates normally with either the pattern or amount of melatonin secretion being disturbed.

These findings indicate that care must be taken in choosing the photoperiodic changes an animal is exposed to

since some changes in photoperiod are incompatible with normal functioning of the internal mechanisms regulating pineal function. In subsequent experiments in this thesis artificial photoperiods similar to the external lighting environment were chosen in an attempt to eliminate this problem.

FIGURE 6

The melatonin profiles (mean ± S.E.) in Spring of Suffolk rams (group 1) which were housed in an animal house but exposed to natural photoperiod at all times.

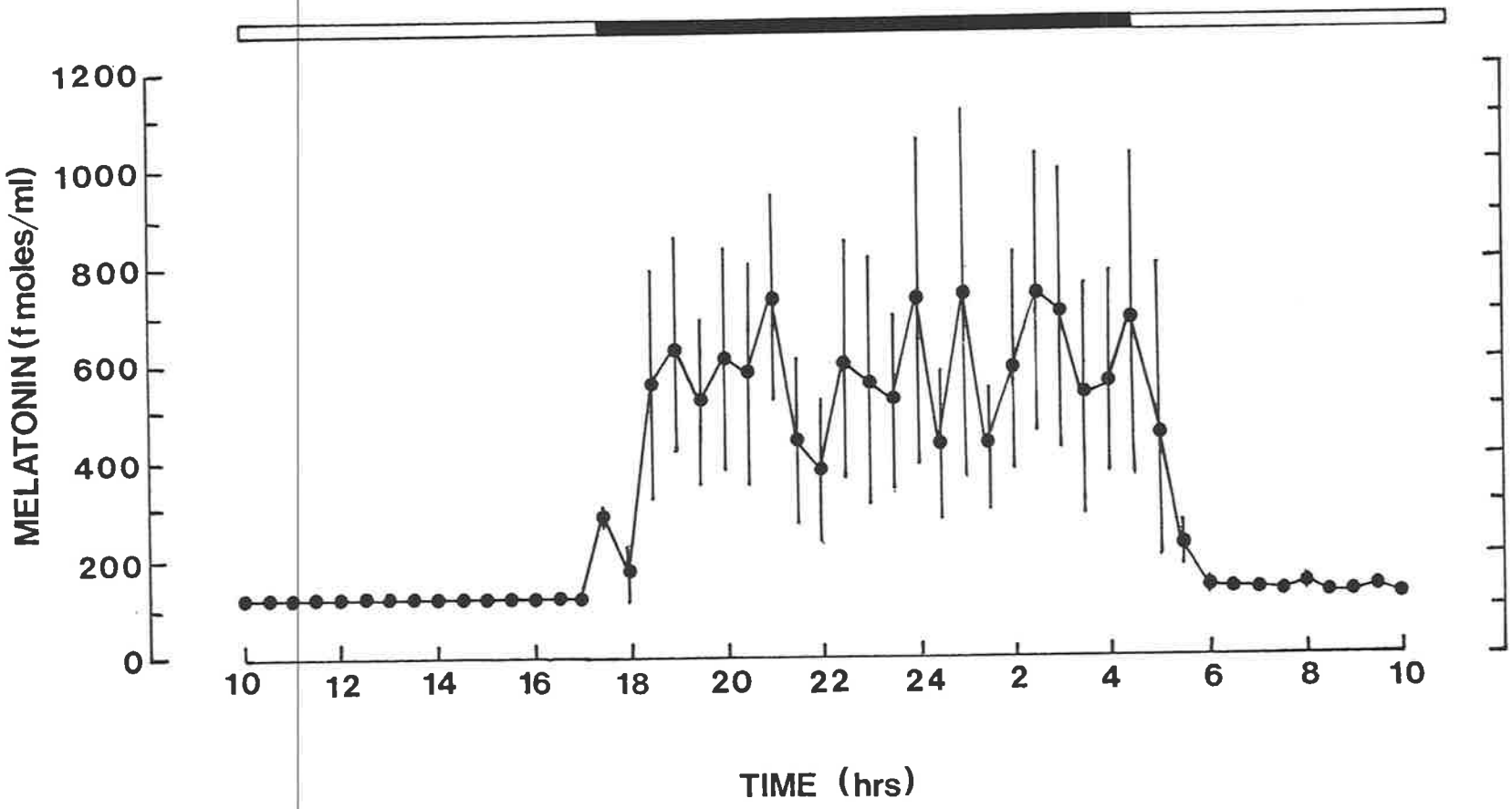

MELATONIN (f moles/ml)
1200
1000
800
600
400
200
0
10
12
14
16
18
20
22
24
2
4
6
8
10
TIME (hrs)

FIGURE 7

Figures 7 (a)-(d) display the melatonin profiles of animals in group 2 which were exposed to L:D, 6.5:17.5 for 8 weeks in Spring.

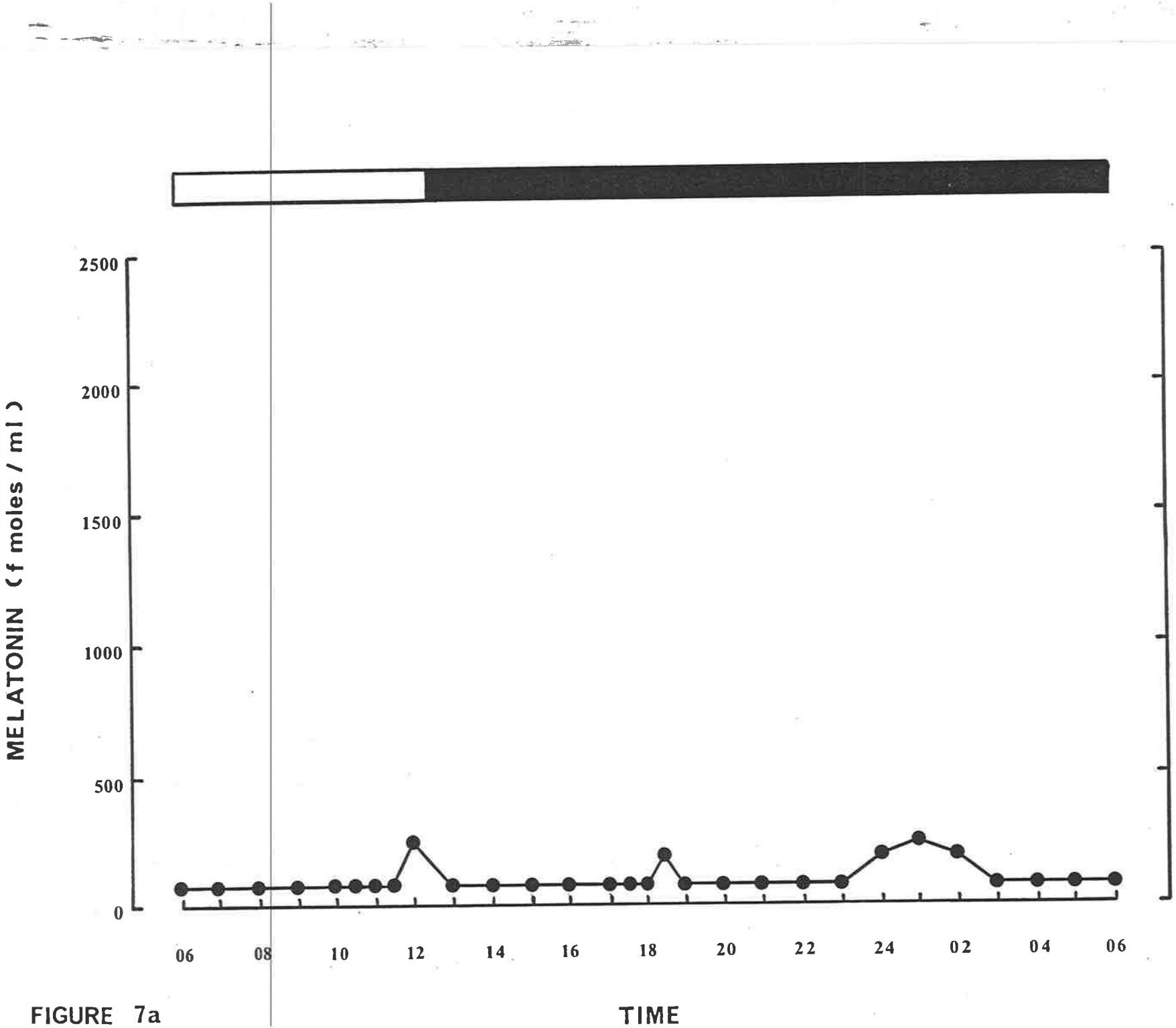

MELATONIN (f moles / ml)
2500
2000
1500
1000
500
0
06
08
10
12
14
16
18
20
22
24
02
04
06
TIME

FIGURE 7a

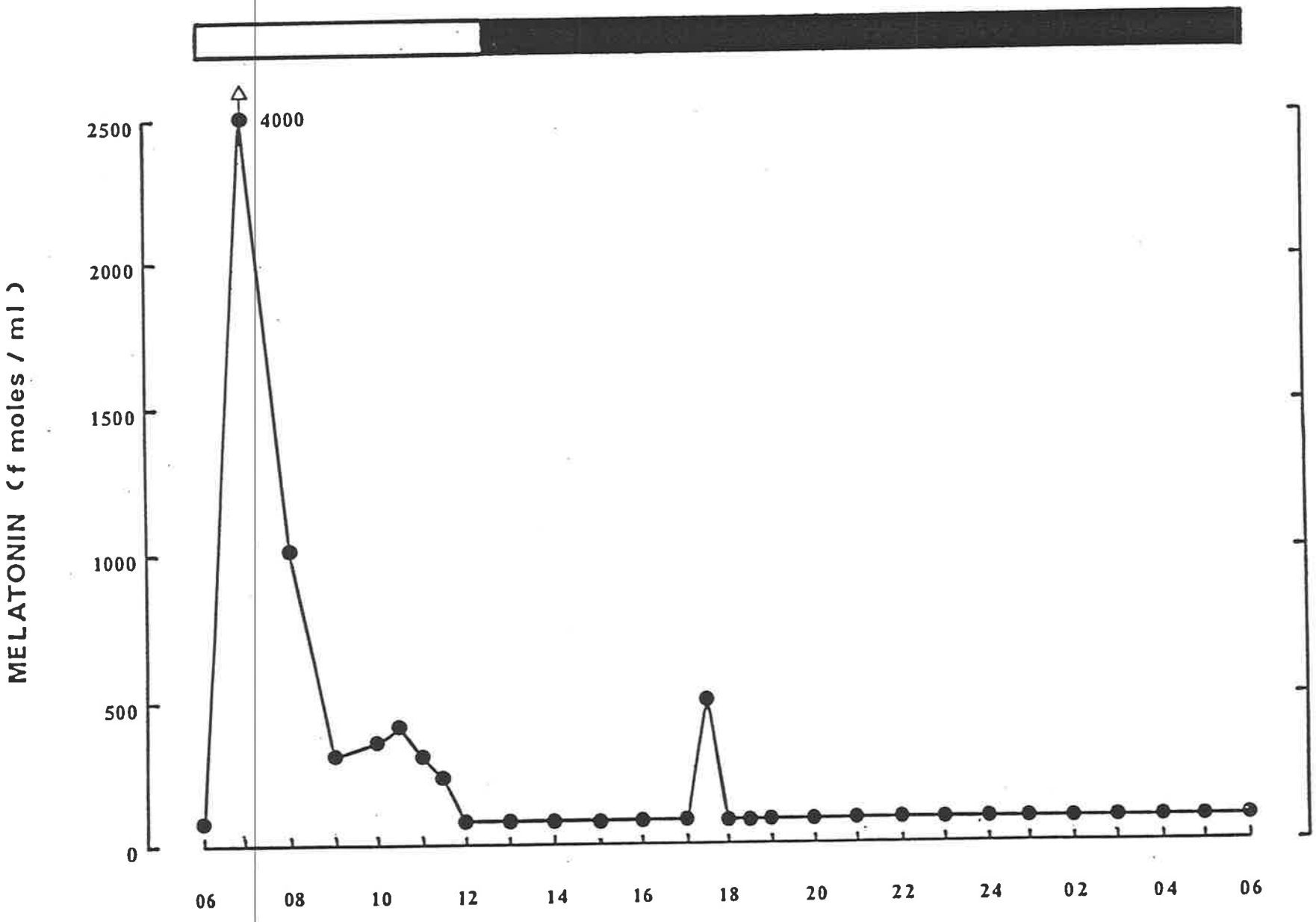

4000
2500
2000
1500
1000
500
0
MELATONIN (f moles / ml)
06
08
10
12
14
16
18
20
22
24
02
04
06
TIME

FIGURE 7b

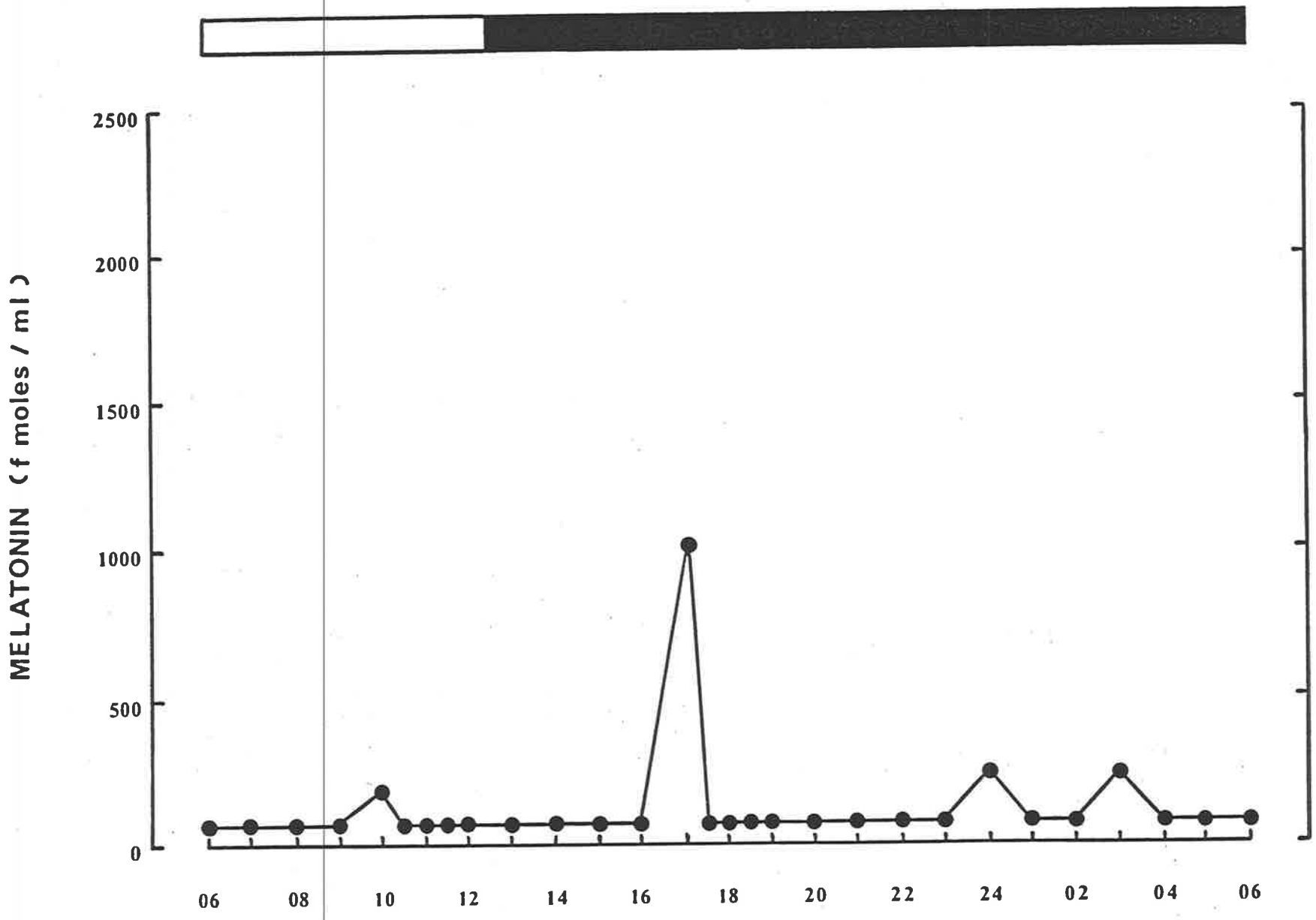

MELATONIN (f moles / ml)
2500
2000
1500
1000
500
0
06
08
10
12
14
16
18
20
22
24
02
04
06
TIME

FIGURE 7c

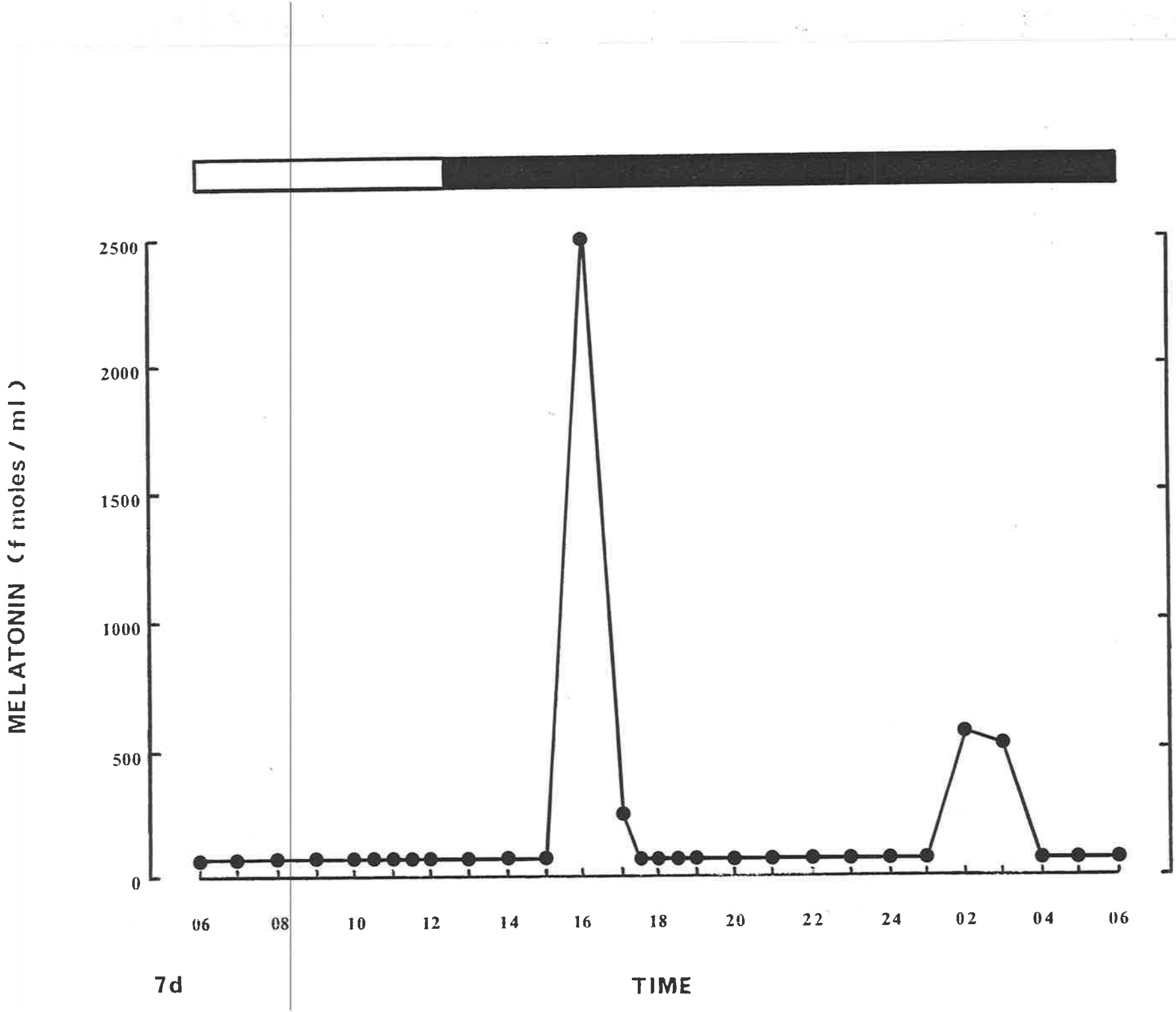
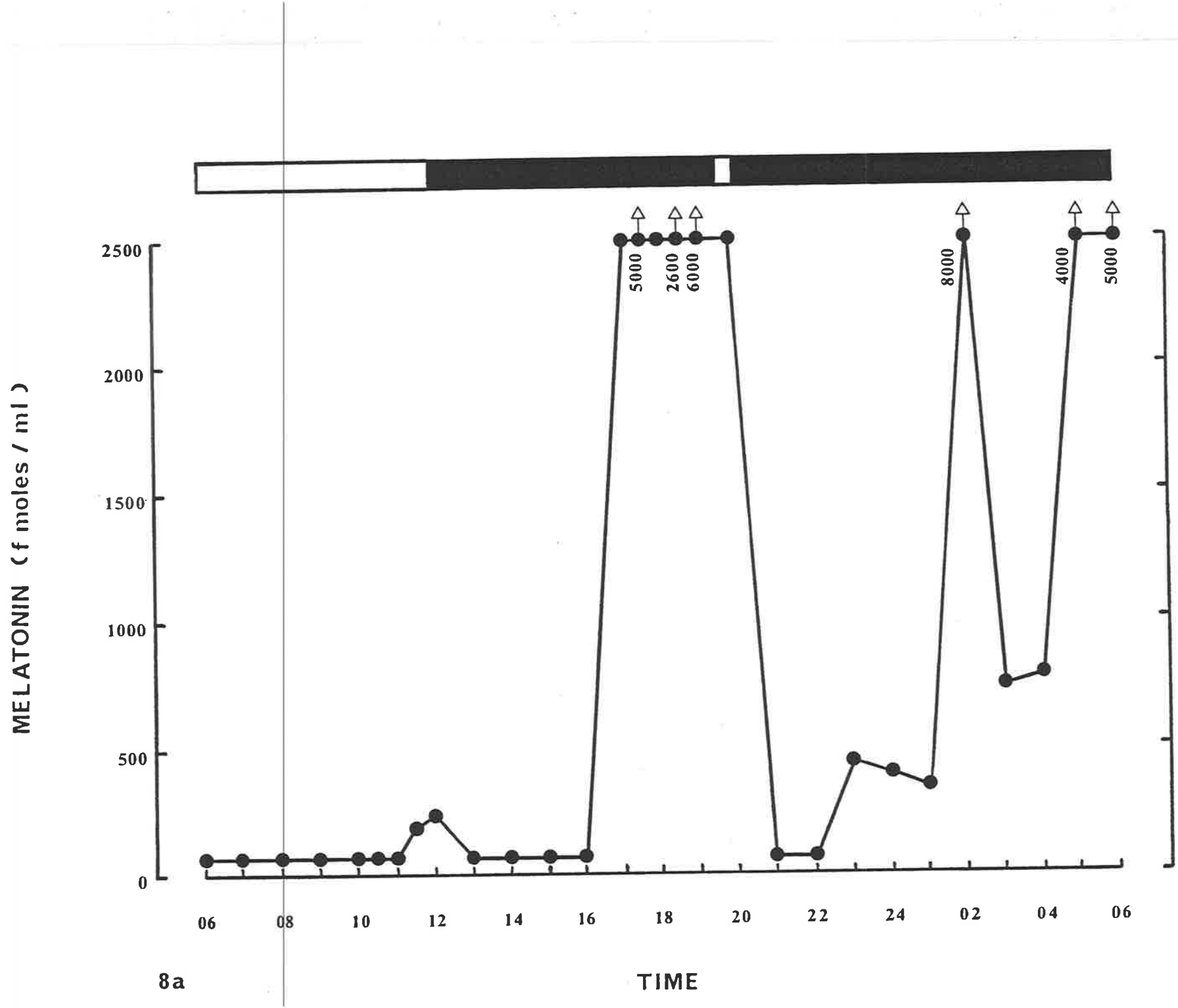
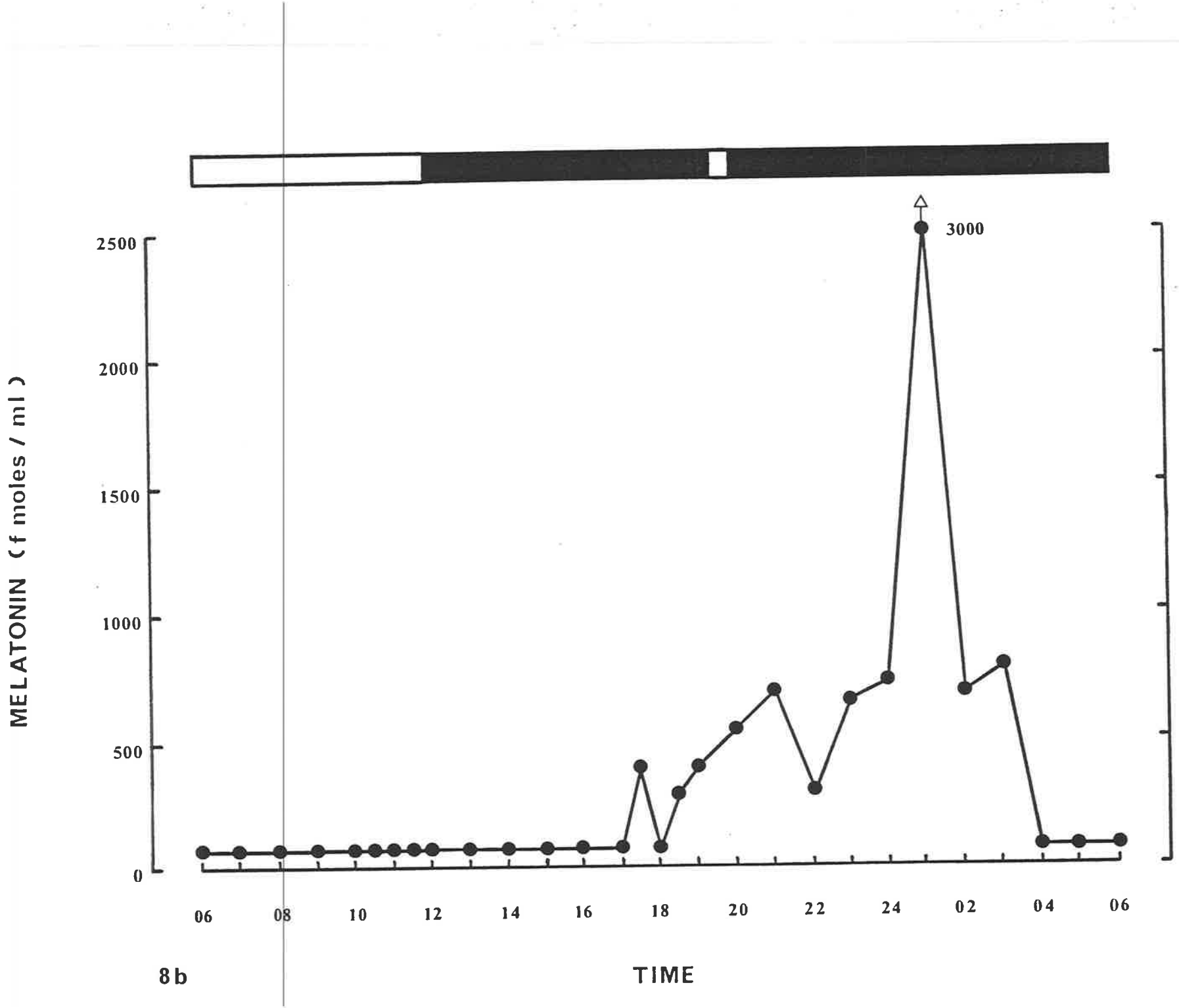

MELATONIN (f moles / ml)
2500
2000
1500
1000
500
0
06
08
10
12
14
16
18
20
22
24
02
04
06
7d
TIME

FIGURE 8

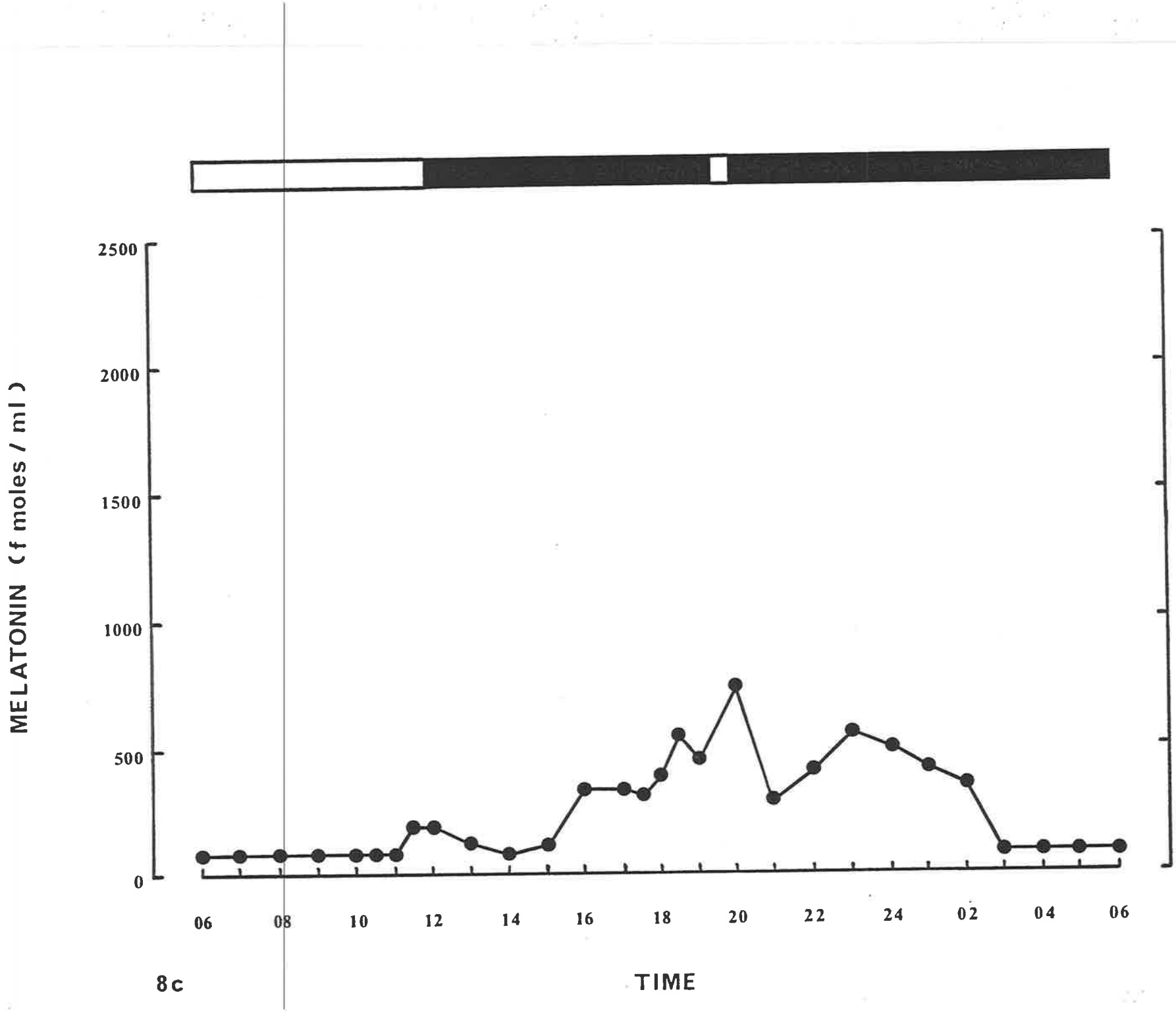
Figures 8 (a)-(d) display the melatonin profiles of group 3 rams which were exposed to 6.5L : 17.5D for four weeks and then to 6L:7.5D:.5L:10D for the following four weeks.


MELATONIN (f moles / ml)
2500
2000
1500
1000
500
0
5000
2600
6000
8000
4000
5000
06
08
10
12
14
16
18
20
22
24
02
04
06
TIME

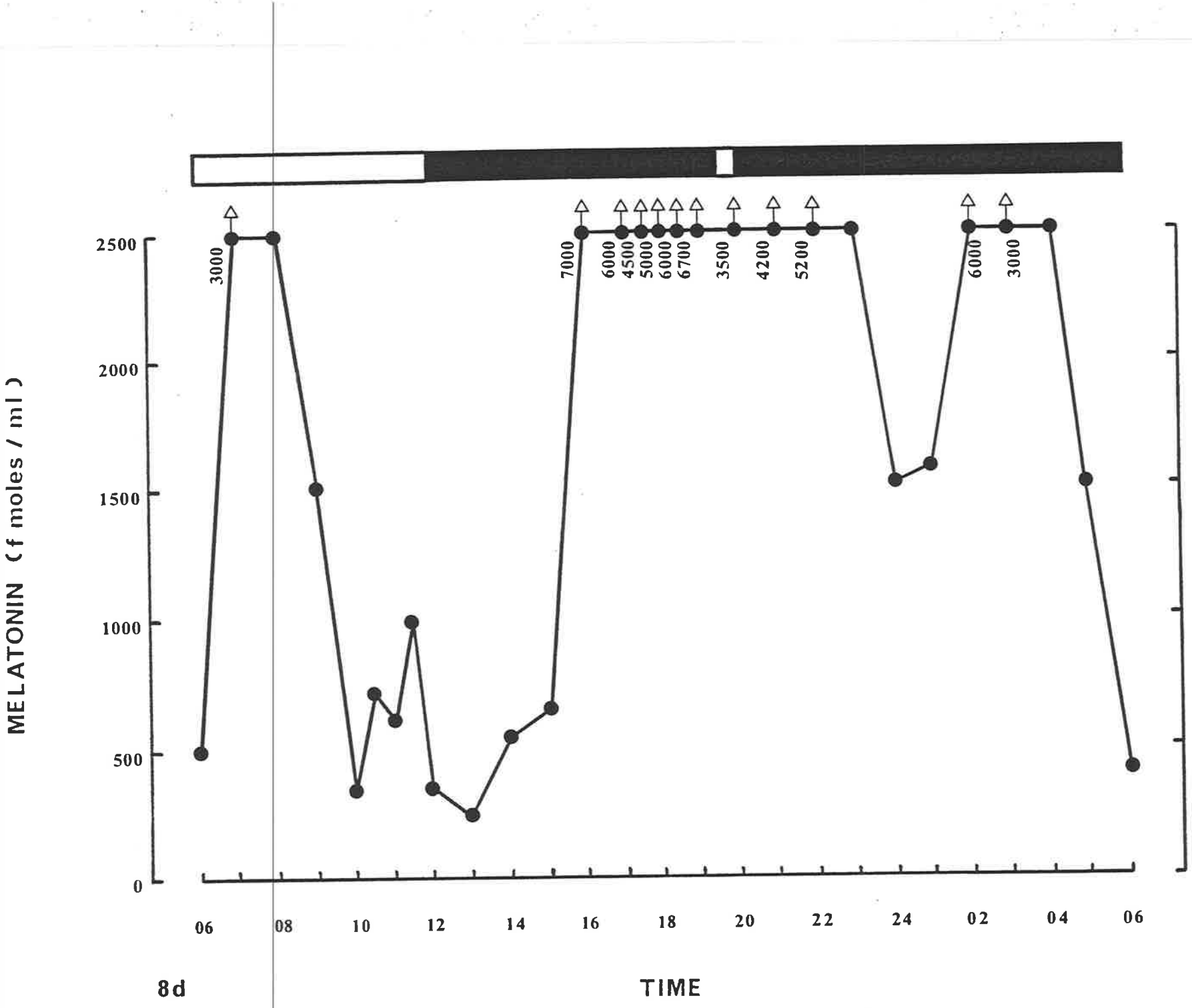
8a


MELATONIN (f moles / ml)
2500
2000
1500
1000
500
0
3000
06
08
10
12
14
16
18
20
22
24
02
04
06
TIME

8b


MELATONIN (f moles / ml)
2500
2000
1500
1000
500
0
06
08
10
12
14
16
18
20
22
24
02
04
06
TIME

8c


MELATONIN (f moles / ml)
2500
2000
1500
1000
500
0
3000
7000
6000
4500
5000
6000
6700
3500
4200
5200
6000
3000
06
08
10
12
14
16
18
20
22
24
02
04
06
8d
TIME

5.0 EXPERIMENTS 3(a) and (b)

ENTRAINMENT OF THE PACEMAKER CONTROLLING MELATONIN SECRETION BY THE LIGHT DARK CYCLE.

5.1 INTRODUCTION

The results presented in the previous section raised questions concerning the interaction between the photoperiod an animal is exposed to and the pineal pacemaker in generating a particular pattern of melatonin secretion.

In this section this interaction was further investigated by examining the melatonin profiles of animals under short and long daylengths both under entrainment and extended darkness where the influence of light on this pacemaker had been removed.

5.2 Materials and Methods

Expt 3a

A group of 4 two year old Suffolk ewes (Group 1), were brought indoors in summer (4/11/83) and acclimatised to L:D,14:10 for 4 weeks with subjective dawn occurring at 0600h. On 4/12/83 while the animals were still exposed to L:D,10:14 hourly blood samples were collected from 1800 until 2200h and from 0500h until 0800h to monitor the offset and onset of melatonin secretion respectively. On the following day the lights were turned off at 1200h and remained off for the rest of the experiment. Hourly sampling commenced at 1600h and finished at 2000h. Sampling continued the following morning from 0600h until 1000h.

Expt 3b

A group of 6 two year old Suffolk ewes (Group 2) were brought indoors on in autumn (6/4/84) and acclimatised to

L:D,10:14 for 4 weeks with subjective dawn occurring at 0700h. After 4 weeks hourly samples were collected from 1200 to 1700h and 20min samples were collected until 1900h. Further 20min samples were collected between 0600h and 0900h on the following day. On the next day the lights in this room were turned off at 1200h and remained off for the rest of the experiment. Hourly samples were collected from 1200h until 1800h and further samples taken between 0700h and 1200h the following morning.

The method for determining the onset and offset of melatonin secretion in each animal has been described in the general methods section.

5.3 Results

Expt 3a

The onset of melatonin secretion of Group 1 animals occurred approximately 1 hour earlier ($P<.05$) in animals exposed to continuous darkness relative to those maintained under L:D,14:10 whereas the offset of melatonin secretion in animals exposed to extended darkness occurred approximately 3 hours later ($P<.05$) relative to those under entrainment.

Expt 3b

The onset of melatonin secretion of Group 2 animals also occurred 1 hour earlier ($P<.05$) in animals which experienced an earlier time of lights off. The offset of melatonin secretion in animals under extended darkness also occurred 3 hours later ($P<.05$) relative to those under entrainment.

5.4 Discussion

The onsets and offsets of melatonin secretion were closely related to the times of lights off and lights on under both photoperiods. Thus the duration of melatonin secretion in animals exposed to short days was longer than animals under long days on ambient photoperiods.

Under extended darkness, the offset of melatonin secretion for animals on L:D,10:14 and L:D,14:10 occurred approximately 3 hours later than under entrainment whereas the onset of melatonin secretion occurred slightly earlier in both groups. Both groups of animals therefore showed advances in onset and delays in offset on exposure to continuous darkness with little difference in the magnitude of the shifts between groups previously entrained to L:D,10:14 or L:D, 14:10.

These results can be interpreted in a variety of ways. In Illnerova and Vanecek,s model (1982), lights off is postulated to entrain the position of the pacemaker controlling the onset of pineal activity and lights on, to entrain the offset of pineal activity. Accordingly the small advance in the onset, seen on exposure to continuous darkness implies that either, light was masking the position of this pacemaker when the animal was entrained or that its position was delayed under entrainment. The delay in the offset of melatonin secretion under extended darkness could therefore be interpreted as a masking effect of light on the position of the pacemaker controlling the offset of pineal activity or postulating that light advanced the pacemaker controlling the offset of pineal activity under the light dark cycle.

These findings are not so easily accommodated within Lewy,s hypothesis (1983) as this hypothesis assumes that the length of the photoperiod to which the animal has been entrained will not influence the duration of melatonin secretion under extended darkness.

A similar challenge to this hypothesis is provided by studies in the rat which indicate that the entraining photoperiod influences the duration of pineal activity under extended darkness (Illnerova and Vanecek 1982). In defending his hypothesis, Lewy (personal communication) suggested that the offset of melatonin secretion under normal photoperiods may be caused by dawn or possibly a limit of available enzyme or substrate whereas under under extended darkness only the latter could act. Using this interpretation secretion would continue under extended darkness if both enzyme and substrate were still available. If the length of the photoperiod is able to influence the enzyme or substrate concentrations involved in melatonin secretion then this hypothesis needs only small adjustments to make it compatible with the results observed in this experiment.

It should also be noted that these results can also be explained by reference to Wevers (1965) single oscillator model. In this model the times of onset and offset of activity are determined by the time the rhythm moves above and below a certain threshold. If this model is applied to the control of pineal activity, it must also include the masking effects of light on pineal activity. Thus placing the animal in continuous darkness allows the true time of onset or offset of pineal activity to be observed. Exposure to shorter photoperiods as in this experiment, may cause a

lowering of the threshold which would result in an increase in the duration of pineal activity when the animals are placed in continuous darkness.

The findings of the experiments described in this section are accomodated by both the single and multiple oscillator models currently favoured.

FIGURE 9

Melatonin profiles (mean ± S.E.) under artificial photoperiod and under extended darkness of ewes previously entrained to L:D,14:10.

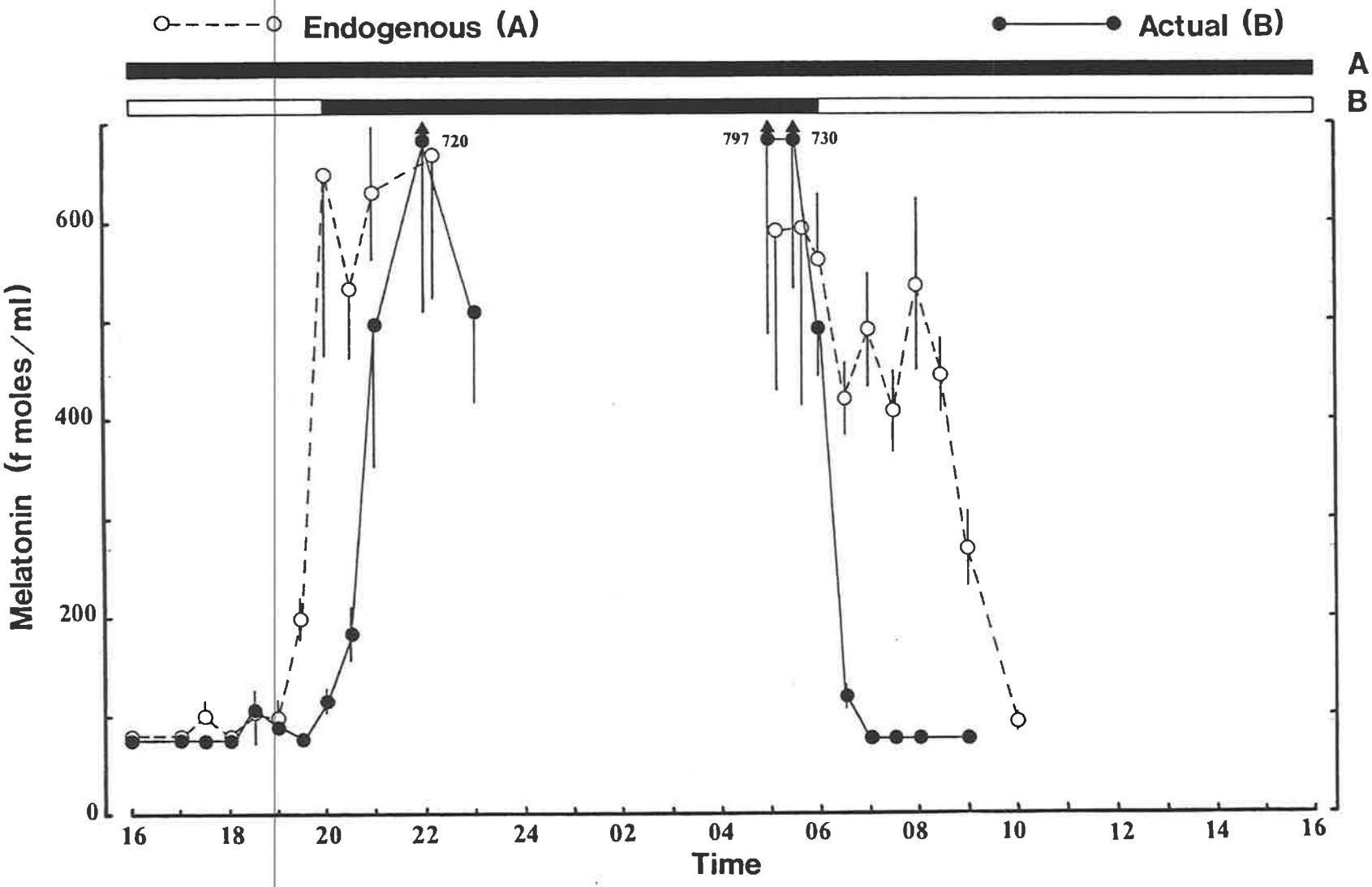
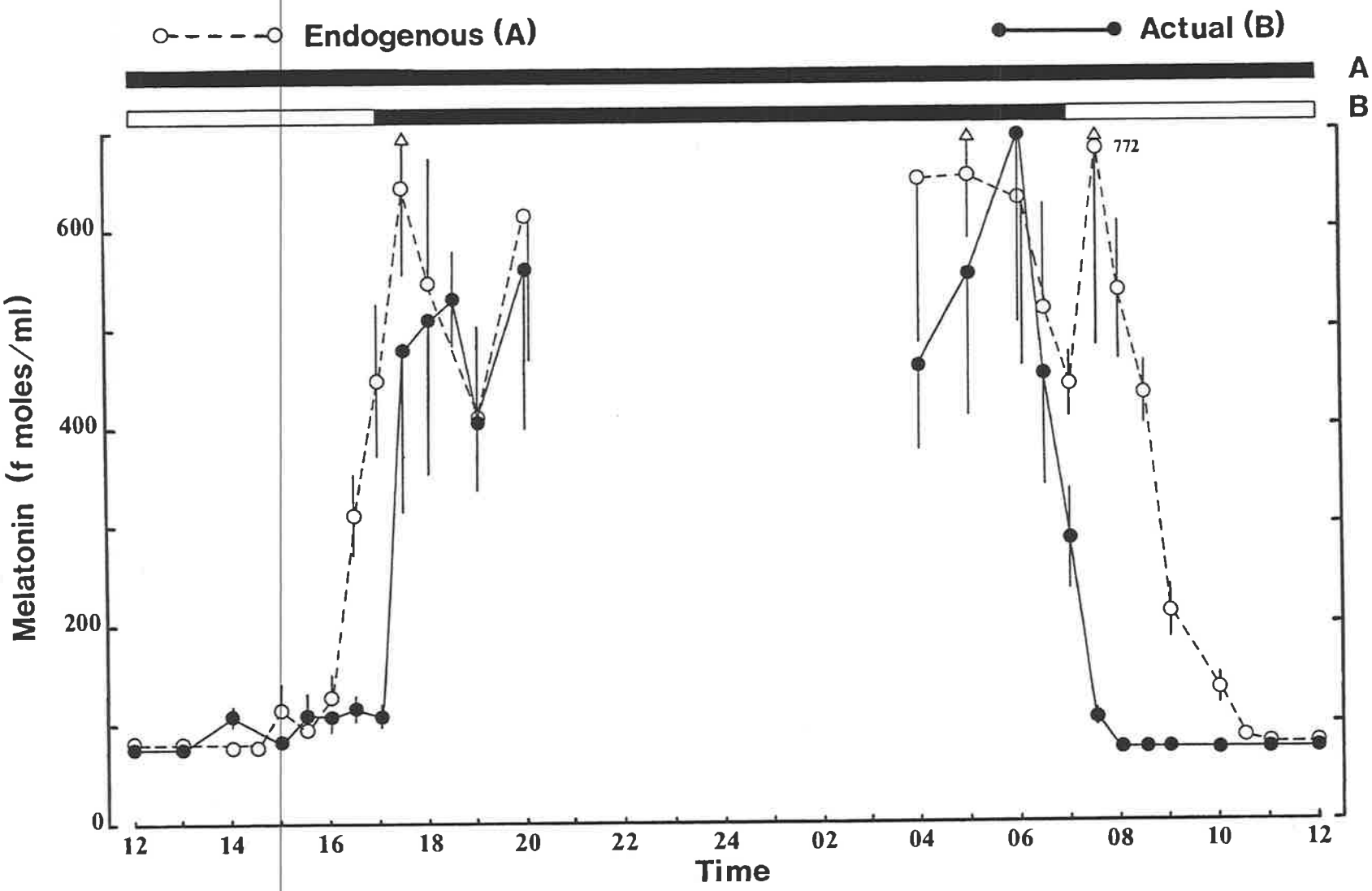

Endogenous (A)
Actual (B)
A
B
720
797
730
600
400
200
0
Melatonin (f moles/ml)
16
18
20
22
24
02
04
06
08
10
12
14
16
Time

FIGURE 10

Melatonin profiles under artificial photoperiod and under extended darkness of ewes previously entrained to L:D,10:14 (mean ± S.E.).


Endogenous (A)
Actual (B)
A
B
772
600
400
200
0
Melatonin (f moles/ml)
12
14
16
18
20
22
24
02
04
06
08
10
12
Time

6.0 EXPERIMENT 4

THE EFFECT OF DELAYING DUSK ON THE TIMING OF THE OFFSET OF MELATONIN SECRETION

6.1 INTRODUCTION

The results of the previous section indicate that exposure to photoperiods of different lengths alters the duration of melatonin secretion of animals under extended darkness. One of the possible interpretations of these findings is that the onset and offset of melatonin secretion may be controlled by separate but interacting pacemakers.

To further investigate this hypothesis an experiment was conducted in which the onset of melatonin was delayed and the effects on the timing of the offset of melatonin secretion under extended darkness monitored.

It was anticipated that this experiment may provide information which could help determine whether these two sections of the rhythm were under the control of separate pacemaking centres.

6.2 Materials and Methods

Twelve 2 year old Suffolk ewes were brought indoors in Spring and allocated to one of two groups which were maintained in separate light control rooms.

Ewes in both rooms were acclimatised to a lighting schedule of L:D,12:12, with the dark phase occurring from 1800h to 0600h. At the end of 4 weeks all ewes were bled at 15 minute intervals from 0400h to 0800h and from 1600h to 2000h to establish the times of termination and initiation of melatonin secretion, respectively. On day 2, subjective dusk for Group 1 ewes remained constant (1800h) while the period of darkness was extended from 0600h to 1100h. In Group 2 ewes

on day 2, subjective dusk was delayed by 4 hours until 2200h and the period of darkness was also terminated at 1100h. To characterise onset and offset of melatonin production on day 2, ewes in Group 1 were bled at 15 minute intervals from 1600h to 2000h and those in Group 2 from 1600h to 2400h. Both groups of ewes were bled at 15 minute intervals from 0400h to 1100h on day 2.

6.3 Statistical Analyses

The method for determining the time points for onset and offset of melatonin secretion have been outlined in the materials and methods section and comparisons within and between treatments were made using analysis of Variance and Students t-test.

6.4 Results

Melatonin profiles of ewes in Groups 1 and 2 are shown in Figures 11 and 12 respectively. On day 1 the times of onset and offset of melatonin production did not differ between ewes in Group 1 (1812 $\pm$ 0.1h and 0615 $\pm$ 0.3h, respectively) and Group 2 (1812 $\pm$ 0.1h and 0636 $\pm$ 0.2h). A delay in subjective dusk of 4 hours on day 2 delayed onset of melatonin production in group 2, (2148 $\pm$ 0.2h) by more than 3.5 hours ($P<0.05$) compared with Group 1 (1845 $\pm$ 0.1h). On day 2, termination of melatonin secretion in Group 1 (0842 $\pm$ 0.3h) occurred later than on the previous day. Termination of melatonin secretion could not be ascertained for Group 2 on day 2 since only 2 of the 6 ewes had ceased melatonin

secretion at the end of sampling (Fig. 2).

6.5 Discussion

In the current experiment the offset of melatonin secretion under extended darkness was delayed by approximately two and a half hours under extended darkness whereas the time of onset of melatonin secretion did not change. The behaviour of offset in this experiment is therefore similar to that observed when animals were released from L:D,10:14 and L:D,14:10 to extended darkness (Experiments 3(a) and (b)) and together these studies suggest that the position of the pacemaker regulating the offset of melatonin secretion is masked by light over a range of photoperiods.

When dusk was delayed the offset of melatonin secretion under extended darkness occurred at least 2 hours later than in control animals. These results suggest that the mechanisms determining the time of offset of melatonin secretion are influenced by the mechanisms controlling the onset of melatonin secretion.

The results of this study are again readily accommodated by both the single and multiple pacemaker models. If pineal rhythms are controlled by a single pacemaker it is possible that delaying the time of lights off allows light to cause a phase delay this single pacemaker resulting in a delay in the time of cessation of melatonin secretion. Alternatively pineal rhythms may be controlled by a regulatory mechanism comprised of two interacting pacemakers. In this model the delay in the time of lights off may allow light to delay the pacemaker controlling the time of offset of secretion or it may cause a delay in the pacemaker

controlling the onset of secretion and this pacemaker may interact with the pacemaker controlling offset and delay it. Pineal rhythms may also be controlled by a regulatory mechanism which contains more than two pacemakers and the delay in the time of lights off may cause a complex set of interactions which results in a delay in the timing of the offset of melatonin secretion.

The present study provides further information on the manner which the pineal pacemaker in the sheep behaves when acted on by light. All that can be concluded from the present study is that if the melatonin rhythm is controlled by the previously described two pacemaker systems, then under the conditions of this study the two pacemakers are not independent since the position of the pacemaker controlling onset could not be altered without changing the position of the pacemaker controlling the offset of melatonin secretion.

FIGURE 11

The melatonin profiles (mean ± S.E.) of 6 Suffolk ewes under entrainment to L:D,12:12 (Day 1) and also subjected to delayed dawn (Day 2).

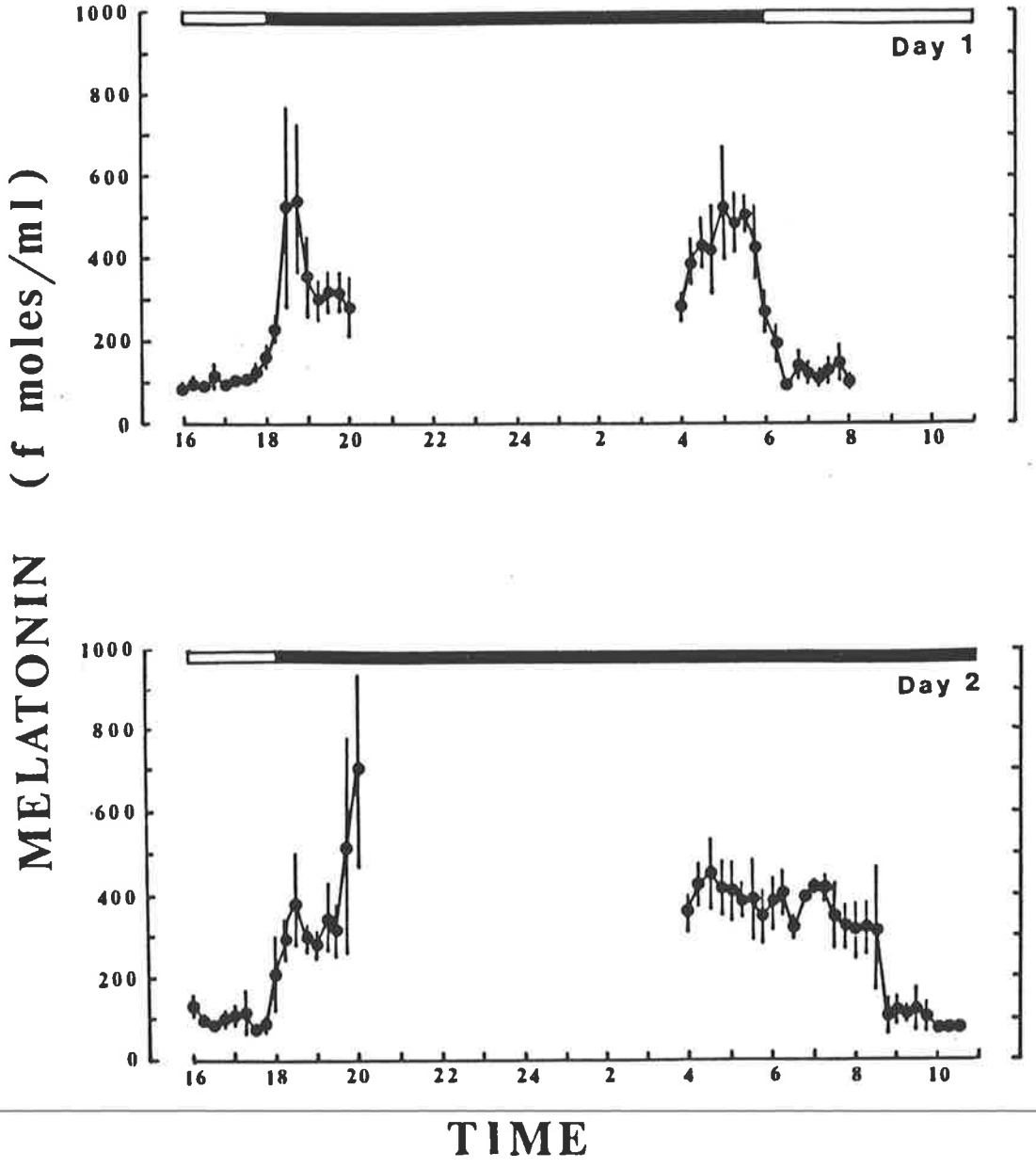
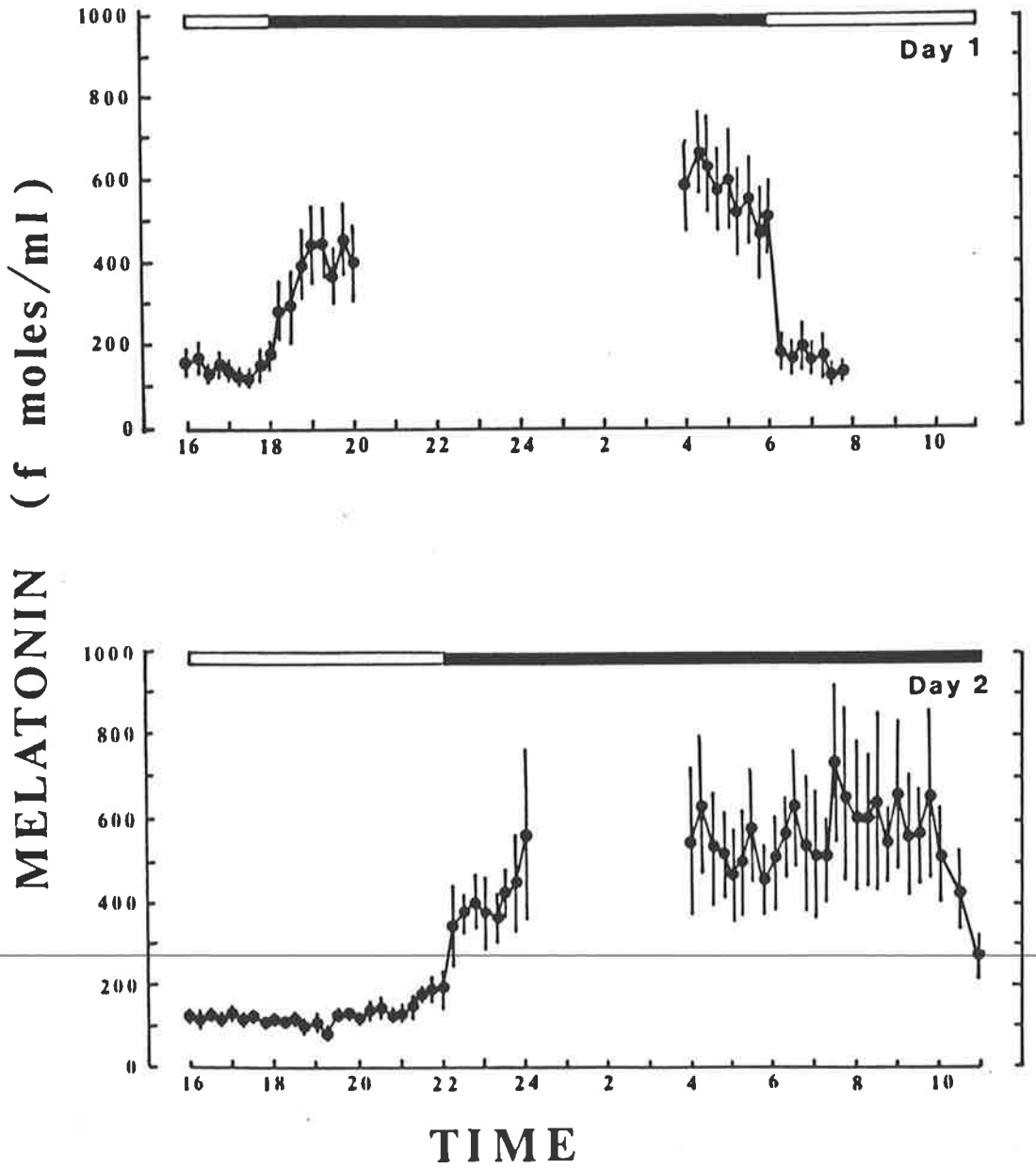

MELATONIN (f moles/ml)
Day 1
Day 2
1000
800
600
400
200
0
16
18
20
22
24
2
4
6
8
10
TIME

Figure 12

The melatonin profiles (mean ± S.E.) of 6 Suffolk ewes in which the timing of dusk was delayed by 4 hours (Day 1) and also on the following day when dawn was also delayed (Day 2).


Day 1
Day 2
1000
800
600
400
200
0
16
18
20
22
24
2
4
6
8
10
MELATONIN (f moles/ml)
TIME

7.0 EXPERIMENTS 5, 6(a)and (b) and 7

STUDIES OF THE ONSET AND OFFSET OF MELATONIN SECRETION UNDER CONTINUOUS DARKNESS

7.1 EXPERIMENT 5

THE OFFSET OF MELATONIN SECRETION UNDER CONTINUOUS DARKNESS

7.1.1 INTRODUCTION

The results obtained so far in this thesis suggest that the offset of melatonin secretion delays when the inhibiting effect of light is removed irrespective of whether the animals were previously entrained to short or long photoperiods. In all of these studies the behaviour of offset was only monitored for one cycle. If offset of melatonin secretion is controlled by a simple single pacemaker then it should continue to show consistent delays over subsequent cycles in continuous darkness.

This proposition was investigated by monitoring the offset of melatonin for several cycles under continuous darkness.

7.1.2 Materials and Methods

Animals -

Six 2 year old Suffolk ewes maintained under field conditions were transferred into a light control room in autumn (1/5/84) and housed in individual pens.

Lighting -

During a 4 week acclimatisation period ewes were entrained to L:D,10:14 with subjective dawn occurring at 1100h. On the 30/5/84 lights were switched off at 2100h and remained off for the remainder of the experiment.

Blood sampling -

On 30/6/84 (day 0) blood samples were taken at 20 minute intervals from 0900h to 1300h to define the offset of melatonin production. During continuous darkness samples were taken as follows: day 1, 20 minute intervals from 0900 to 1600h; day 2, 20 minute intervals from 0900 to 1700h; day 3,

30 minute intervals from 1100 to 1600h and 20 minute intervals from 1600h to 1900h; day 6, 30 minute intervals from 1200h to 1200h the following day.

7.1.3 Results

Melatonin profiles for individual ewes are shown in figures 11(a)-(f). The mean interval between offsets of melatonin secretion during the first, second and third cycles were 25.4 $\pm$ .03 (mean $\pm$ SE; n=6), 24.8 $\pm$ .3 and 27.1 $\pm$ .4 hours respectively. The mean interval between offsets of the second and third cycle were significantly longer ($P<.05$) than that of the preceding cycles. The period of the mechanisms controlling the offset of melatonin secretion over the first six days in continuous darkness were calculated by regression analysis to be 25.8h, 27.6h, 25.4h, 25.4h, 27.4h, and 25.7h for animals 1-6 respectively.

7.1.4 Discussion

The decline in melatonin levels under continuous darkness were as abrupt as that observed when the lights were turned off on the first day of the experiment. If this decline were caused by a rundown in substrate or enzyme as Lewy (personal communication) has suggested a more gradual drop in concentration would have been expected.

This raises questions concerning the mechanisms which act to cause such an abrupt decline in melatonin concentration. Under normal photoperiods it is known that light acts to reduce the turnover of norepinephrine resulting in a decline in (N.A.T.) activity and melatonin secretion (Deguchi and Axelrod 1972, Klein and Weller 1972). Moreover this happens so rapidly that it is thought that N-acetyl transferase is actively broken down in some manner (Brownstein 1977). Under continuous darkness the rapid decline cannot be explained as an effect of light. It must then be caused by some endogenous mechanism. Whether this mechanism controls the whole rhythm or just the offset of melatonin secretion cannot be answered by this study alone.

The melatonin profiles of the six ewes studied show that the rhythm in the offset of melatonin secretion persisted for six days in continuous darkness. The period of this pacemaker appeared to be greater than 24 hours since the offset in melatonin secretion occurred later each day in all animals. The melatonin profiles of sheep maintained in continuous darkness have also been monitored by Lincoln and coworkers (Lincoln et al 1985). In their studies the onset of melatonin secretion was used as a marker for the melatonin

rhythm. Their results indicated that the period of the offset of secretion was initially longer than 24 hours but shorter than 23 hours after the third consecutive day in continuous darkness. The period of the offset of melatonin production was not calculated by Lincoln et al (1985), however it is apparent from their data that the period of the offset was not greater than 24 hours since after 10 days in continuous darkness there was no delay in the times of cessation of melatonin production in any of the four animals in relation to the times of cessation on day 1. There are several possible explanations for the differences in period length of the melatonin rhythm between the current study and that of Lincoln et al (1985).

Firstly there may be species differences in period of the mechanisms controlling melatonin secretion between the Soay and the Suffolk.

Alternatively the mechanisms controlling melatonin secretion may have been influenced by the preceding photoperiod as it is known that prior exposure to photoperiods of different lengths can alter the period of a rhythm (Pittendrigh et al 1976). The rams used in the study of Lincoln et al (1985) were previously exposed to L:D,16:8 whereas the ewes in the current study were previously on L:D,10:14.

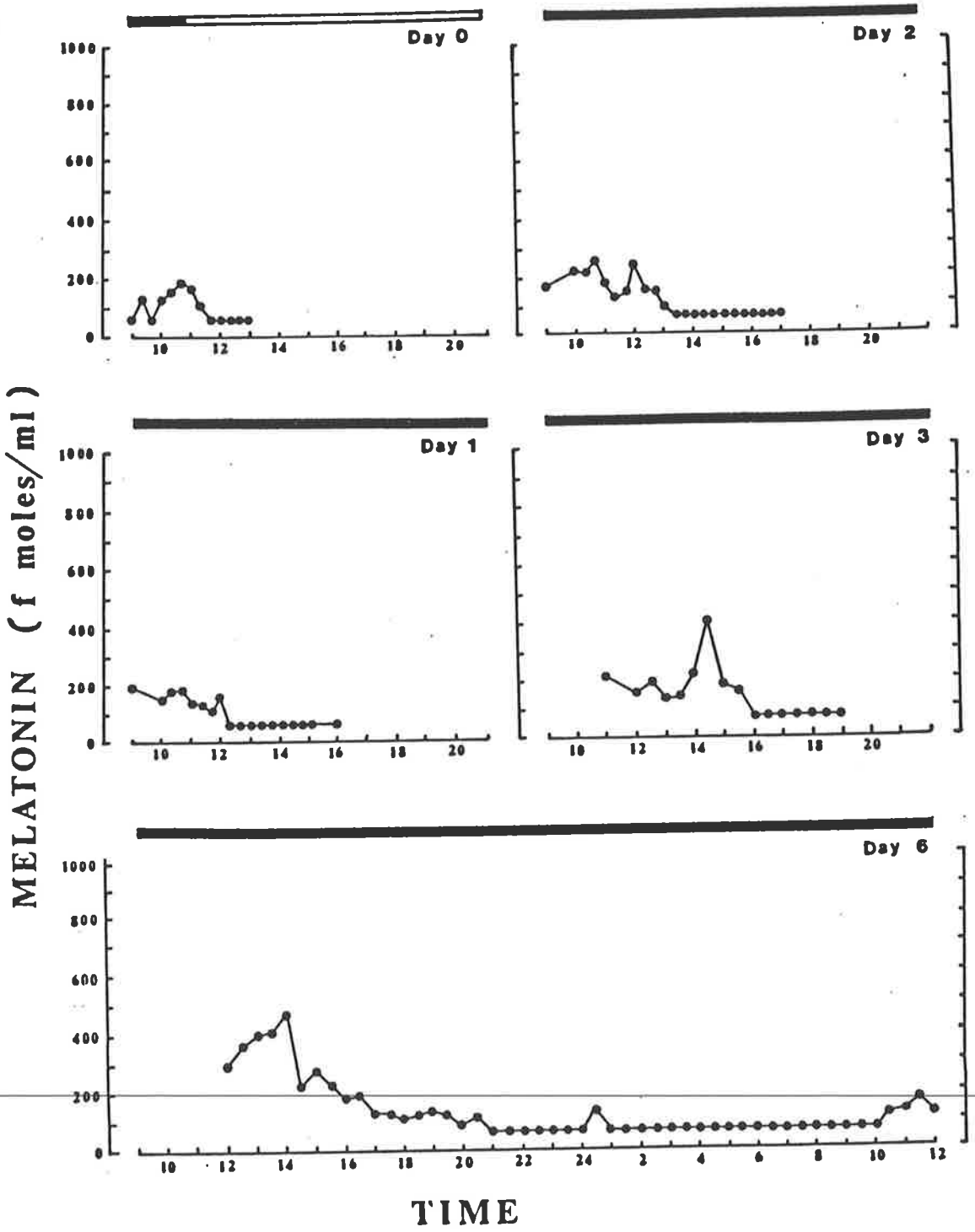
Differences in the period of the melatonin rhythm were noticed soon after the animals were placed into continuous darkness in both studies. In the current study the periods of the first two cycles were similar (25.4 .03 and 24.8 .3) but the third cycle (27.1 .4 P>.05) was longer. In Lincoln,s experiment the period changed from being greater than 24 hrs

during initial cycles to less than 24 hrs after the third cycle. These observations can be readily explained by the two oscillator model. In this model the removal of the constraining influence of light could allow the pacemakers controlling onset and offset to express the nett result of the forces acting on them. Each pacemaker,s movement would then be influenced by its intrinsic period and its interaction with the other pacemaker. The changes in the period of onset and offset during the first few cycles under continuous darkness may therefore provide valuable information about the pacemaking system which controls this rhythm.

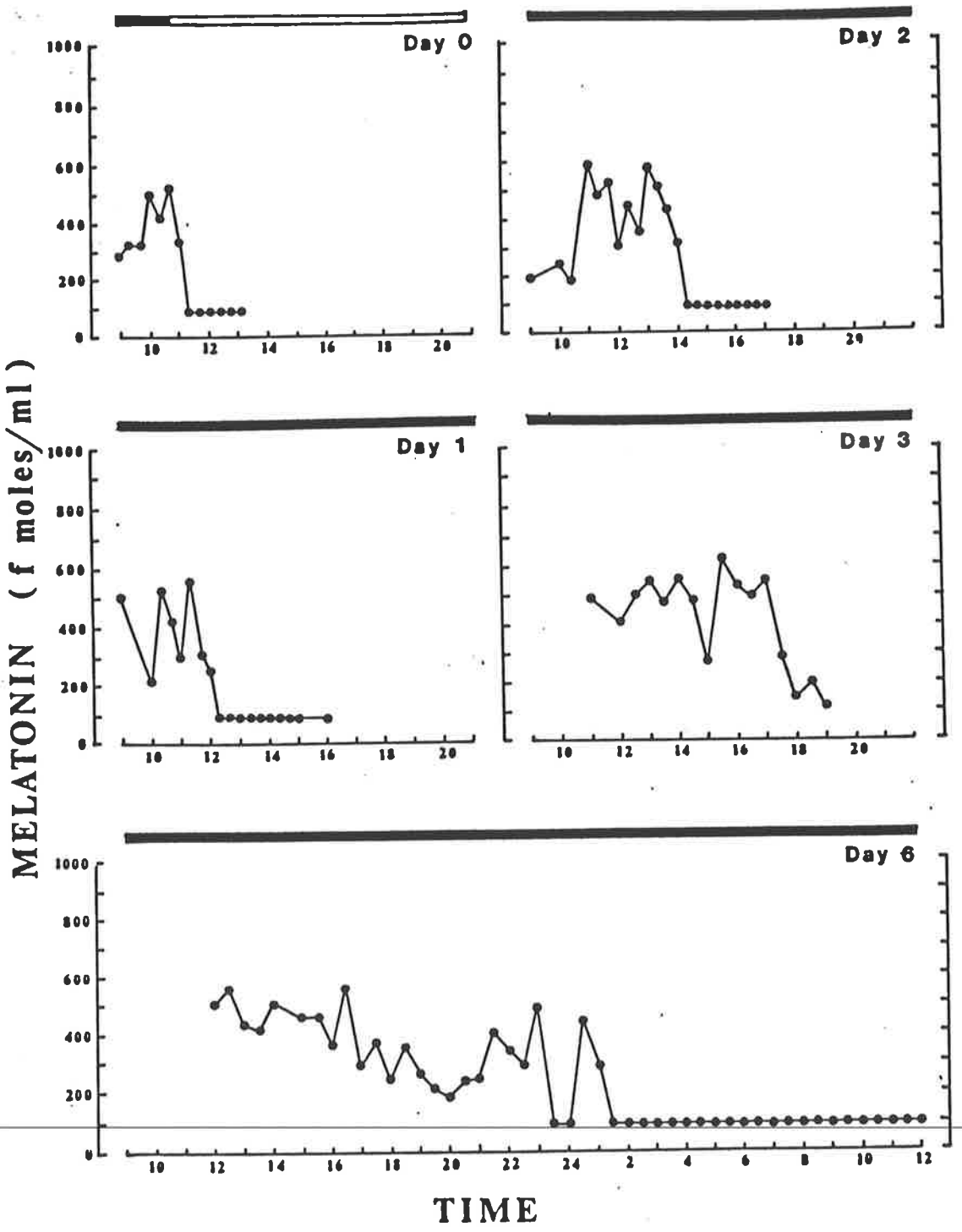
The explanations given above present the simplest explanations for the findings of these studies. More complex models in which many interacting pacemaking centres are involved could also explain these observations.

FIGURE 13

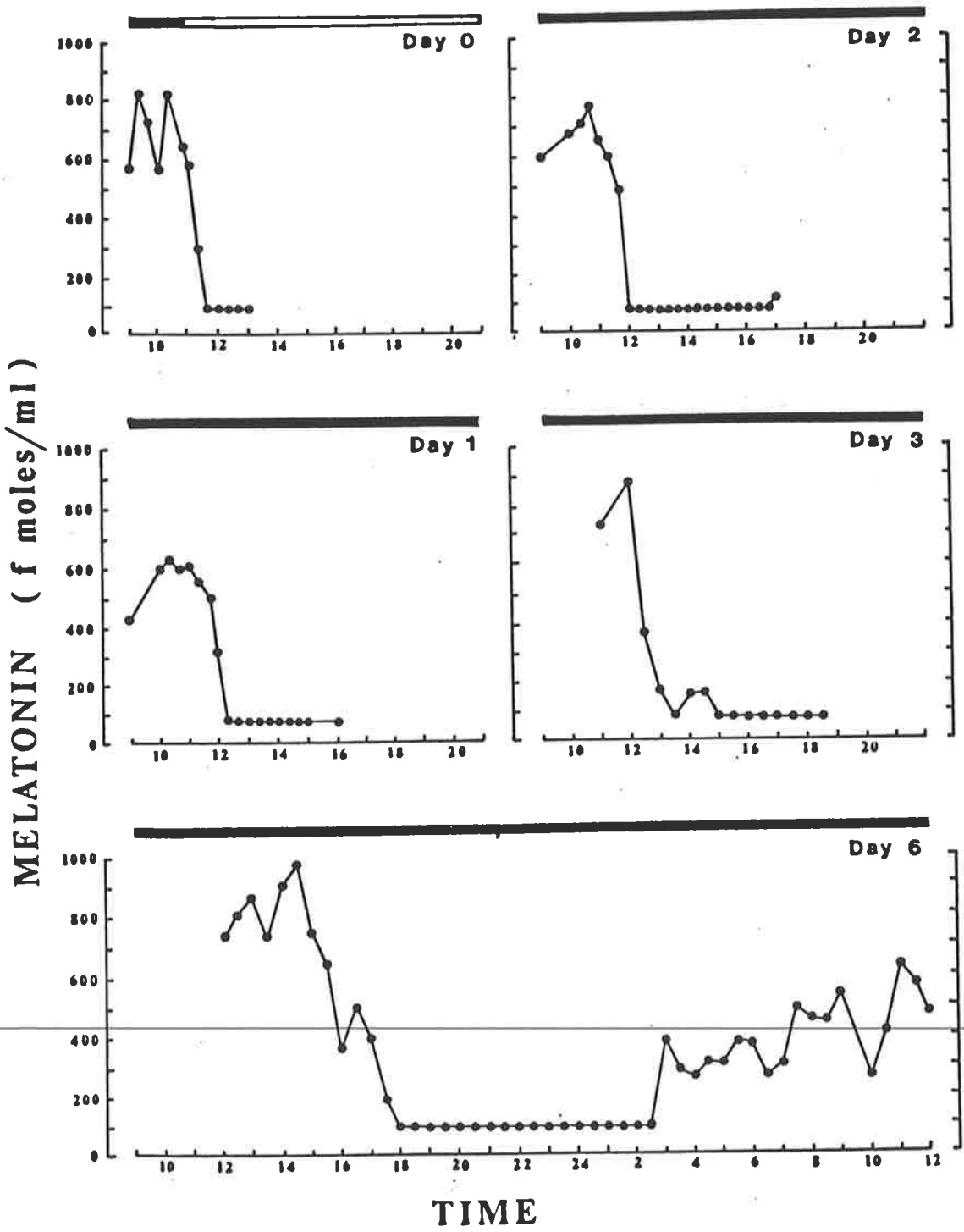
Figures 13 (a)-(f) display the offset of melatonin secretion of 6 Suffolk ewes over the first three days and also on day 6 under continuous darkness.



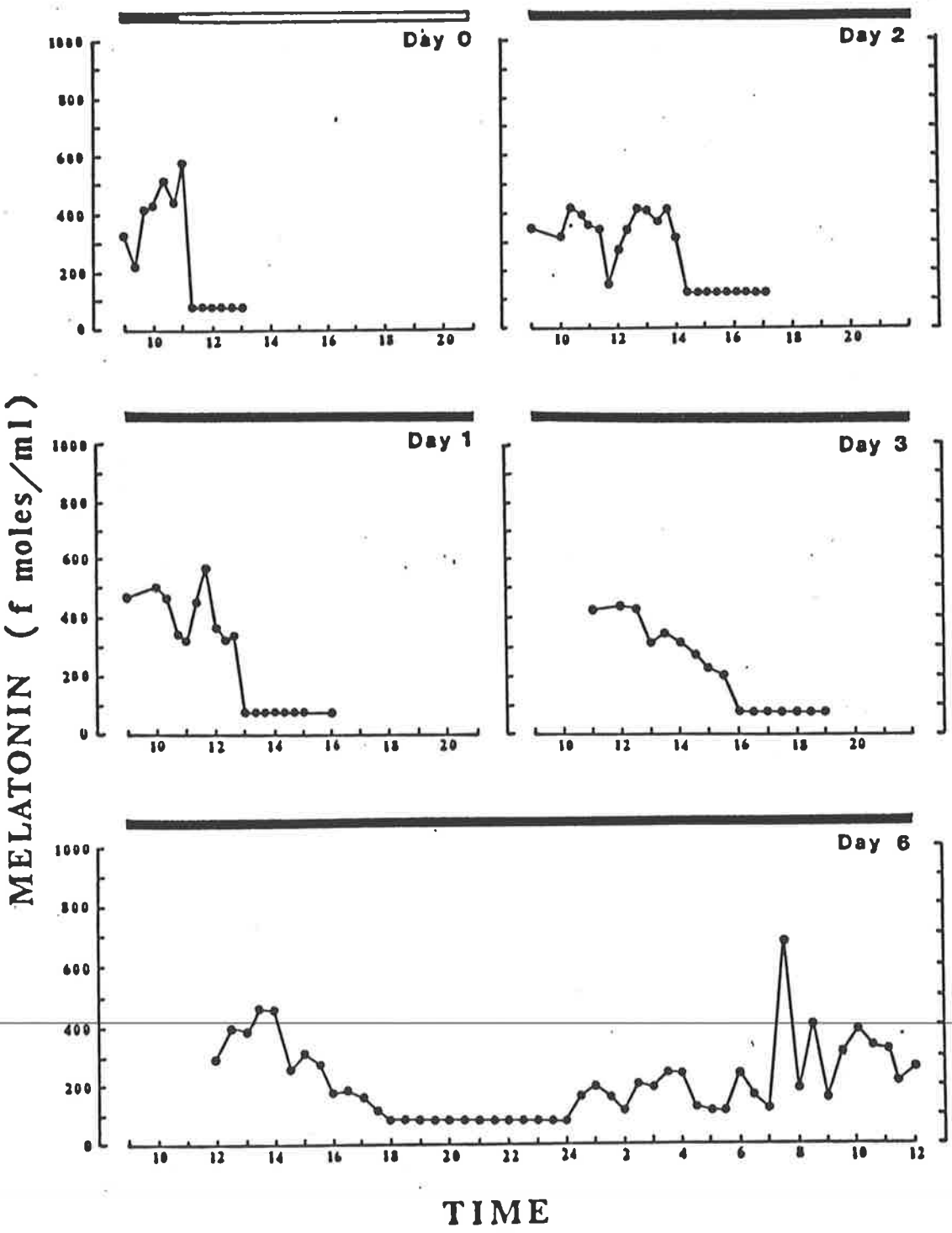
13a



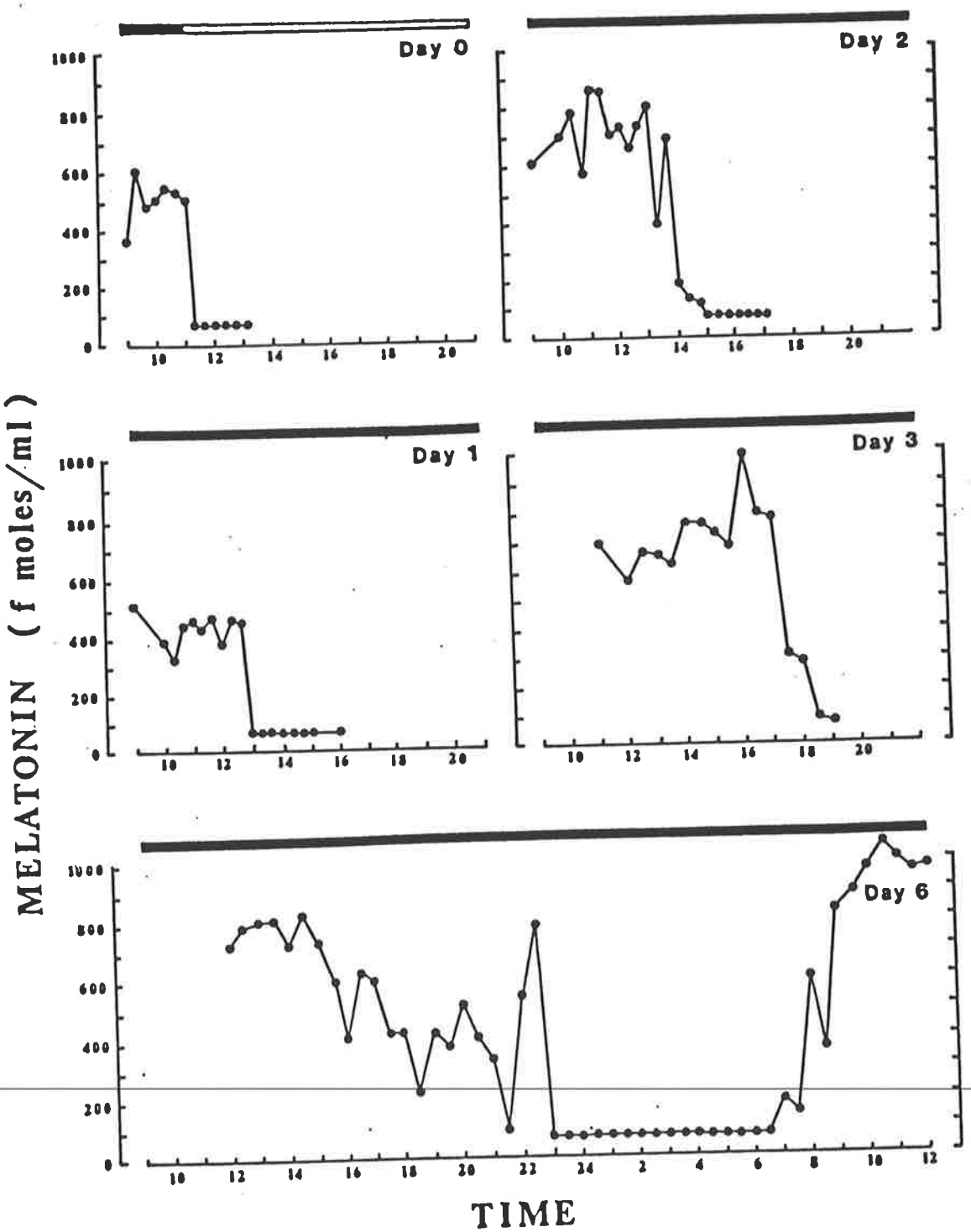
13b



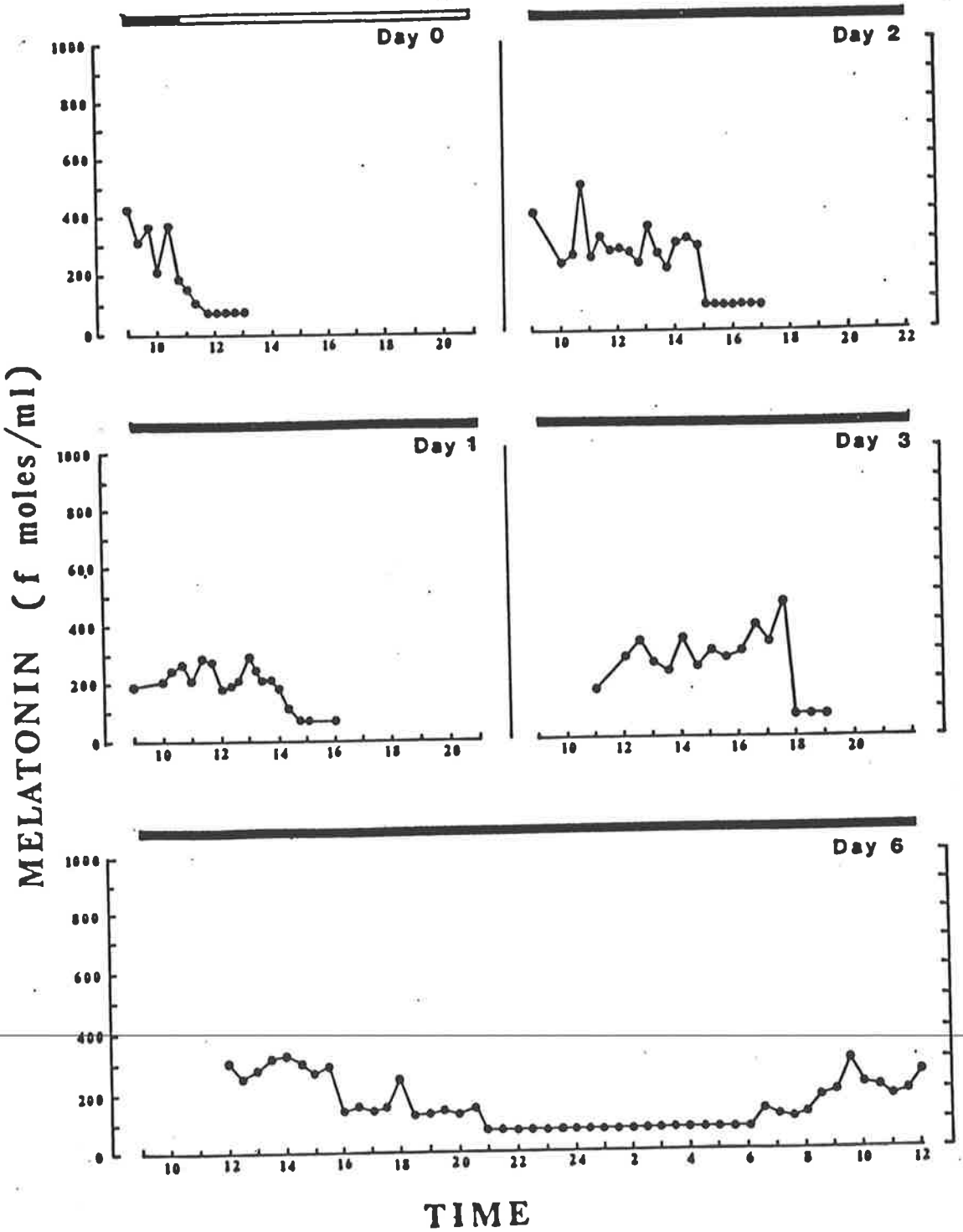
13c



13d



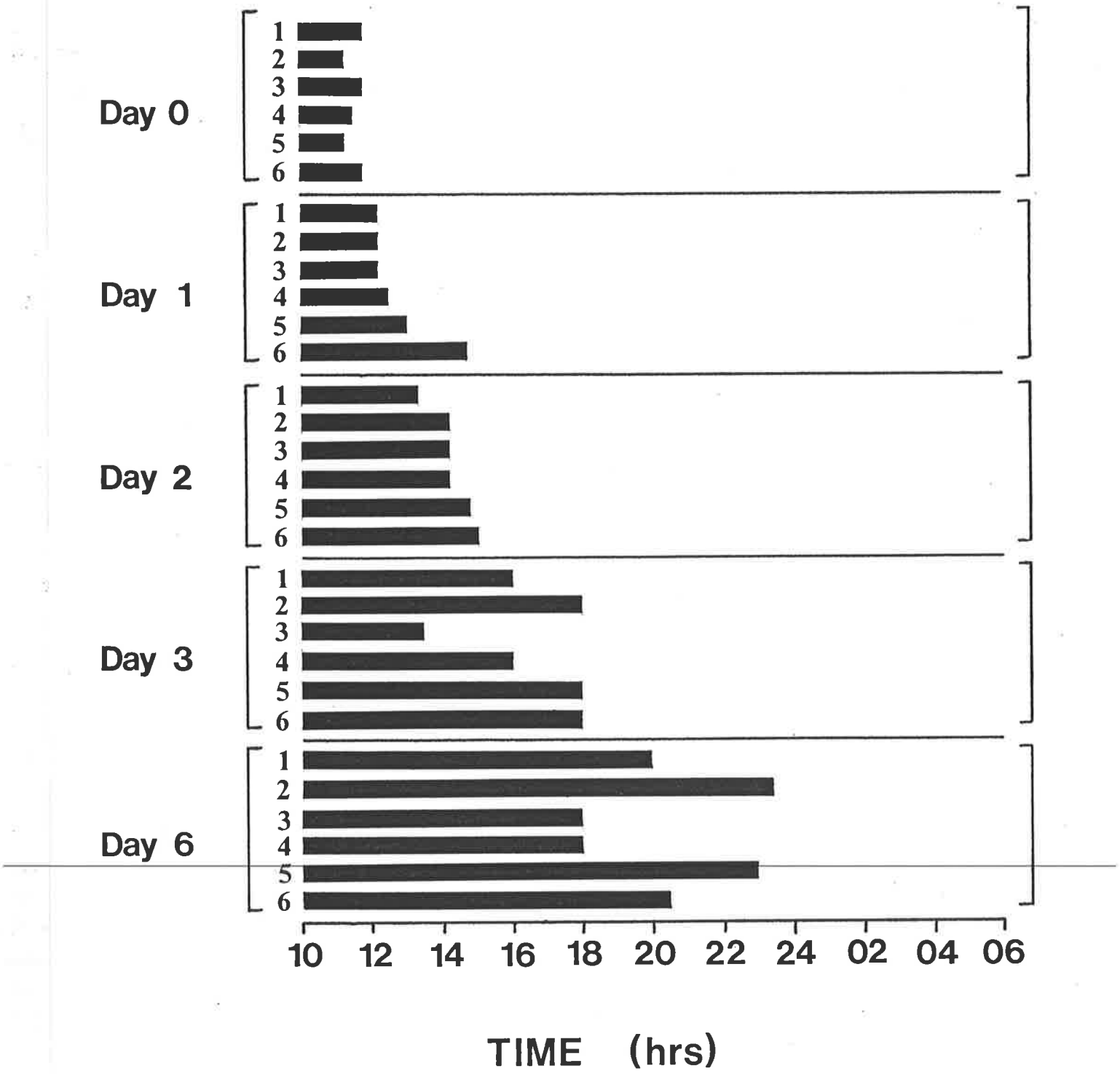
13e



13f

Figure 14

Figure 14 provides a visual display of the times of the offset of melatonin secretion for each animal over the course of the experiment. The right hand end of each black bar is the time of offset of secretion for each animal. The time of onset of secretion for each animal was not determined in this experiment.


Day 0
Day 1
Day 2
Day 3
Day 6
1
2
3
4
5
6
10
12
14
16
18
20
22
24
02
04
06
TIME (hrs)

7.2 EXPERIMENTS 6(a) and (b)

ON THE ONSET OF MELATONIN SECRETION IN EWES RELEASED INTO CONSTANT DARKNESS DURING AUTUMN AND WINTER.

7.2.1 INTRODUCTION

In the previous section the offset of melatonin secretion in sheep appeared to be controlled by a circadian regulatory centre which had a period greater than 24h.

To gain information on the behaviour of the onset of melatonin secretion in sheep exposed to continuous darkness, the onset of melatonin production was monitored under continuous darkness at two different times of the the year.

7.2.2 Materials and Methods

Six 2 year old Suffolk ewes were brought indoors in autumn and housed in individual pens. During a 4 week acclimatisation period the ewes were entrained to LD, 10:14 with dawn occurring at 0800h. In the following winter (19/7/84) the experiment was repeated but in this group subjective dawn occurred at 0100h. After lights off on the first night the animals remained in darkness for the remainder of the experiment.

To determine more precisely the time of onset, 20min. samples were taken over short intervals when it was anticipated onset would occur. On the last day 30 minute samples were taken for a 24h period to determine the position of the rise after a period under continuous darkness.

In the autumn group sampling commenced at 1400h and finished at 2000h on day 0. On day 1 sampling occurred between 1300h and 1900h; day 2 from 1000h until 1600h with two further samples at 1700h and 1800h; on day 3 from 0800h to 1400h with hourly samples until 1800h. On day 4, 30

minute samples were taken from 0700h until 1800h.

In the winter group in which subjective dusk occurred at 1100h, sampling commenced at 0800h on Day 0 and continued until 1400h. On day 1 sampling occurred between 1000h and 1500h; day 2 from 1000h until 1600h; day 3 from 1100h until 1700h and on day 4, 30 minute sampling was carried out from 0700h until 1800h.

7.2.3 Results

In the initial experiment conducted in winter the sampling regime was directed at more precisely monitoring the advance in the onset of melatonin secretion indicated by previous research (Lincoln et al 1985, Bittman et al 1983). The onset of melatonin secretion in this group did not appear to be as closely associated with the time of lights off as that observed in experiments 3(a) and 3(b). The onsets of melatonin secretion therefore could not be analysed because they did not fall within the sampling period. The results have only be included because the differ markedly from those obtained when this experiment was repeated the following autumn

Since the onset of melatonin secretion obviously did not advance under extended darkness it was decided to repeat the experiment to monitor an expected delay in the onset of melatonin secretion under continuous darkness in the following autumn..

In three of the four ewes studied (1,3 and 4) the onset of melatonin secretion advanced over the course of the experiment. In ewe 2 the onset of melatonin secretion was

delayed. In ewe 5 onset was delayed on day 4 but its behaviour in the intervening period is unclear. The remaining animal exhibited continuously high melatonin levels and this abnormal animal was excluded from the group.

7.2.4. Discussion

In animals brought indoors in winter the sampling times were designed to monitor an expected advance in the onset of melatonin secretion. This advance was expected from the results obtained in the experiments reported in section 5.0. However no advance in the onset of melatonin secretion was observed and the entrainment of the animals in this experiment to the light dark cycle was less obvious than in previous experiments. Another experiment was therefore set up in the following autumn and this time the sampling periods were timed to monitor an expected delay in the onset of melatonin secretion. On the last day of the experiment a prolonged sampling was conducted in case the rhythm moved in an unexpected direction.

The onset of melatonin secretion in animals brought indoors in autumn was closely associated with the time of lights off. However in continuous darkness there was considerable differences in the way the animals behaved under continuous darkness. Animals 1,3, and 4 showed a clear advance in the onset of melatonin secretion whereas animal 2 clearly delayed. It has been suggested that light acts to delay the regulatory mechanism for melatonin onset so that it advances as soon as the suppressive effect of light is removed. In animal 2 however onset occurs at lights off but in continuous darkness the onset is delayed. In this situation, also observed by Lincoln (1985) it would be of interest to know why onset still occurs at lights off. Possibly in individuals such as this ewe the response is determined by the period of the regulatory entraining

mechanism such that the onset of melatonin production is delayed to entrain to lights off if the regulatory mechanism has a period less than 24h and advanced to entrain to lights off if the period is greater than 24h.

The difference between the groups observed in the onset of melatonin is of special interest because it suggests that although animals may display the same duration of melatonin secretion, the mechanisms acting to generate this melatonin signal may be quite different. More information on these underlying factors may be important to our understanding of how a particular duration of darkness can be interpreted as long days or short days, depending on the prior photoperiodic history of the animal (Robinson 1985).

In the current study the differences in the course of the onset of melatonin secretion could have been caused by a number of factors. Firstly, the photoperiodic history of the ewes prior to the period of entrainment were different. The autumn group were experiencing shortening days while the winter group had already passed the winter solstice. The effects of prior photoperiodic history on period length have been considered in the review and may explain why the onset behaved differently in each case. Secondly the ewes were at different stages of their annual reproductive cycle and would therefore have had different levels of reproductive hormones which may have altered the behaviour of this rhythm since it has previously been shown that period length can be influenced by the level of these hormones (Ellis and Turek 1979, Morin et al 1977).

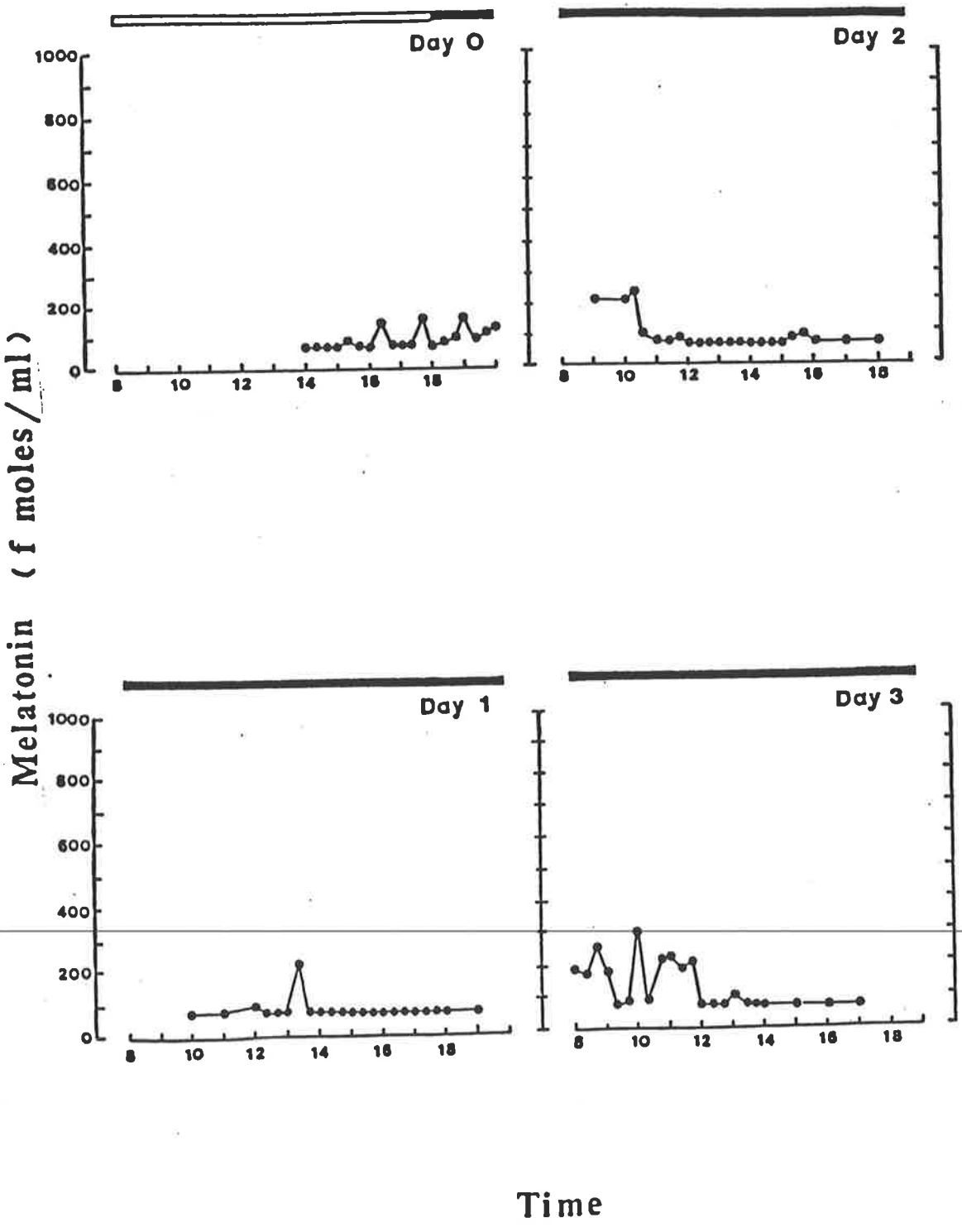
Thirdly the autumn group were subjected to a large

change in the time of lights off at the beginning of the entrainment period. The after effects of phase shifts have been reported to persist for several cycles but would not be expected to persist for four weeks (Pittendrigh and Minnis 1964). However this possibility cannot be excluded.

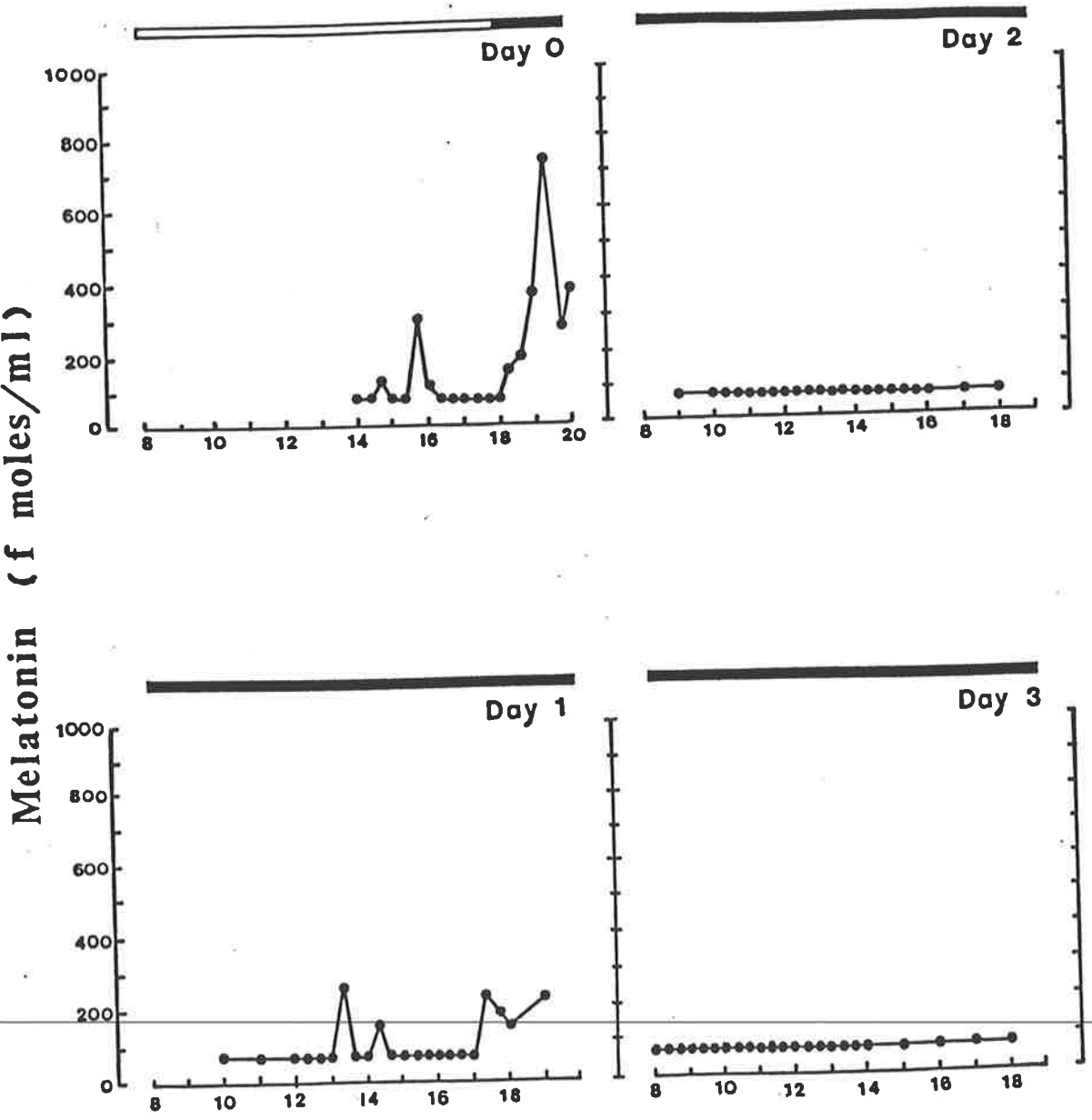
These two experiments indicate that the behaviour of the onset of melatonin production is highly variable and is not solely determined by the length of the photoperiod to which the animals have been entrained.

FIGURE 15

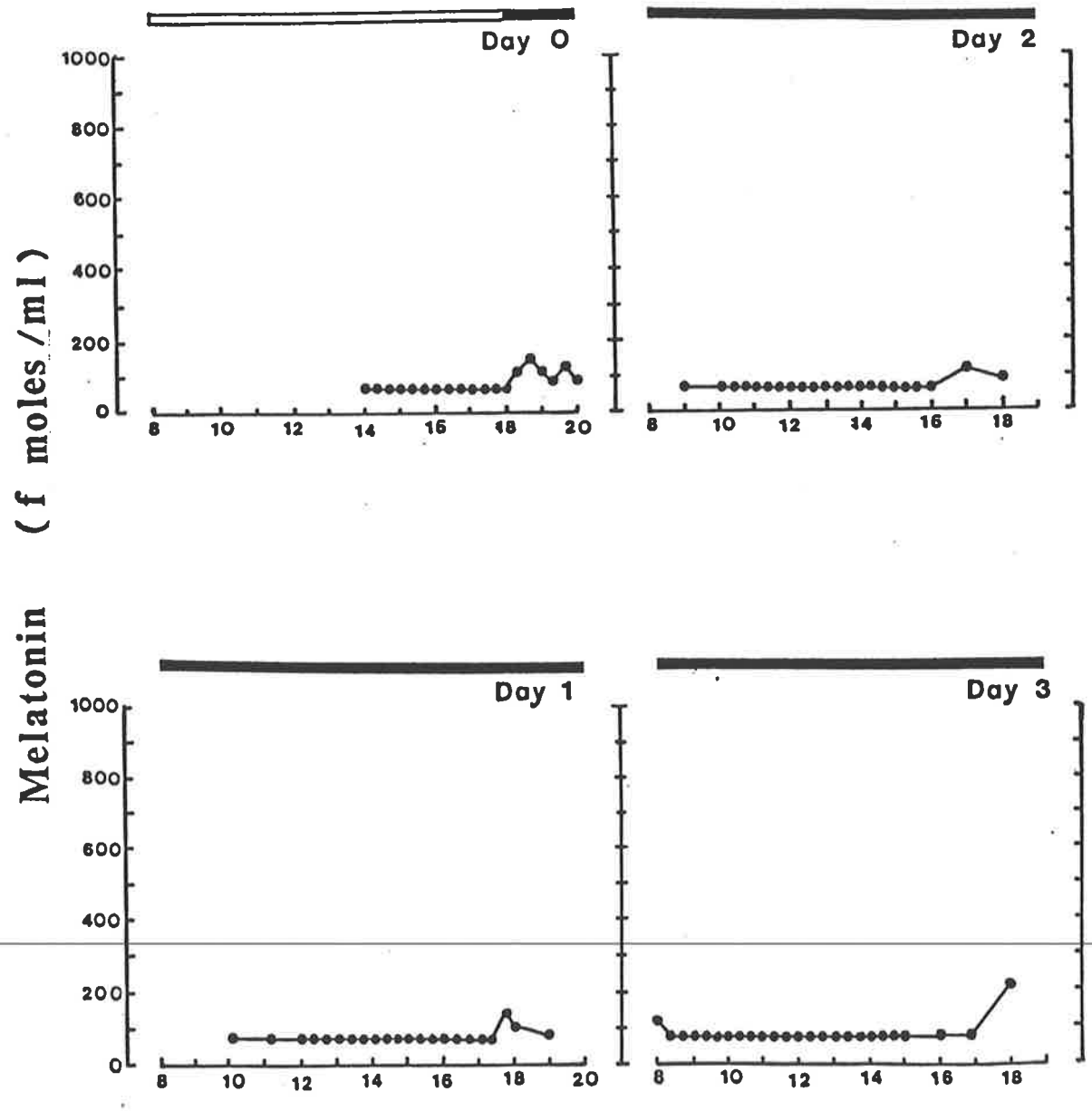
Figures 15 (a)-(e) display the onset of melatonin secretion of Suffolk ewes which were entrained to L:D,10:14 in autumn and then placed in continuous darkness.


Day 0
Day 2
Day 1
Day 3
1000
800
600
400
200
0
8
10
12
14
16
18
Melatonin (f moles/ml)
Time

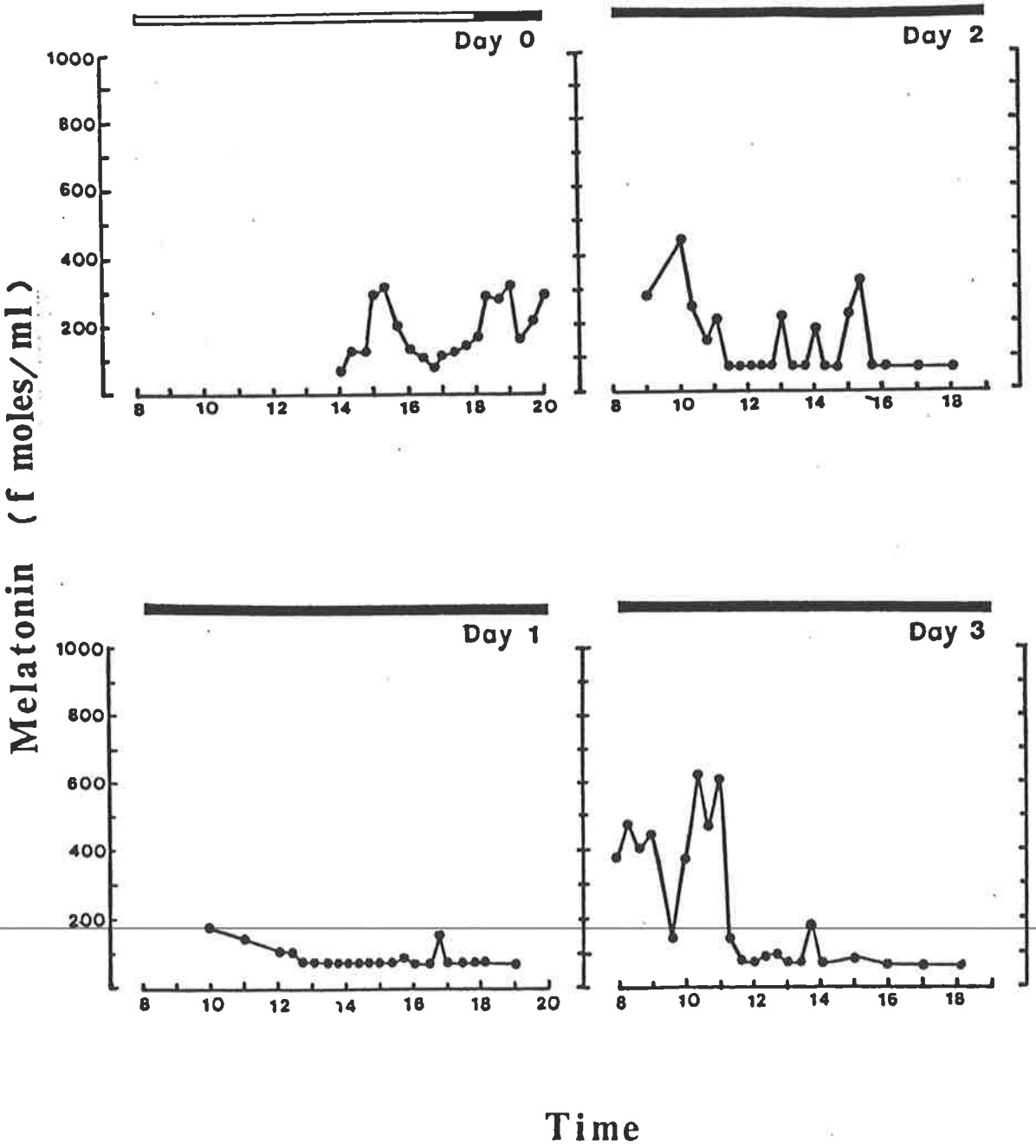
15a


Day 0
Day 2
Day 1
Day 3
1000
800
600
400
200
0
8
10
12
14
16
18
20
Melatonin (f moles/ml)
Time

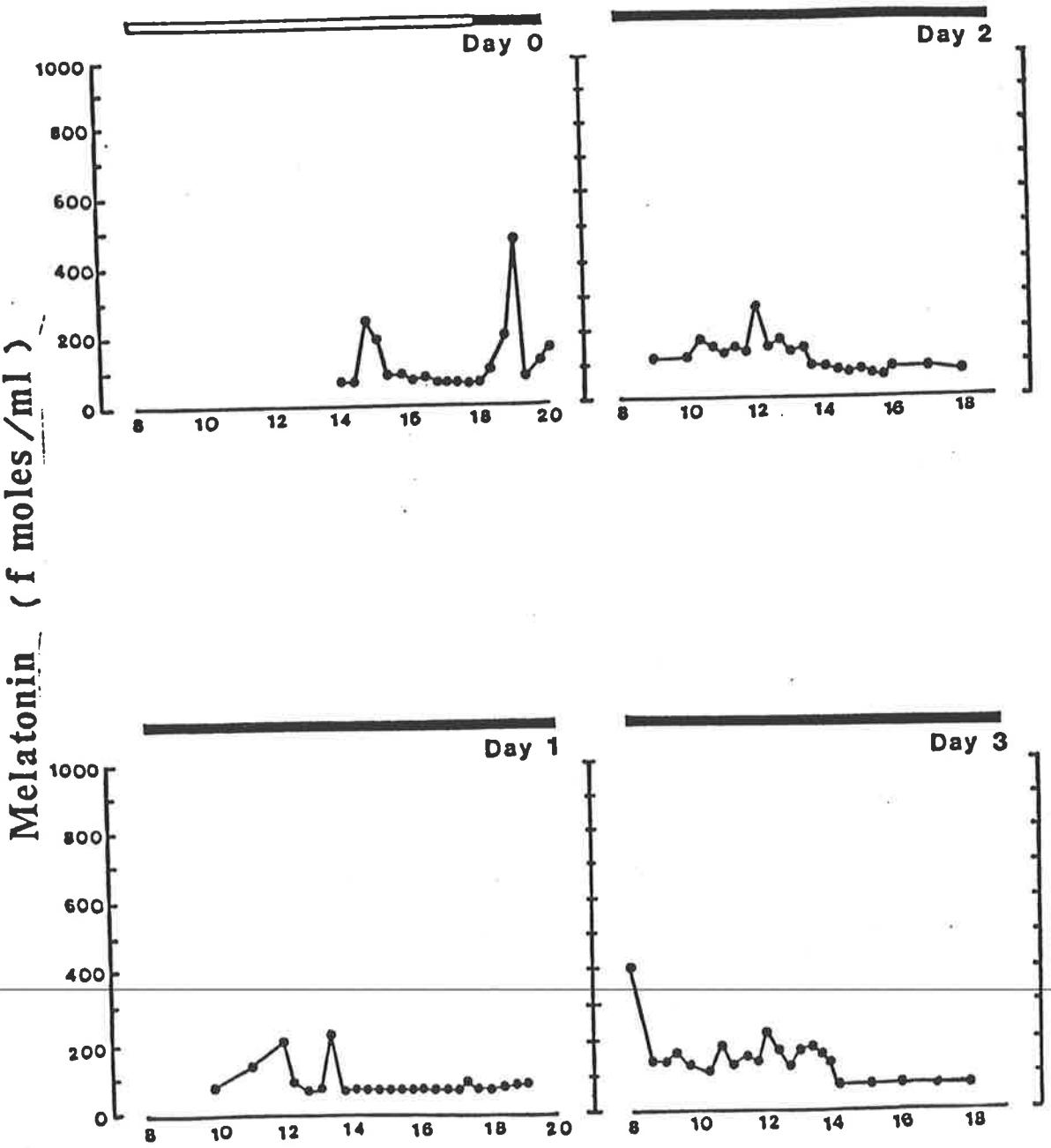
15b


Day 0
Day 2
Day 1
Day 3
1000
800
600
400
200
0
8
10
12
14
16
18
20
Melatonin (f moles/ml)
Time

15c


Day 0
Day 2
Day 1
Day 3
1000
800
600
400
200
8
10
12
14
16
18
20
Melatonin (f moles/ml)
Time

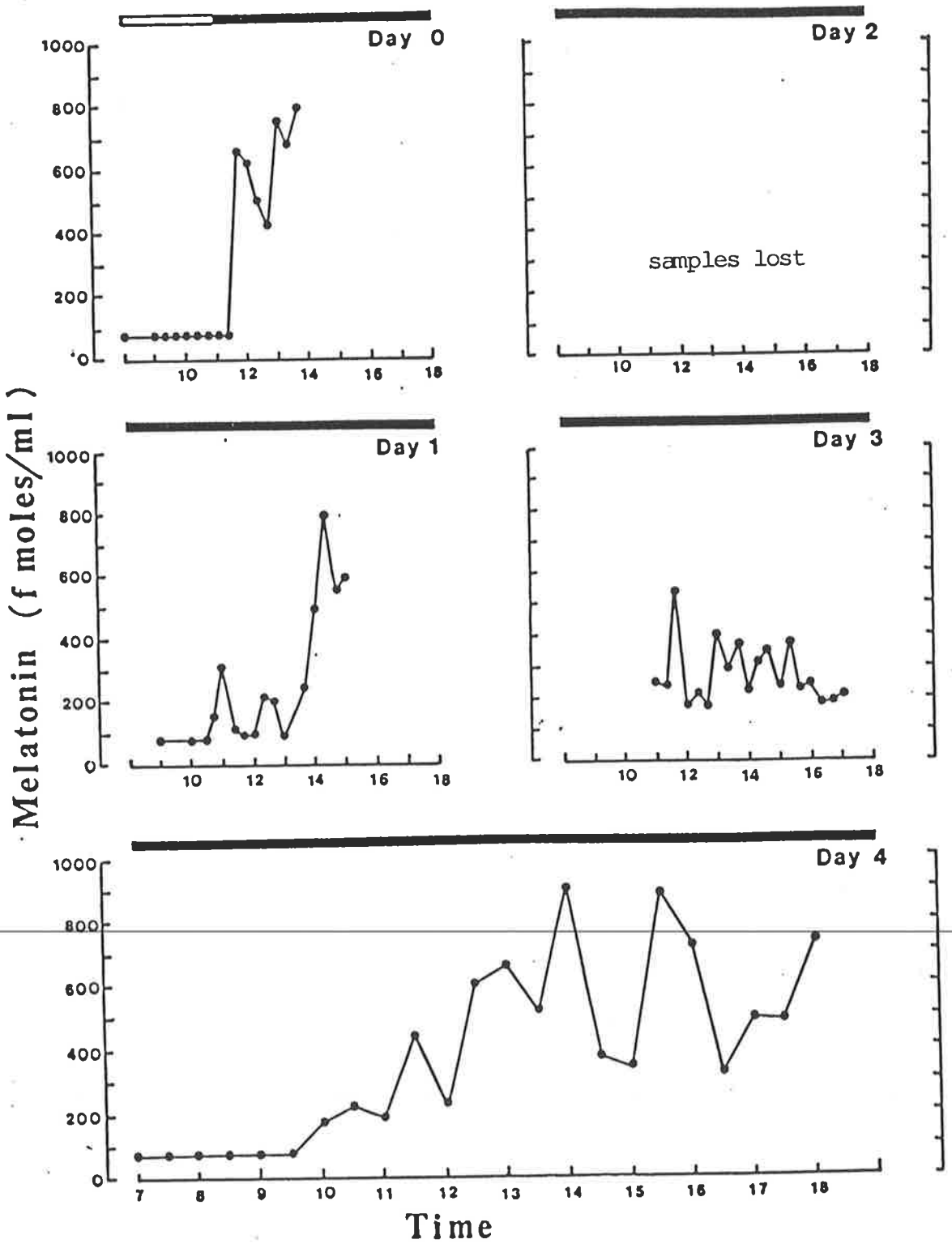
15d


Day 0
Day 2
Day 1
Day 3
1000
800
600
400
200
0
8
10
12
14
16
18
20
Melatonin (f moles/ml)
Time

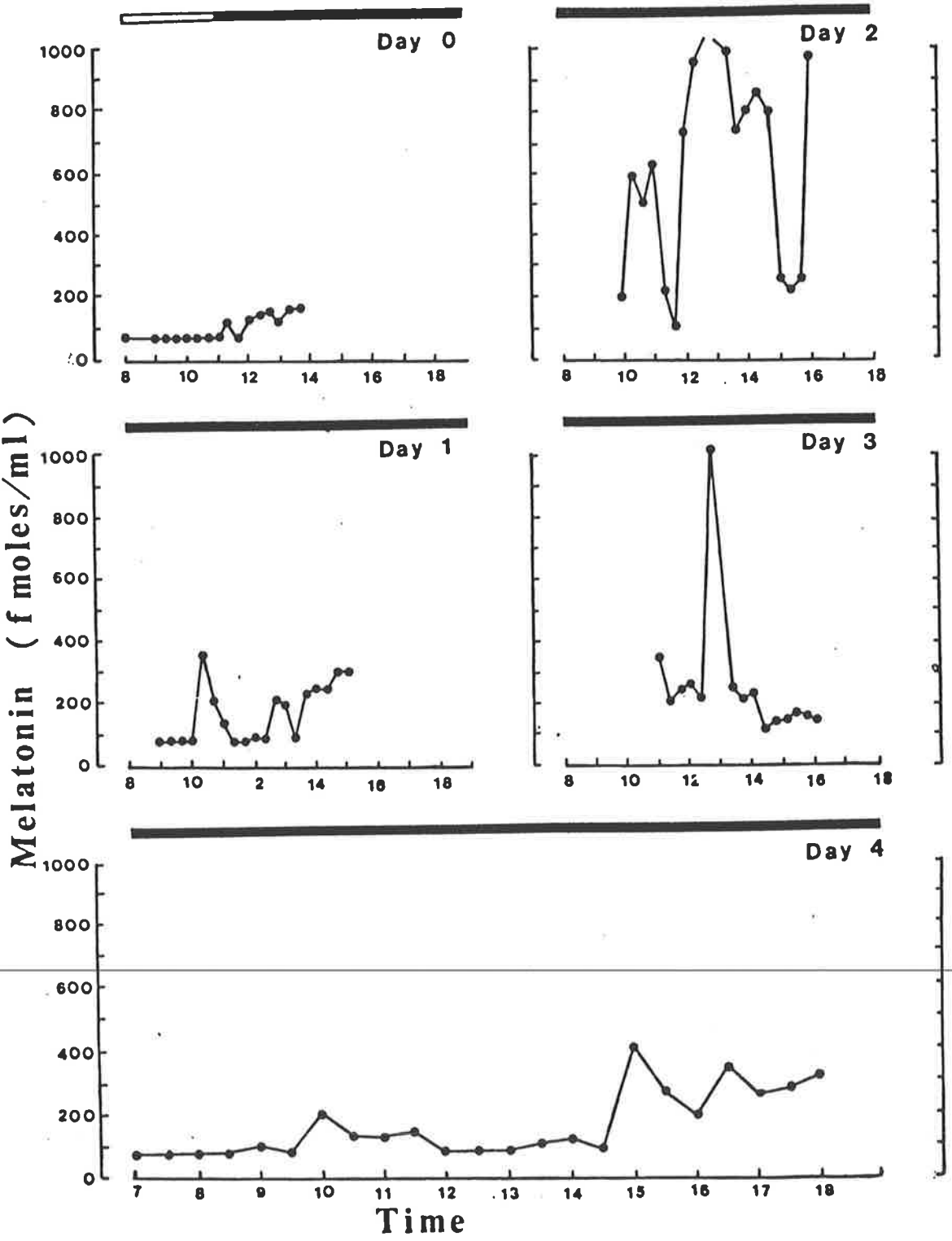
15e

FIGURE 16

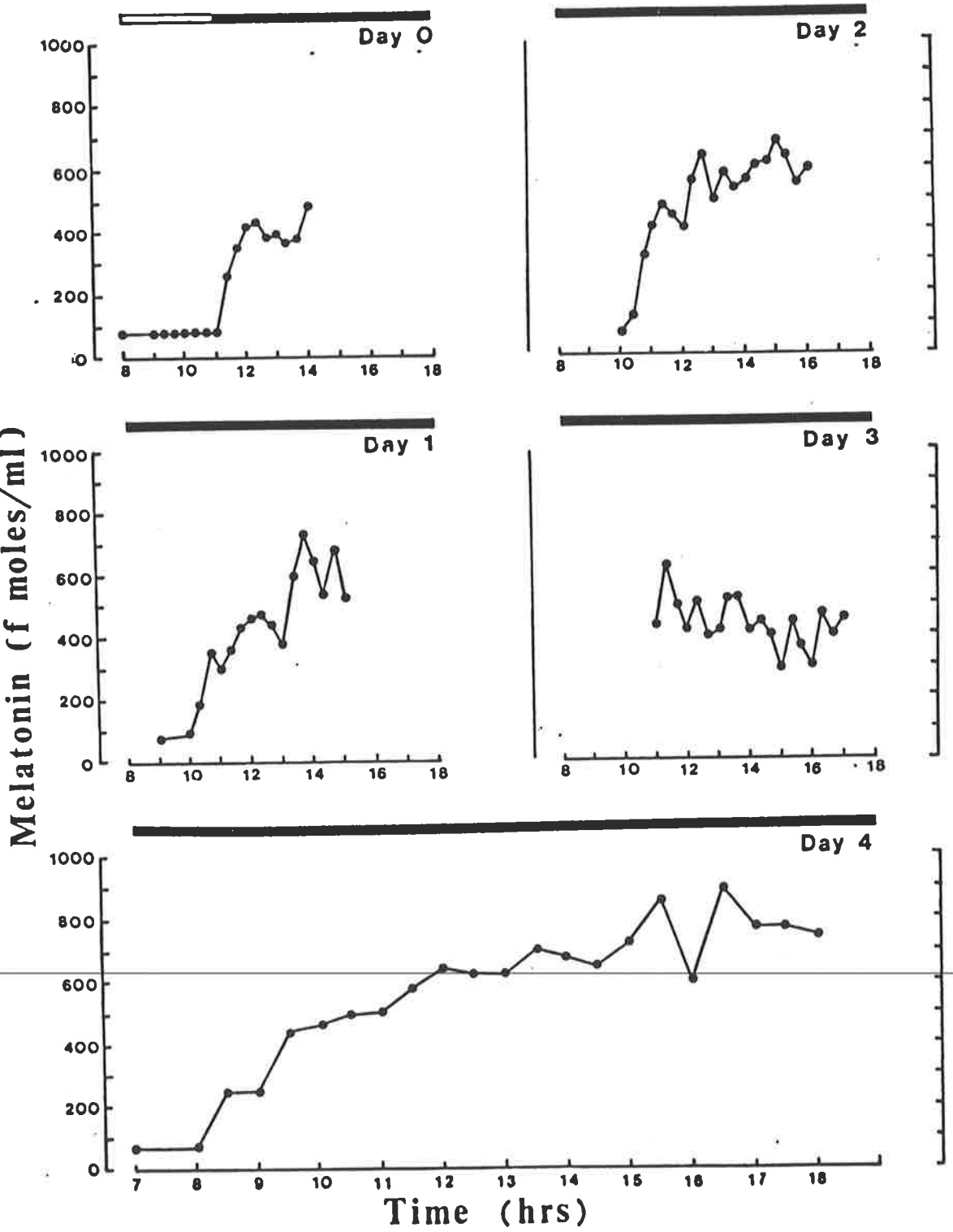
Figures 16 (a)-(e) display the onset of melatonin secretion in Suffolk ewes which were entrained to L:D, 10:14 in winter and then placed in continuous darkness.


Day 0
Day 2
samples lost
Day 1
Day 3
Day 4
Melatonin (f moles/ml)
Time
0
200
400
600
800
1000
7
8
9
10
11
12
13
14
15
16
17
18

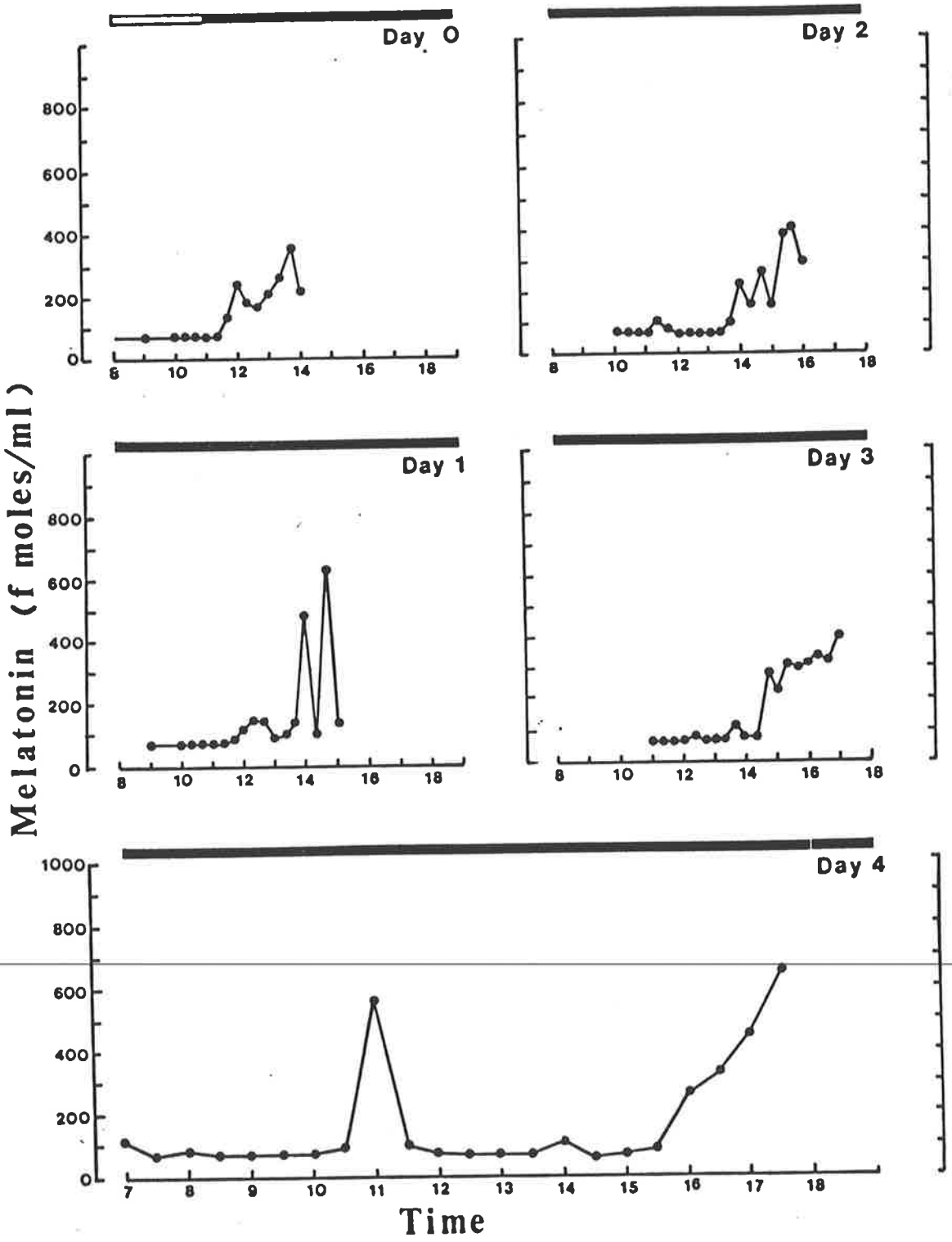
16a


Day 0
Day 2
Day 1
Day 3
Day 4
Melatonin (f moles/ml)
Time
0
200
400
600
800
1000
8
10
12
14
16
18
7
9
11
13
15
17

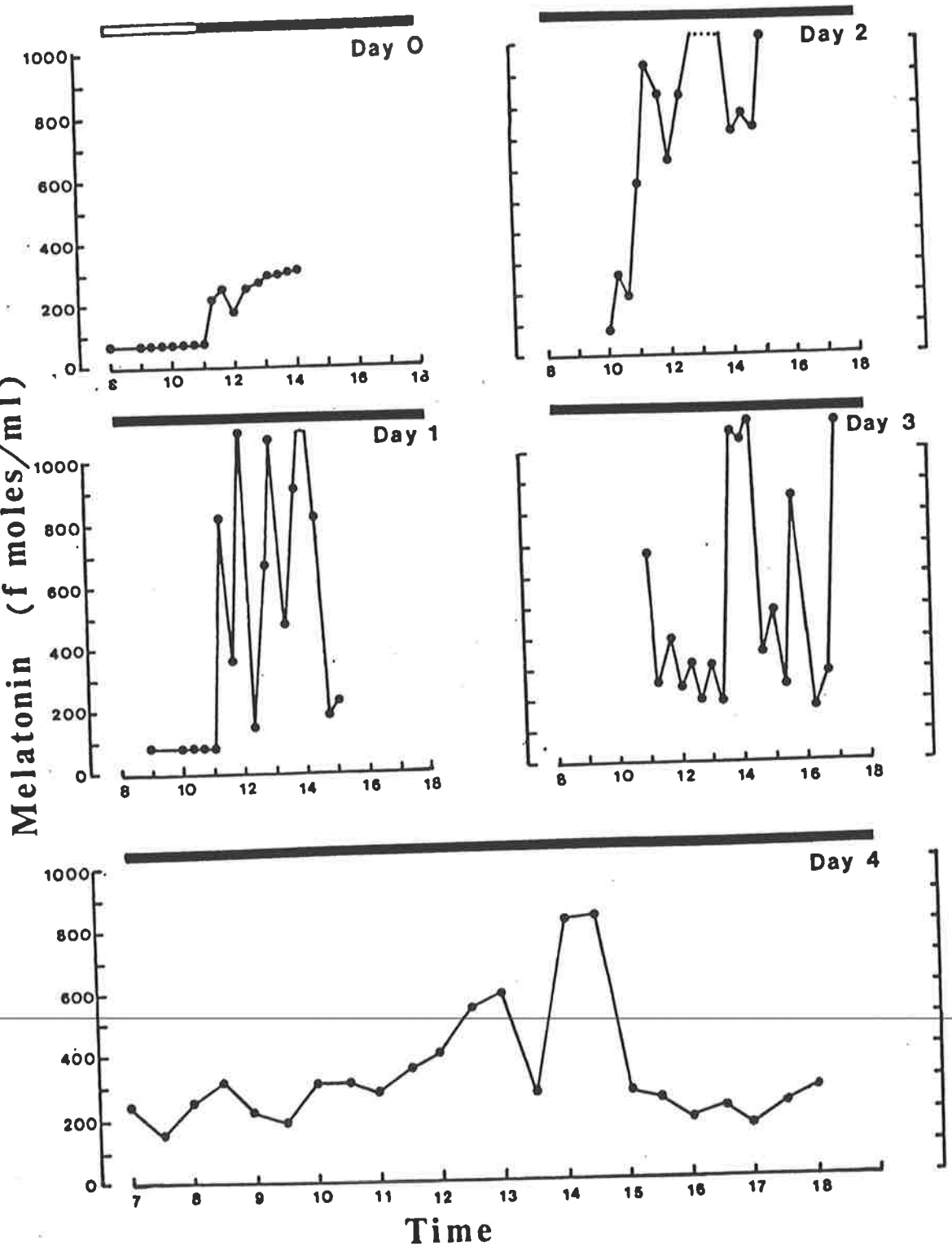
16b


Day 0
Day 1
Day 2
Day 3
Day 4
Melatonin (f moles/ml)
Time (hrs)
0
200
400
600
800
1000
7
8
9
10
11
12
13
14
15
16
17
18

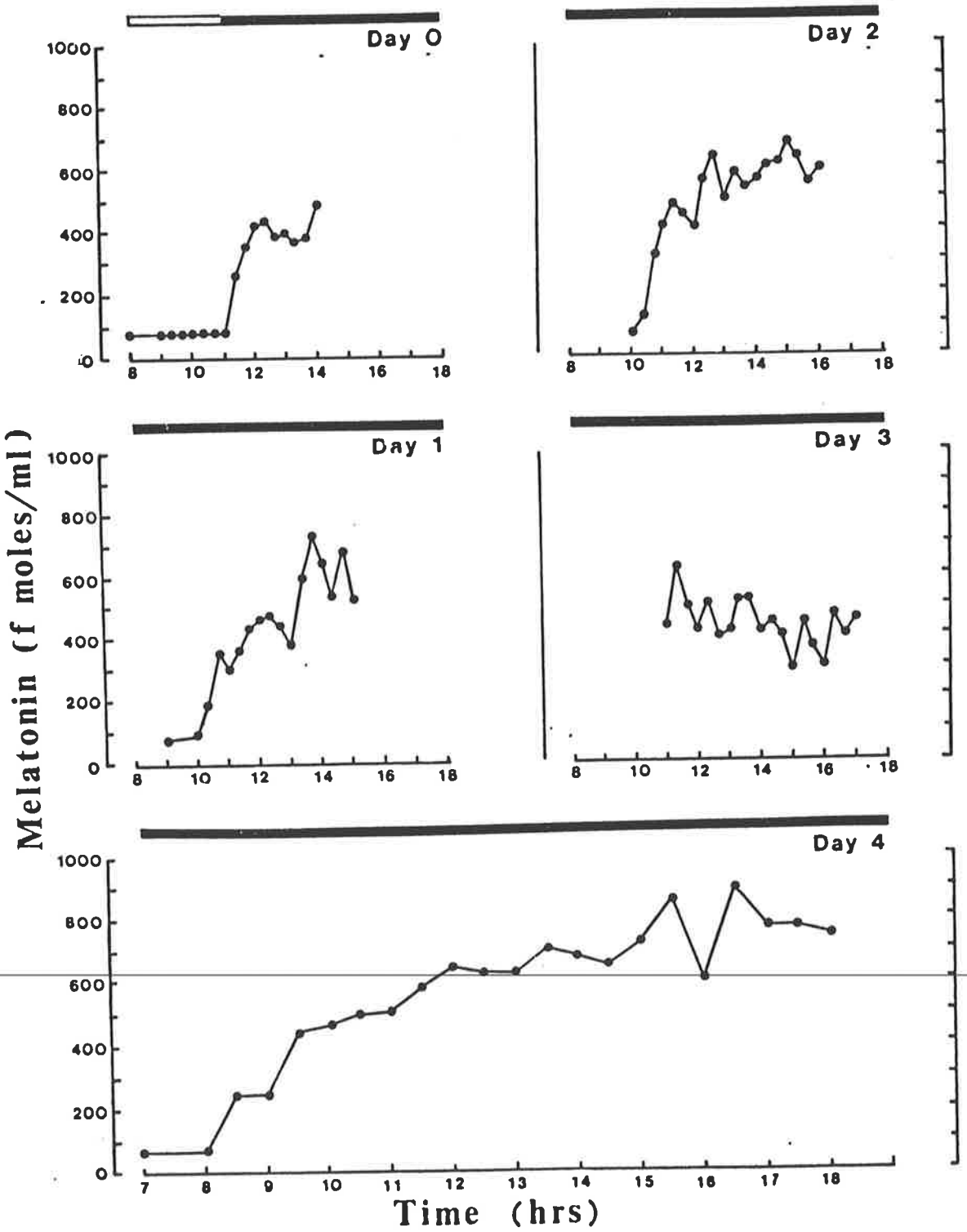
16c



16d


Day 0
Day 1
Day 2
Day 3
Day 4
Melatonin (f moles/ml)
Time
0
200
400
600
800
1000
7
8
9
10
11
12
13
14
15
16
17
18

16e


Day 0
Day 1
Day 2
Day 3
Day 4
Melatonin (f moles/ml)
Time (hrs)
0
200
400
600
800
1000
7
8
9
10
11
12
13
14
15
16
17
18

16f

7.3 EXPERIMENT 7

TEMPORAL CHANGES IN THE PATTERN OF MELATONIN SECRETION IN SHEEP HELD IN CONSTANT DARKNESS.

7.3.1 INTRODUCTION

Data obtained in initial studies of this section of the experimental work showed differences in the periodicity of onset and offset of melatonin secretion. Offset appeared to be controlled by a circadian pacemaker with a period greater than 24 hours. The studies of onset of melatonin secretion showed much greater variation with some animals showing advances and others delays within the same experiment. There was also an indication that the behaviour of the onset of melatonin secretion could be influenced by past photoperiodic treatment

These studies provided useful information on the behaviour on the behaviour of these two parts of the rhythm but did not help to determine whether they were controlled by separate mechanisms.

A further experiment was therefore conducted in which both parts of the rhythm were monitored in the same experiment. This allowed the behaviour of onset and offset to be compared within the same animals and therefore exposed to the same photoperiodic history. Any differences in offset and onset under these conditions may be due to differences in the way these two parts of the rhythm are controlled.

7.3.2 Materials and Methods

Six two year old Suffolk ewes were used in the experiment. They were brought indoors on the 3/12/85 and acclimatised to LD 14:10 for 4 weeks with subjective dawn occurring at 0600h. On day 1 of the experiment, 20 minute

blood sampling commenced at 0400h and finished at 0800h, recommencing at 1800h to terminate at 2200h. The lights were turned off at 2000h on day 1 and remained off for the rest of the experiment. On day 2, 20 minute sampling occurred between 0400h and 1300h and also between 1600h and 2200h. On day day 3, 30 minute samples were taken between 0600h and 1100h and between 1400h and 2000h. On day 4, 30 minute samples were collected between 0800h and 2100h and on day 5 between 0900h and 1700h. The final sampling period commenced at 0900h on day 9 and finished at 1200h on day 10.

7.3.3 Analysis and Results

The mean period between onset and offset on day 1, 2, 3 and 8 was 10.2 $\pm$ 0.3h (mean $\pm$ S.E.) 11.9 $\pm$ 0.3h, 15.0 $\pm$ 1.2h and 14.4 $\pm$ 0.8h respectively. The mean period between offsets for the 1st, 2nd, 3rd and 4th cycles was 25.8 $\pm$ 0.03, 24.7 $\pm$ 0.03, 24.9 $\pm$ 0.8 and 26.5 $\pm$ 0.3 respectively. The mean period over 8 cycles was 25.2 $\pm$ 0.2h. The mean period between onsets was 24.3 $\pm$ 0.1 and 22.0 $\pm$ 0.8 for the first and second cycles respectively. On day 3 some onsets did not occur during the sampling period, however, on day 4 all onsets of melatonin secretion occurred earlier than on day 2. On day 9 of the experiment the onset of melatonin secretion in all animals occurred later than on day 4.

7.3.4 Discussion

Under the entraining light regime, the duration of the daily episodes of melatonin secretion was constrained by light as indicated by an immediate and continuing expansion which occurred when light was withheld. During the time the ewes were held in constant darkness, the time of offset of melatonin secretion was delayed each day by about an hour relative to the preceding day, consistent with offset being under the control of a putative circadian pacemaker with a period greater than 24 hour. A similar daily delay was obtained in the previous experiment carried out with ewes which were entrained to short days (L:D,10:14) prior to their transfer to darkness.

By contrast, the initial impression gained of the onset of melatonin production was that it was determined by a separate pacemaker with a period of less than 24h, as during the first 4 days of darkness melatonin secretion was initiated at an earlier time on each successive day. The net result of the successive daily delay in offset and the advance in onset was that by the fourth day the period of melatonin secretion was extended to about 19-20hrs. However, when assessed on the eighth day, the period of melatonin secretion had been reduced to about 14hrs, largely due to an apparent change which occurred between days 5 and 9 in the pattern of the onset of melatonin secretion which changed from a daily advance to a delay. An additional effect of this change was a rapid advance (6.5h) on days 5-9 in the clock time of the mid-point in the period of melatonin secretion. A similar temporal change in the regulation of the

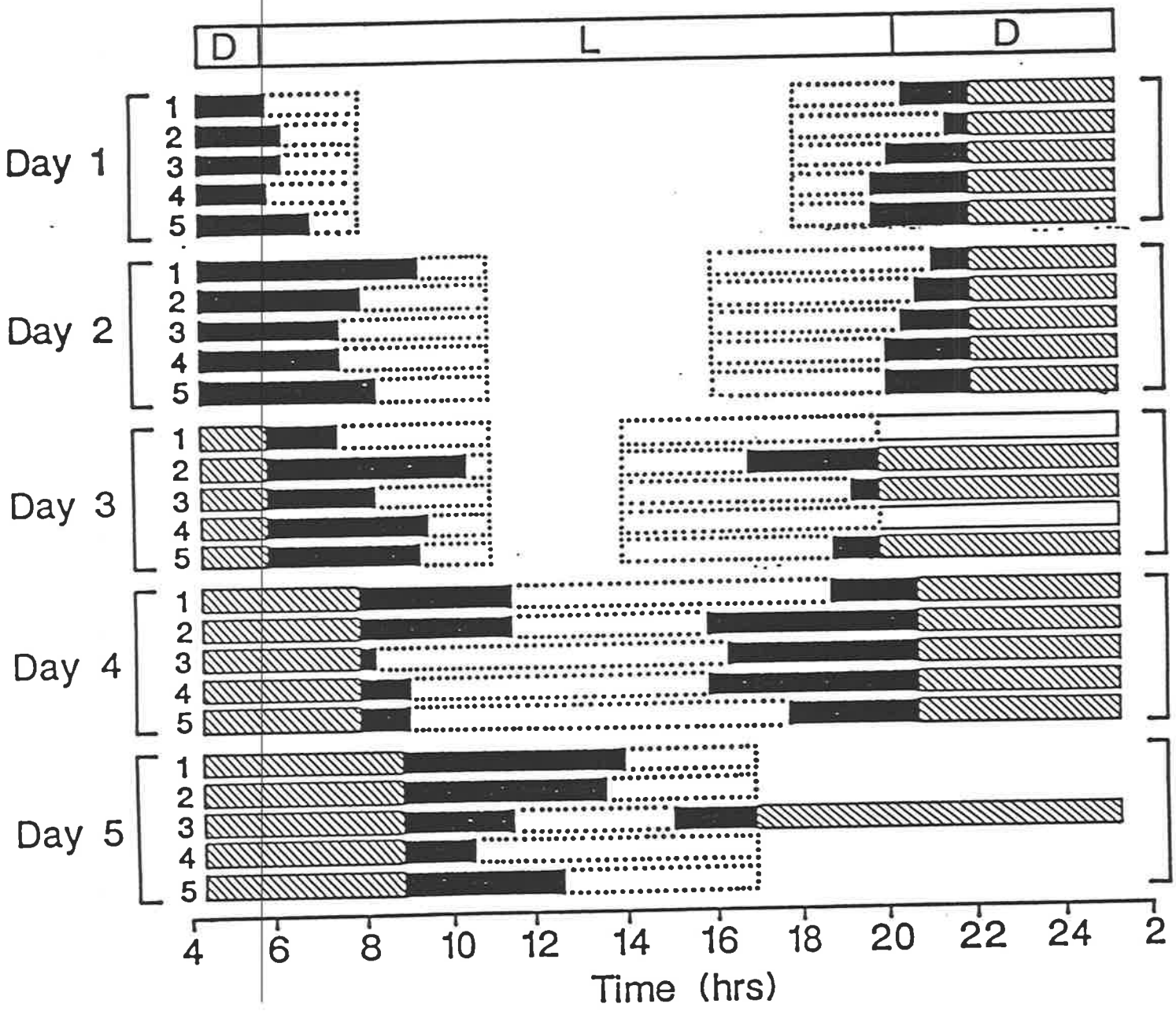
onset of melatonin secretion in sheep exposed to continuous darkness is seen in data presented by Lincoln and his associates, (Lincoln et al, 1985). However, in marked contrast to the present study, the Edinburgh researchers found that in the highly seasonal Soay breed, the onset of melatonin secretion was delayed each day during initial cycles in continuous darkness, then changed to occur earlier each day after the third cycle.

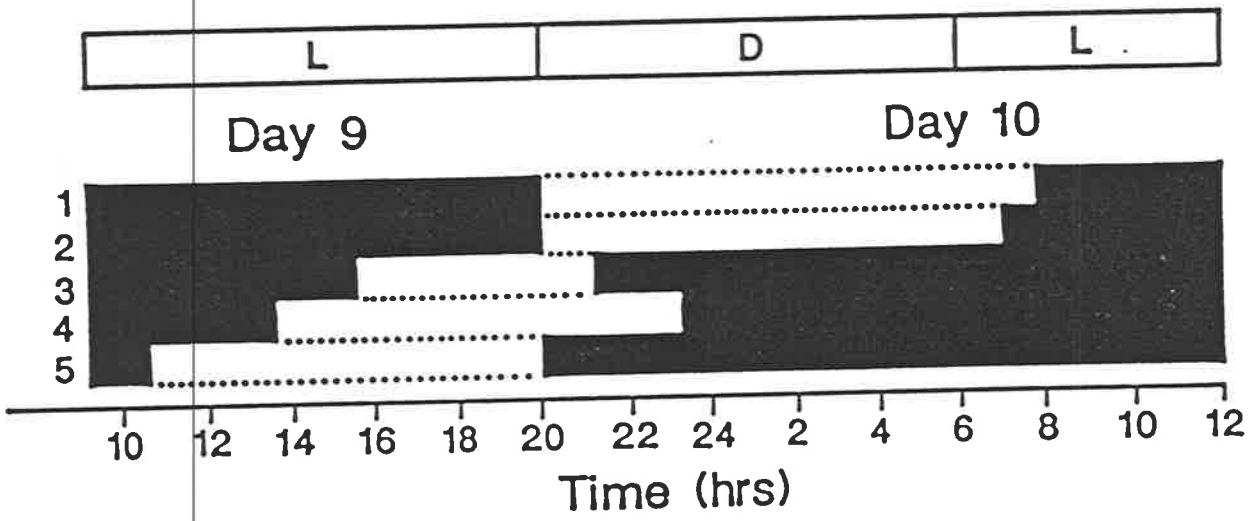
In respect to the nature of the pacemaker centre(s) determining the period of melatonin secretion, the data could be interpreted in a number of ways. Possibly both the onset and offset are determined by a single pacemaker but during the entraining period, there is an interaction with light prior to dusk and after dawn which prevents melatonin secretion and it is simply the normalising adjustments occurring during the first few days following removal of the lighting constraint that gave the appearance that onset was advancing and that this data was not in itself sufficient evidence to suggest a separate control mechanism. An alternative interpretation, based on a hypothesis stemming from data derived from studies of the circadian rhythms in pineal N-acetyl-serotonin activity of rats (Illnerova and Vanecek 1983), is that the timing of the onset and offset of melatonin secretion are determined by two independent but interacting pacemaker centres. According to this interpretation, when the ewes were placed in darkness, the resultant difference in the pattern in the onset and offset of melatonin secretion seen during initial cycles compared with later cycles is due to the activity of separate pacemaker centres which, being no longer constrained by

light, can express their independent properties. If offset, determined by a pacemaker with a period of greater than 24h, continues to delay and onset, because it is determined by an indirect pacemaker with a period of less than 24h, continues to advance, as seen in the initial cycles, the production of melatonin would eventually become continuous. The finding that it did not is provided for in the two pacemaker hypotheses, by hypothesising that under such circumstances there is an interaction between the two pacemakers with a dominant pacemaker, that is the one controlling offset, causing a change in the period of the subservient pacemaker. The interaction of the pacemakers may provide an explanation why it is that under natural photoperiod the extension of melatonin secretion in sheep is limited and does not extend to the full scotophase in winter (Guerin et al 1989). In this and in previous studies (Shaw et al 1988), individual ewes showed differences in the pattern of melatonin secretion which reflects variation in the activity and interaction of the putative pacemakers and further investigation is required to differentiate conclusively between hypotheses based on one or two pacemakers and other possible interpretations (Shaw et al. 1988). The study confirms the usefulness of this type of approach for studying the nature of the centre(s) controlling pineal function. However, it also highlights the need to make detailed observations over an extended period of time to avoid misinterpretations.

FIGURE 17

The times of melatonin secretion for five Suffolk ewes brought indoors in summer and entrained to L:D,14:10 before being placed in continuous darkness are shown by the darkened areas. The hatched areas represent those periods during which it is predicted that melatonin secretion would occur.


D
L
D
Day 1
Day 2
Day 3
Day 4
Day 5
1
2
3
4
5
4
6
8
10
12
14
16
18
20
22
24
2
Time (hrs)


L
D
L
Day 9
Day 10
1
2
3
4
5
10
12
14
16
18
20
22
24
2
4
6
8
10
12
Time (hrs)

8.0 Discussion

8.1 Introduction

The experimental section reports the results of a series of experiments conducted to investigate the nature of the pacemaker controlling melatonin production in the sheep. The results of each experiment are presented and discussed separately in relation to two of the models of the pineal pacemaker presented in the literature.

The following provides further general discussion of the main points raised in the separate experiments.

8.2 A consideration of Lewy and Illnerova and Vanecek's models

These two models which purport to provide a basis for understanding the nature of the pineal pacemaker were presented in detail in the literature survey. By gathering further information on both the onset and offset of melatonin output in the sheep it was hoped to identify whether in this species the data best fitted a single oscillator model described by Lewy (1983) or the two oscillator model of Illnerova and Vanecek (1982). However as the discussions of each experiment point out, the behavior observed could in all cases be explained by reference to any of these models. This reflected not simply limitations in the experimental design but the fact that the models allow, with slight modifications both alternatives. The value of models which allow several possible outcomes can be questioned.

This point is well illustrated by reference to data

obtained in experiment 7 (pg 152) where the behaviour of the onset and offset of melatonin production under continuous darkness is exactly that which could be predicted from the two pacemaker model of Illnerova and Vanecek (1982). On release from long days into continous darkness the dusk oscillator (E) and the dawn oscillator (M) shift separately indicating they may be controlled by pacemakers with different periods. However in Pittendrigh and Daan,s modified two pacemaker model (1976) the two pacemakers cannot move independently for very long before they begin to interact and evidence for such interaction is clearly seen after the fourth cycle when M is forced to change its period from less than 24 hrs to greater than 24hrs.

This data is easily accommodated by the two pacemaker model however it can also accommodated by a single pacemaker model if certain assumptions are made. For example if it is assumed that the rhythm is controlled by the single pacemaker described by Wever (1965) in which onset and offset occur when a certain threshold is achieved, then the behaviour observed in this experiment can be explained by a the lowering of the threshold over the first few cycles in continous darkness causing a lengthening of the duration of melatonin production which would appear as if onset was advancing and offset was delaying. The interaction seen in the initial cycles could also be explained by assuming that the threshold stabilises and the subsequent delay after several cycles reflects the period of the single pacemaker.

The single pacemaker model of Lewy (1983) could be defended in a similar way. However the results of experiment 3(a) and 3(b) indicate that the duration for which pineal activity occurs under darkness is affected by the length of

photoperiod the animal is exposed to in conflict with the prediction from Lewy,s model.

The possible complexity of pacemaker structure has been covered in the literature review. This section considers how many different outcomes may be produced by even the most simple pacemaker. This means that it is unlikely that from simple measurements of activity or hormonal output alone we will ever be able to definitively state that the pacemaker controlling a single rhythm is comprised of a single, two or many oscillators. All we can say is that the behaviour observed on this rhythm to this point of time is best explained by reference to a particular model.

The information collected by Illnerova and Vanecek (1982), by Lincoln (1985) and in this thesis on the behaviour of the pineal pacemaker can all readily be explained by reference to the two pacemaker model described by Illnerova and Vanecek (1982). Furthermore the remarkable consistency with which the results obtained in this thesis can be predicted from Illnerova and Vanecek,s model adds support to their hypothesis.

8.3 The offset of melatonin secretion

All of the observations on the offset of melatonin production in this thesis are consistent with this section of the rhythm being controlled by an endogenous circadian pacemaker. Furthermore when animals were released into continuous darkness as in experiments 4 and 6 the period of the offset of melatonin production was always greater than 24 hours. When animals were released from L:D,12:12 or L;D,10:14

into extended darkness offset was similarly delayed supporting the hypothesis that the offset of melatonin production is controlled by an endogenous pacemaker with a period greater than 24 hours.

When the time of lights off was delayed by 4 hours as in experiment 4 the offset of melatonin production was delayed by 2 hours longer than animals which experienced lights off at the normal time indicating that light around the time of lights off can influence the phase of the pacemaker controlling the offset of melatonin production.

8.4 The onset of melatonin secretion

Although less consistent than the offset of melatonin production there was evidence that the onset of melatonin was controlled by a separate endogenous circadian pacemaker. The best evidence for this was shown in experiment 7 when onset and offset were observed to display different behaviour during initial cycles under continuous darkness. In experiment 7 the onset of melatonin production advanced for the first few cycles under continous darkness but then delayed. In contrast the offset of melatonin production delayed during initial and subsequent cycles. The reasons why this may occur are consistent with the two pacemaker hypothesis and have already been discussed in previous sections.

8.5 The disorganisation of the melatonin rhythm

Exposure to very short days at a time of the year when

ewes were experiencing lengthening days had the interesting result of causing a breakdown in the mechanisms which generate the melatonin rhythm (experiment 2). This resulted apparently from the sudden change in photoperiod since normal melatonin profiles have been observed in sheep under photoperiods as short as that employed in the present experiments.

If the two pacemaker model is accepted then it is possible to provide an explanation for the observed disorganisation as entrainment after a sudden change in photoperiod would require that the period of both pacemakers must be compatible under the new photoperiod. Following a sudden change in photoperiod the period of the pacemakers or the manner in which they interact may have been changed so that they are not able to cope with the new set of conditions.

An interesting example of how this might occur has recently been provided by Mrosovsky et al (1986). In this study of hamsters free running in dim light they found there were two bouts of activity one around onset and another around offset which moved slightly further apart each cycle. After approximately fifty cycles their rhythm became disorganised indicating perhaps that the pacemakers had moved so far apart that they could no longer interact successfully. This experiment is important because it clearly demonstrates that two observable components of a circadion rhythm can show different periods and that the rhythm can only be sustained while these components are in reasonable proximity to each other. Once these two components move too far apart the rhythm becomes disorganised.

If the two pacemaker model is accepted then it is likely that

there will be changes in photoperiod with which the pineal pacemaker cannot cope and a disturbance in the pattern of melatonin will result.

8.6 Species differences in melatonin secretion

The results obtained in this thesis suggest that there may be species differences in the way in which pineal activity is regulated. In the sheep the duration of melatonin production coincides with that of the dark period under both short and long days. After exposure to extended darkness the onset of melatonin production either advances a little or remains the same whereas the offset of melatonin production delays substantially .

This contrasts with the rat where the duration of pineal activity only coincides with the duration of darkness under long photoperiods (Illnerova and Vanecek 1982, 1983, 1985). On photoperiods such as L:D,12:12 and L:D,8:16 melatonin onset does not occur immediately the lights go off but follows a lag time which increases as the photoperiod is shortened. The offset of pineal activity in the rat is more closely related to the times of lights on and appears to precede it by approximately 1 hour except under very short photo periods when it occurs at lights on.

Assuming that pineal activity in the sheep and the rat is controlled by 2 pacemakers then differences in their behaviour may be due to species differences in the way these two pacemakers interact. If the forces acting to keep E and M closer together are stronger in the rat than in the sheep then this may explain why the duration of pineal activity in

the rat never reaches the length seen in the sheep.

A similarity between the sheep and the rat is that the rhythm under extended darkness after exposure to LD 12:12 tends to move into the morning hours which indicates that the period of compound pacemaker is also greater than 24 hours.

8.7 Conclusions

The experiments conducted in this thesis provide new information on the behaviour of the onset and offset of melatonin secretion under different photoperiodic conditions. They provide no definitive evidence on the nature of the pineal pacemaker but I believe the results are most plausibly interpreted by reference to the two pacemaker hypothesis.

In the final experiment it was observed that the period of the offset of melatonin secretion changed after three days in continuous darkness. This may be of significance to researchers who wish to use the melatonin rhythm to monitor the period length of humans with suspected circadian disorders as initial observations may not give a true indication of period length in some circumstances. Indeed while our understanding of the nature of the pineal pacemaker are so limited it is highly speculative to use this rhythm to probe disorders of the circadian system. However this rhythm provides more hope than many other rhythms and further studies of the type conducted in this thesis coupled with investigations of the biochemical basis of the rhythm should reveal its true nature.

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