A STUDY OF CERTAIN FACTORS
AFFECTING REPRODUCTION
IN THE MARE

by

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SUMMARY

This thesis describes a study of the behavioural, hormonal and ovarian changes recorded in groups of housed mares during late autumn / spring over three years. In the first year the investigations concerned larger mares but these were replaced by pony mares during the subsequent two years. In the third year, mares demonstrating positive oestrous behaviour were mated. Variations from normal cyclical activity are discussed.

Incorporated in this study was an investigation into the effects of different environmental lighting on melatonin patterns and on reproductive activity in the pony mares. A radioimmuno-assay for the estimation of plasma melatonin concentration was established and the assay validated by recognised reliability criteria.

No correlation was found between environmental lighting, patterns of melatonin secretion and reproductive activity in these mares. Factors which may have affected reproductive activity in this study, other than light and plasma melatonin concentration, are suggested and discussed.

CHAPTER I

GENERAL INTRODUCTION

1.1 - INTRODUCTION

Domestication of the horse is thought to have occurred initially in the Turkestan area around 2,500 - 2,000 B.C. From Turkestan, horses migrated to Macedonia, to Egypt and so to Western Europe (Short, 1975).

Horses became indispensable to man, playing a role in war and peace - as transport, as a source of power in agriculture, and in sport. Such a variety of uses resulted in the breeding of certain types for specific purposes. This selective breeding has resulted in the wide variation of breeds of horses found today.

In Britain now, horses are mainly associated with sport and leisure.

In 1833 the Jockey Club decreed that the official birthday of the Thoroughbred should be the 1st of January - this is still so today. In order to have foals born at this time of year, mares must conceive outwith their apparently normal breeding season. This has led to numerous investigations into methods of manipulating the breeding patterns of the species. Although much progress has been made in our understanding of the control of breeding in the mare, the mechanisms involved are, as yet, not fully understood.

1.2 - ANATOMY OF THE REPRODUCTIVE TRACT

The reproductive tract of the mare lies within the pelvic and abdominal cavities. The vagina, cervix and part of the body of the uterus lie along the pelvic floor, with the rest of the uterine body, the uterine horns and the ovaries situated within the abdomen.

The uterus, suspended in the abdomen by the broad ligament, adopts a Y-shape, with the uterine horns, forming the gently curving arms of the Y, lying just in front of the pelvic brim. The ovaries are situated adjacent to the tips of the uterine horns. The tract, however, is not held rigidly in place by its ligamentous attachments and so the position of the uterus, ovaries and even cervix may vary from mare to mare, and in the same mare on different occasions.

The mares' ovaries are bean-shaped with the ovulation fossa situated in the lesser curvature. This forms, and can be recognised as, a deep depression in the surface of the ovary. In the mare each ovary is covered in peritoneum except at the ovulation fossa (Ginther, 1979). The average length and diameter of the ovary of the Thoroughbred mare are 5 - 8 cm and 3 - 4 cm respectively (Rossdale and Ricketts, 1980). Similar variations in length and diameter occur in the different breeds.

Developing follicles can be palpated on the surface of the ovaries with a range in diameter of up to 10 cm (Hughes, Stabenfeldt and Evans, 1975). Follicles of less than 3 cm in diameter are usually regarded as being unlikely to ovulate

(Rossdale and Ricketts, 1980).

Follicles first present as turgid, thin-walled structures which become softer as ovulation approaches and they migrate towards the ovulation fossa. Ovulation, in the mare, always occurs at the ovulation fossa (Witherspoon and Talbot, 1970).

After ovulation the area occupied by the follicle fills with blood to form the corpus haemorrhagicum. This can be palpated as a softening, of sponge-like consistency, occupying almost the whole or part of the ovary. As this becomes organised, the corpus luteum forms. The corpus luteum can be felt in some mares as a firm but 'rubbery' structure in approximately the same place as the follicle was situated previously.

The corpus luteum in the mare is not as easily identified as it is in the bovine. In the mare there is no rupture of the surface of the ovary and no development of an ovulatory papilla which can be palpated in the cow (Witherspoon and Talbot, 1970).

The ovary of the mare differs from the other domestic animals in that it has no distinct cortex and medulla. In the mare the vascular zone is very superficial and follicles are situated throughout the ovary (Sissons and Grossman, 1975).

The right ovary of the mare lies anatomically in front of the left (Sissons and Grossman, 1975). The left ovary often lies among the loops of the small intestine and must be differentiated from faecal balls.

The fimbriae of the cranial edge of the infundibulum of the oviduct are attached to the cranial pole of the ovary to form the

cranial margin of the ovulation fossa. During ovulation, the infundibulum covers the ovulation fossa to facilitate the entry of the ovum into the oviduct (Ginther, 1979).

The oviducts in the mare are tortuous tubes, 20 - 30 cm in length (Rossdale and Ricketts, 1980). They are not normally palpable on rectal examination.

The uterine body lying partly in the pelvis, partly in the abdomen, can usually be felt as a smooth, rounded tubular structure lying on the floor of the pelvis. This can be traced forward into the abdomen with the uterine horns curving gently upwards and forward on either side. The position and consistency of the uterus varies within and between mares.

By palpating along the normally soft, tubular horns of the uterus, one usually finds the ovaries situated slightly above and in front of the tip of each horn.

The body of the uterus is separated from the vagina by the cervix. The cervix in the mare is a tubular structure, on average 6 - 7 cm long and 3 - 4 cm wide (Rossdale and Ricketts, 1980). Size and consistency of the cervix may be judged on rectal palpation. The consistency of the cervix varies with the stage of the cycle.

The external os of the cervix and folds of the vagina may be examined per vaginum using a vaginascope. On examination per vaginum the cervix often appears rosette-shaped, suspended in the vagina by one or more membraneous ligaments, or frenuli.

The external os of the cervix may point caudally or to either side. The shape and position of the cervix, however, varies with the sexual status of the mare.

The vagina is continuous with the vestibule which originates at the level of the urethral orifice and is bounded externally by the vulvar lips. In most mares there is a slight constriction just cranial to the vestibulo-vaginal junction which may make passage of a vaginascope difficult. This is the site of the hymen when present.

The vulvar lips reach from just below the base of the anus to the clitoris which they enclose ventrally. The optimum conformation of the vulva is one in which the vulvar lips are in a vertical plane with 80% below the pelvic floor (Rossdale and Ricketts, 1980). The position and shape of the vulvar lips is important, as together with the constriction at the vestibulo-vaginal junction and the cervix they maintain a barrier against air and infection reaching the vagina and the uterus. The position of the vulvar lips may become altered by trauma at foaling, or with age, as the anus retracts anteriorly and the upper part of the vulva becomes more horizontal.

1.3 - THE OESTROUS CYCLE OF THE MARE

The mare is generally considered to be a seasonally polyoestrous animal, cycling regularly throughout the spring and summer months and becoming sexually quiescent during the winter (Arthur, Noakes and Pearson, 1982).

As in most domestic species, the oestrous cycle in the mare can be subdivided into the stages of pro-oestrus, oestrus, metoestrus and dioestrus. Pro-oestrus and metoestrus are not usually detected clinically in the mare.

In the mare, the oestrous cycle lasts approximately 21 days (Hughes et al, 1975). Oestrus, dioestrus and anoestrus are associated with specific changes in behaviour and in the reproductive tract. These changes occur due to variation in circulating hormone levels.

1.3A   -   OESTRUS

The oestrous period in the mare lasts from 5 - 8 days but will vary from mare to mare and with the time of year (Rossdale and Ricketts, 1980; Ginther, 1979).   Oestrus tends to last longer at the beginning of the breeding season, being shortest at the optimum breeding time.

In oestrus, the mare is both attractive and receptive to the stallion.   On teasing, with a stallion, she will adopt the oestrous posture where the tail is raised and often held to one side and the legs are splayed and slightly crouched as in a urination stance. The mare will often present her hind-quarters towards the stallion. There is a rhythmic constriction of the vulvar lips with evertion of the clitoris referred to as 'winking'.   The mare, in oestrus, will usually pass variable amounts of urine which may vary from a clear, straw-coloured to a more opaque, creamy-yellow-coloured fluid.

Before or after the oestrous period, many mares will, for a few days, exhibit some of these positive oestrous signs together with some negative oestrous signs.   Negative oestrous signs include flattening of the ears against the head, swishing of the tail, squealing and kicking.   In some in-season mares, it may take some 5 - 10 minutes before positive oestrous signs are shown on teasing.   This happens most often with maiden mares or those with a foal at foot. Some mares show the phenomenon of split oestrus where positive behavioural signs are seen for a few days, followed by negative signs for 1 - 2 days and then a return to positive oestrous behaviour (Ginther, 1979).

During standing oestrus, follicles are developing in one or both ovaries. The ovaries become larger in size due to the presence of these follicles. One or more follicles may develop in any oestrous period. However, one follicle usually becomes larger than the others and it is this follicle which will ovulate. A mature follicle in the Thoroughbred mare may range from 2.5 - 10.0 cm (Rossdale and Ricketts, 1980).

Multiple ovulations do occur in the mare. The incidence has been reported to vary from 4 - 44% (Hughes, Stabenfeldt and Kennedy, 1980).

Ovulation takes place approximately two days before the end of oestrus. In one survey, (Hughes et al, 1975) 78% of ovulations, in 11 mares examined over two years, occurred within 48 hours of the end of oestrus.

Follicles do not always ovulate but may regress or become luteinised without ovulation. This tends to occur towards the beginning and end of the breeding season, (Ginther, 1979).

The consistency of the uterus in the mare in oestrus is variable. However, there is never the marked turgidity of the uterus in the mare as occurs in the oestrous cow. Generally, the uterus of the oestrous mare is soft and flaccid.

During oestrus the cervix is soft and not easily palpated per rectum. On examination per vaginum, it is generally found resting on the floor of the vagina, pinky-red in colour, relaxed, oedamatous and covered in a glistening fluid (Lieux, 1970; Rossdale and Ricketts, 1980).

1.3B - DIOESTRUS

The dioestrous mare usually exhibits aggressive behaviour towards the stallion when teased.   The ears are flattened back against the side of the head and the tail is swished vigorously from side to side.   The mare may squeal very volubly and attempt to get away from the stallion or kick out at him when teasing is continued.   Dioestrous behaviour varies from mare to mare.   Some mares do not adopt such aggressive behaviour but may only flatten their ears and occasionally swish their tail.

During the dioestrous period a corpus luteum is present in the ovaries.   The corpus luteum may or may not be palpable. Since ovulation occurs only at the ovulation fossa, most corpora lutea develop deep in the ovary and the only indication of their presence is that one ovary may be considerably larger than the other.   However, corpora lutea may be palpated as soft or rubbery structures at the site which the follicle occupied previously. Arthur and Allen (1972) record the average period during which the corpus luteum was palpable in a Welsh pony mare as 8 days.

As more than one follicle may ovulate during oestrus, there may be more than one corpus luteum present in dioestrus (Urwin and Allen, 1983).

Follicles continue to develop in the ovaries during dioestrus. These follicles may regress, luteinise or ovulate.   It is not known

whether these ova produced in the luteal phase are fertile. Ovulations in the luteal phase do not affect the length of dioestrus (Stabenfeldt, Hughes, Evans and Geschwind, 1975).

The consistency of the uterus in dioestrus again varies from mare to mare. Hughes et al (1975) could make no correlation between the state of the uterus and the stage of the oestrous cycle in the mare. However, Ginther (1979) reports that the tone of the equine uterus is greater during dioestrus.

1.3C   -   ANOESTRUS

When teasing the anoestrous mare, the response may vary from aggression in the mare in shallow anoestrus to indifference when in deep anoestrus with the mare appearing totally unconcerned by the presence of the stallion.

The ovaries of the anoestrous mare are small and firm, approximately 3 - 6 cm long (Rossdale and Ricketts, 1980). Follicles may or may not be present in the ovary at this time. Follicles, when present, do not usually exceed 3 cm in diameter, remain firm and regress.

The cervix in the anoestrous mare may be palpable, tubular and closed, or soft and open.   In all instances the cervix and vagina are pale and dry.   Any mucous present is scant and sticky.   When the cervix is open, the interior of the uterus can be seen per vaginum.

1.4 - CONTROL OF REPRODUCTION IN THE MARE

Our understanding of the control of reproductive function in the mare is still incomplete. However, several investigations have been undertaken and a number of steps in the complex process have apparently been determined.

As in all the domestic species, the cyclical changes in the behaviour and reproductive tract in the mare are thought to occur due to the interaction of certain higher brain centres and the sex hormones.

Mares generally cycle regularly, with approximately twenty-one day cycles, throughout the spring and summer months. This cyclicity in the mare appears to be regulated via the hypothalamus, the pituitary and the ovaries (Fig. 1.1).

The hypothalamus, situated on the undersurface of the brain (Ginther, 1979), produces a number of small peptide regulatory hormones which control the release of pituitary hormones. These may be stimulatory as in the case of gonadotrophin releasing hormone (GnRH) or inhibitory as with prolactin inhibitory factor (P.I.F.) (Austin and Short, 1979).

GnRH exerts its influence on the anterior pituitary via a vascular portal system which links the anterior pituitary with the hypothalamus (Ginther, 1979).

The pituitary, which is located at the base of the brain, lying in close proximity to the hypothalamus (Ginther, 1979) produces and stores the hormones follicle stimulating hormone (FSH) and
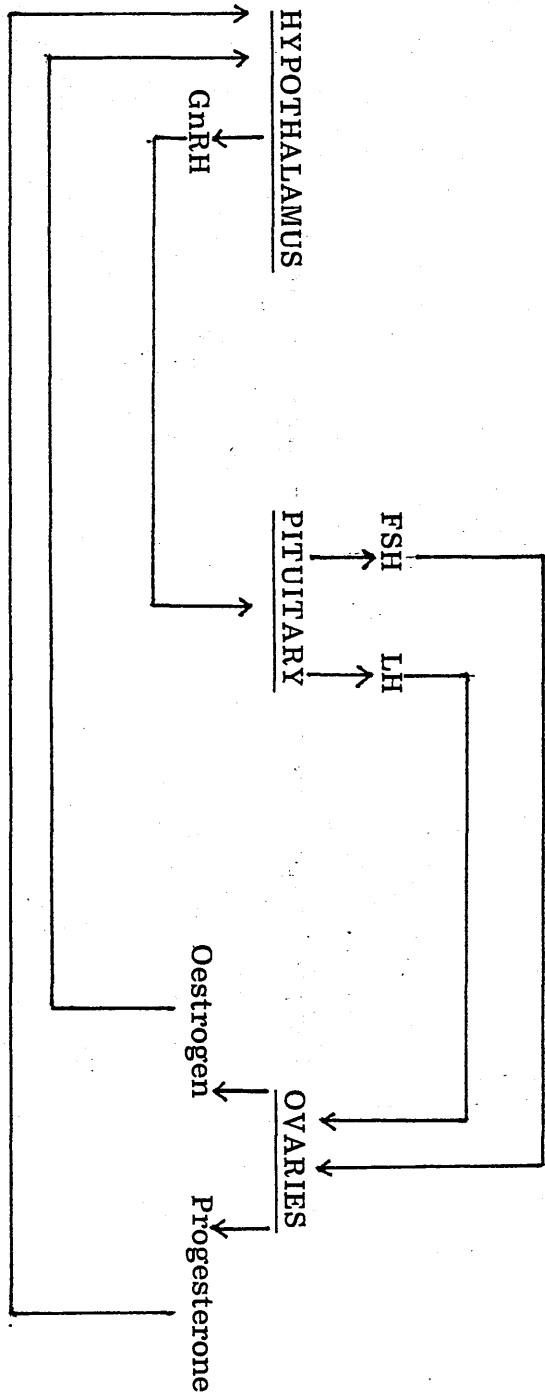
Fig. 1.1 - SUMMARY OF THE ENDOCRINOLOGY OF THE OESTROUS CYCLE

luteinising hormone (LH). These hormones, referred to as gonadotrophins, are released into the bloodstream in response to GnRH from the hypothalamus.

FSH promotes the growth of follicles in the ovary. Hughes et al (1980) reported that a distinct ten-day rhythm of FSH surges occur in the mare throughout the breeding season. This results in two waves of follicular development during each oestrous cycle. A surge of FSH occurs in late oestrus - early dioestrus and in mid-dioestrus (Fig. 1.2) and these workers suggest that it is this second surge which stimulates the growth of those follicles which go on to ovulate. Evans and Irvine (1975) also report a ten to eleven day rhythm of FSH surges. They note, however, that almost invariably, the dioestrous FSH surge preceded ovulation by ten to thirteen days. Thus, in mares exhibiting short dioestrous intervals, the pre-ovulatory surge occurs in early - rather than mid-dioestrus. Urwin and Allen (1983) found a definite rise and subsequent fall of FSH around mid-dioestrus, but found an oestrous surge in only some of the mares studied.

LH acts to initiate the latter phases of maturation and ovulation of the follicle or follicles suitably primed by FSH and to stimulate the cells of the follicle to steroid production. The steroid most likely to be produced at this time is progesterone, which is thought to induce the production of an ovulatory enzyme which weakens the collagen framework of the theca interna of the follicle (Ginther, 1979).

LH levels begin to rise just before the onset of oestrus, peaking

about two days after ovulation (Hughes et al, 1980). Evans and Irvine (1975) report the LH peak occurring 1 - 2 days after ovulation and Urwin and Allen (1983) also report a well-defined peak between 0 - 3 days after ovulation. The mare differs in this respect from most other domestic species where the LH peak occurs immediately before ovulation (Noden, Oxender and Hafs, 1975). LH levels in the mare take a further 4 - 5 days to return to baseline levels (Hughes et al, 1980).

As the follicle or follicles develop in the ovary, under the influence of FSH, they secrete increasing amounts of oestrogens.

Oestrogen levels begin to rise 2 - 3 days before the onset of oestrus (Noden et al, 1975; Hughes et al, 1980) and peak about two days before ovulation (Fig. 1.2). Oestrogens then decrease to reach baseline dioestrous levels by about two days after ovulation.

Oestrogens act on the reproductive tract to prepare it for coitus, the transport of sperm and the reception of a fertilised ovum, and on behavioural centres in the brain resulting in the demonstration of oestrous behaviour and acceptance of the stallion.

However, some mares, although a follicle develops and ovulates, do not demonstrate oestrous behaviour. This is known as silent oestrus. The cause of this lack of demonstrable oestrous behaviour is unknown. Munro, Renton and Butcher, (1979) showed no consistent relationship between oestrous behaviour and circulating oestrogen concentrations. Ginther (1979) reports an incidence of 7.5% of silent oestrus in horses. Rossdale and Ricketts (1980) suggest that silent oestrus is, in most cases, an

FIG. 1.2 – Diagramatic representation of hormonal changes in peripheral blood during one 21-day oestrous cycle

Legend:
•••••• – Oestrogen
– – – – LH
–•–•– FSH
——— Progesterone

BLOOD HORMONE LEVELS

OESTRUS
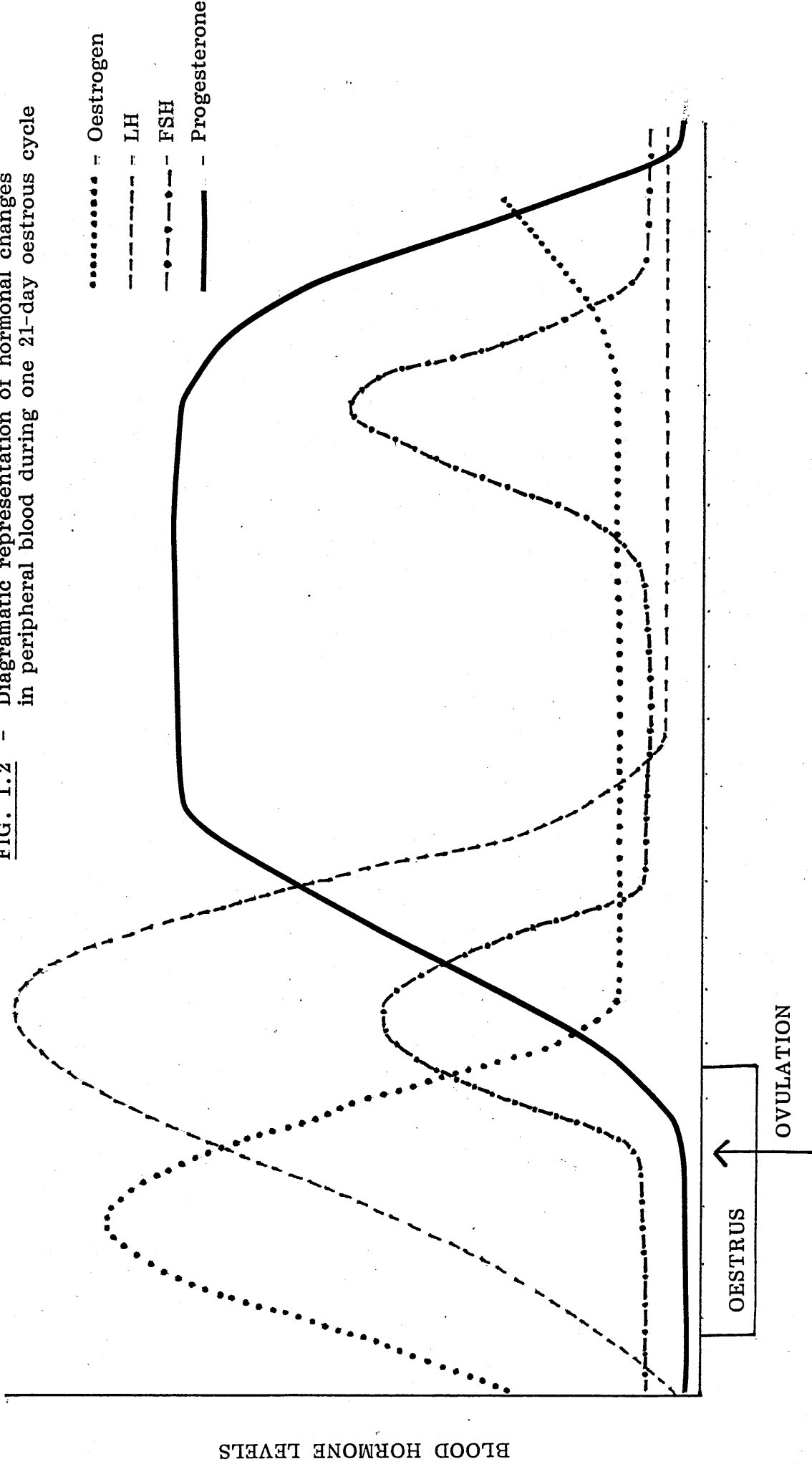
OVULATION

error of interpretation and the mare will, if presented, accept the stallion.

After ovulation occurs, the cavity left by the developing follicle fills with blood to form the corpus haemorrhagicum. This becomes invaded by granulosa cells which organise to form the corpus luteum. The corpus luteum secretes progesterone.

Progesterone levels, in the mare, rise abruptly after ovulation reaching a maximum approximately 6 days after ovulation (Fig.1.2). Progesterone levels remain high until 14 – 15 days after ovulation, when, if the mare is not pregnant, the corpus luteum is lysed by the secretion of prostaglandins from the uterus (Oxender, Noden, Louis and Hafs, 1974).

Prolonged dioestrus, with the continuing production of progesterone by the corpus luteum when there is no pregnancy, does occur spontaneously in the mare. These corpora lutea generally persist for about 2 months. The cause of this spontaneous prolongation of luteal activity is unknown but may result from a failure of the uterus to release adequate amounts of prostaglandin (Stabenfeldt et al, 1975).

Progesterone acts to suppress the action of oestrogens and, although follicles are growing and secreting oestrogens during the luteal phase, oestrous behaviour is not demonstrated. Progesterone also acts on the reproductive tract to ensure that pregnancy is maintained, and on behavioural centres resulting in a marked rejection of the male.

This cycle of events occurs with regularity throughout the

breeding season. During the winter months levels of FSH, LH, oestrogen and progesterone are thought to ramain basal and the mare is said to be in anoestrus (Sharp, 1980).

How does the mare, then, make the transition from anoestrus to oestrus? What is the trigger? How does it work?

Sharp, Grubaugh, Berglund, Seamans, McDowell, Kilmer and Peck (1981) report that the onset of the breeding season of the mare is very precise and state that in Florida the breeding season begins on day 129 (May 7th) $\pm$ 6.7 d.

They also report that towards the beginning of the breeding season there is an increase in the number and size of follicles present in the ovaries associated with elevated plasma FSH concentrations. At this stage, however, although plasma FSH concentrations are high, LH concentrations remain low and thus none of the developing follicles ovulate. Oestrogen secretion appears to lag behind follicular development with plasma oestrogen concentrations remaining low until 2 - 3 weeks before the first ovulation when they rise rapidly. Most of the pony mares studied (Sharp et al, 1981) began to demonstrate positive oestrous behaviour before this marked rise in circulating plasma oestrogens. Plasma LH concentrations then rise sharply and ovulation occurs within 4 - 6 days.

Following this first ovulation the mare becomes polyoestrous, cycling regularly throughout the spring and summer months.

Daylight length has been suggested as playing a major role in the regulation of the breeding season in seasonally polyoestrous animals (Yeates, 1949; Nishikawa, 1959; Menaker, 1971; Turek and Campbell, 1979; Sharp, 1980).

Seasonally polyoestrous animals can be broadly classified into two groups, those which begin to cycle under natural conditions when daylight length is decreasing, i.e. short-day animals which include the sheep and the mink; and those which begin to cycle when daylight length is increasing, i.e. long-day animals, including the hamster, ferret and mare.

Although little detailed knowledge is available about the mechanisms involved in the effect of daylight length on the regulation of the breeding season, the generally accepted hypothesis is that it is mediated in some way, via the pineal gland (Reiter, 1980).

The pineal gland has long been associated with the perception of light and hence with its effect on reproductive function. The pineal gland has often been described as a third eye and indeed may be directly photosensory in fish, amphibians and some reptiles (Firth and Kennaway, 1980). In mammals and other reptiles, the pineal is situated deep in the brain and cannot be reached directly by light. However, neural pathways have been discovered which connect the eye with the pineal gland (Reppert and Klein, 1980).

Environmental light stimulates the retinal photoreceptors and this information is carried to the suprachiasmatic nucleus of the hypothalamus via the accessory optic tracts (Wurtman and

Moskowitz, 1977) or the retinohypothalamic projections (Reppert and Klein, 1980). The suprachiasmatic nucleus is thought to stimulate the pineal by sending sympathetic nerve impulses, via the superior cervical ganglion, which results in the release of noradrenaline at the nerve endings. Light is generally considered to be inhibitory to pineal function and it is during the hours of darkness that sympathetic innervation is increased.

Much of the work on the effect of the pineal gland on reproductive function has been carried out using laboratory rodents in which pinealectomy is a relatively simple procedure (Reiter, 1980). Such studies have produced varying results.

In the male Syrian hamster, a long-day animal, Reppert and Klein (1980) report that pinealectomy prevents the testicular regression normally seen when they are exposed to short photoperiods.

In ferrets (long-day animals) pinealectomised in the autumn, i.e. at the start of their anoestrous period, and kept under natural daylight conditions, the onset of the breeding season in the following spring occurred at the same time as the sham-operated and control animals. However, in the second year after surgery pinealectomised ferrets came into season much later (20 - 30 weeks) than controls (Herbert, 1972).

In sheep (short-day animals) Seamark, Kennaway, Matthews, Sellenberg, Philipou, Kotaras, McIntosh, Dunstan and Obst (1981) reported that the lifetime reproductive performance of two flocks of Merino crossbred ewes, pinealectomised at 7 - 60 days of age, did not

differ from that of sham-operated control animals kept in the same flock.

Pinealectomy and its effects do not only seem to vary between different species of animal but also between different breeds of the same species.

Goldman (1982) working with three breeds of hamster, Mesocricetus auratus, Mesocricetus brandti and Phodopus sungorus, found they had differing responses to pinealectomy. In M. auratus and P. sungorus pinealectomy prevents the testicular regression normally seen in these long-day animals when transferred from long to short photoperiods. This was not the case with M. brandti.

The neural pathways which transmit photic information from the retina of the eye to the pineal, appear to do so via the suprachiasmatic nuclei (S.C.N.) of the hypothalamus (Reppert and Klein, 1980). Destruction of the S.C.N. tends to mimic the effects found with pinealectomy. Rusak (1980) reported that destruction of the S.C.N. in hamsters prevented the testicular regression normally seen in these animals, when transferred from long to short photo-periods. He also reported that S.C.N. lesions enhance testicular recrudescence in hamsters with previously regressed testicles.

Obviously the results of pinealectomy are confusing since it can prevent testicular regression in some species of hamsters but not others, while it causes a delayed inhibitory effect on the onset of oestrus in ferrets and has very little or no effect in the ewe.

The integrated role of the pineal gland and photoperiod is,
therefore, still far from clear.

However, the apparent link between environmental light,
the pineal gland and reproductive function has led to several
investigations into the substances produced by the pineal in an
attempt to find out how they exert their effect.   Most research
has been carried out on the secretion and effect of the pineal
produced indole melatonin (N-acetyl-5-methoxytryptamine).

Melatonin is only one of the compounds produced and secreted
by the pineal gland.   It is secreted with a diurnal rhythm, being
at basal or near basal levels during the hours of daylight and at
peak levels during the hours of darkness (Reppert and Klein, 1980).
This diurnal rhythm of melatonin secretion has been reported in many
species including sheep, humans, rhesus monkeys, sparrows, hamsters,
horses, cattle and marmots.   (Rollag and Niswender, 1975;   Arendt,
Wetterburg, Heyden, Sizonenko and Paunier, 1977;   Jenkin, Mitchell,
Hopkins, Matthews and Thorburn, 1980;   Menaker, Hudson and
Takahashi, 1981;   Goldman, Hall, Hollister, Reppert, Roychoudhury,
Yellon and Tamarkin, 1981;   Kilmer, Sharp, Berglund, Grubaugh,
McDowell and Peck, 1982;   Martin, Cunningham and Saba, 1983;
Florant and Tamarkin, 1984.)

The integrated processes whereby light influences the synthesis
and release of melatonin are thought to be as follows.

The pineal gland manufactures and stores serotonin.   This
involves the uptake of the amino-acid tryptophan from the bloodstream
by the pineal cells, the pinealocytes.   Tryptophan is converted to

serotonin by the action of the enzymes tryptophan hydroxylase and aromatic amino-acid decarboxylase (Reppert and Klein, 1980) (Fig. 1.3).

With the onset of darkness the serotonin is converted to N-acetylserotonin by the enzyme N-acetyltransferase (N.A.T.) This is then converted to melatonin by the enzyme hydroxyindole-o-methyltransferase (H.I.O.M.T.) (Reppert and Klein, 1980). The dramatic increase in N.A.T. and H.I.O.M.T. activity, with the onset of darkness, is thought to be the result of increased sympathetic nerve stimulation and noradrenaline release (Reppert and Klein, 1980).

Noradrenaline is thought to act via beta-adrenergic receptors, on the surface of the pinealocytes resulting in activation of the enzyme adenylate cyclase. The activation of this enzyme results in increased production of cyclic adenosine monophosphate (cyclic A.M.P.). Cyclic A.M.P. may thus mediate sympathetic nervous control of the enzymes controlling melatonin synthesis (Reppert and Klein, 1980).

Melatonin enters the general circulation (Rollag, Morgan and Niswender, 1978) and is metabolised by the liver microsomes via 6-hydroxylation and conjugation to a sulphate or glucuronic acid (Reppert and Klein, 1980). This is then excreted in the urine.

Although melatonin levels appear always to be elevated at night, there is considerable species variation in the pattern of secretion (Reiter, 1980).

In the rat, pineal melatonin increases 3 - 4 hours after the onset

Fig. 1.3 — THE SYNTHESIS OF MELATONIN

TRYPTOPHAN

$\downarrow$

tryptophan hydroxylase

aromatic amino-acid decarboxylase

SEROTONIN

$\downarrow$

N-acetyltransferase (N.A.T.)

N-ACETYL SEROTONIN

$\downarrow$

hydroxyindole-o-methyltransferase
(H.I.O.M.T.)
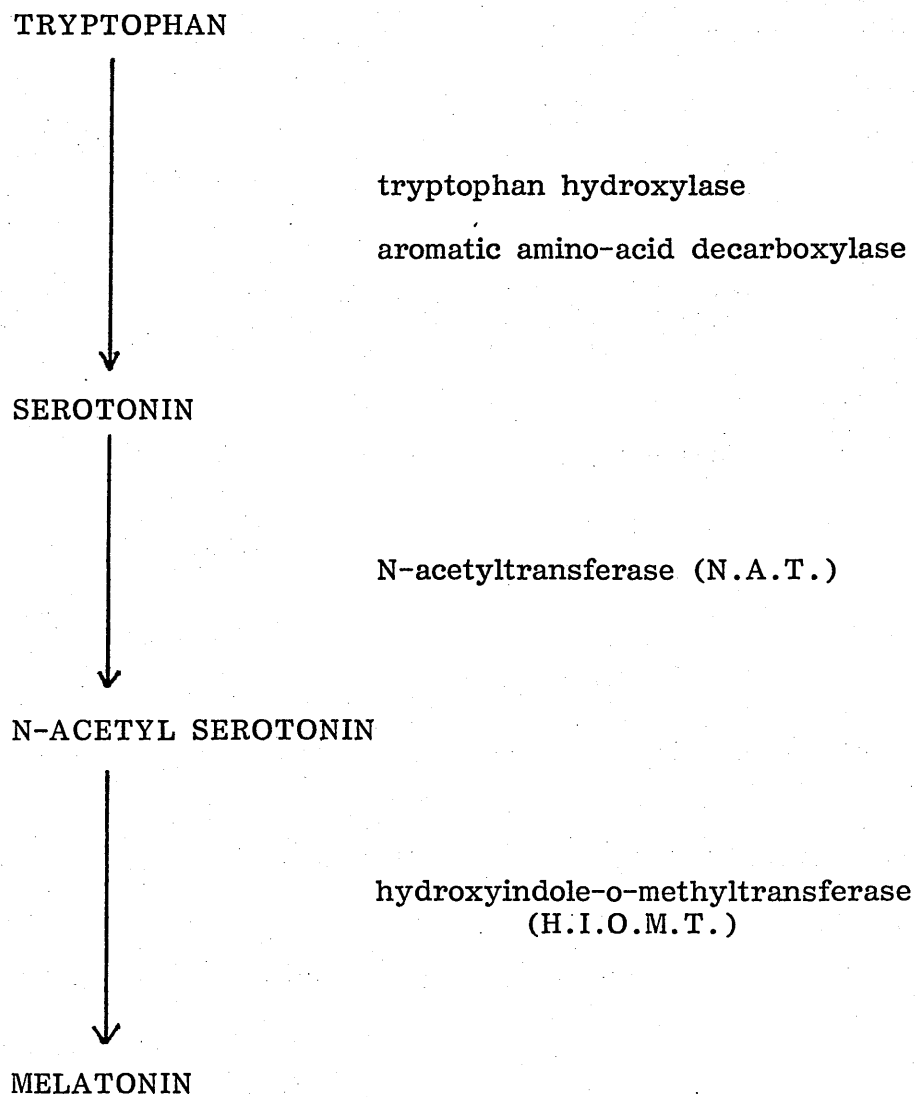
MELATONIN

of darkness, whereas in the Syrian hamster this increase does not occur until 4 - 6 hours after darkness falls. On the other hand, work carried out measuring melatonin in the cerebro spinal fluid (C.S.F.) of rhesus monkeys, sheep and Guernsey calves shows a rise within 2 hours after dark (Reppert and Klein, 1980). Similar daily rhythms of melatonin occur in plasma and C.S.F. (Hedlund, Lischko, Rollag and Niswender, 1977).

The pattern of decreasing melatonin levels with the onset of light also varies. Sheep, monkeys and calves all showed a rapid melatonin decrease with the onset of light. Syrian hamsters maintained on a long photoperiod (14L : 10D) showed an immediate decrease in melatonin levels with the onset of light. However, Syrian hamsters maintained on a short photoperiod (10L : 14D) showed an apparent decrease in melatonin levels 2 hours prior to lights on. There is also one reported situation where, in hamsters maintained on a very long photoperiod (20L : 4D), the normal night-time elevation of melatonin appears absent (Reppert and Klein, 1980).

When melatonin was first measured in correlation with daylight length and reproductive function, in hamsters, it was postulated that melatonin was having an inhibitory effect on reproduction by suppression of hypothalamo-pituitary-gonadal axis function. However, when hamsters maintained on a long photoperiod were first injected with melatonin, in order to mimic a short photoperiod, the exogenous melatonin was found to have no effect on their reproductive status (Reiter, 1980).

Later, Tamarkin, Westrom, Hamill and Goldman (1976) reported that melatonin was antigonadotrophic only if injected late in the light phase. Melatonin injected 6.5 - 13.75 hours after lights on resulted in a decrease in circulating gonadotrophins and circulating prolactin.

It would appear that this antigonadotrophic effect of melatonin requires an intact pineal. Pinealectomy or superior cervical ganglionectomy, which destroys the sympathetic innervation to the pineal, results in afternoon melatonin injections having no measurable effect on any aspect of the reproductive system (Reiter, 1980).

It has since been demonstrated that melatonin can have a gonadotrophic effect in hamster as well as an antigonadotrophic effect.

Subcutaneous implants of melatonin can prevent the testicular involution normally seen in male hamsters exposed to short photo-periods. A similar gonadal effect is also seen in female hamsters and it has been found that continually available melatonin is as effective as pinealectomy in restoring normal vaginal cyclicity in surgically blinded female hamsters (Reiter, 1980).

When hamsters, implanted with melatonin capsules and maintained on a long photoperiod, were given daily late light phase melatonin injections, the gonadotrophic effect of the chronically adminstered melatonin prevailed (Reiter, 1980).

Melatonin patterns, under natural and artificial lighting regimes, have been studied in the sheep by various workers.

Rollag and Niswender (1975) reported a circadian rhythm of melatonin secretion in the ewe. They found that this circadian rhythm persisted under conditions of constant darkness but was abolished when ewes were exposed to constant light.

Lincoln, Almeida, Klandorf and Cunningham (1982) reported marked nocturnal melatonin increases in Soay rams during long days. The mean night-time level during long days was found to be 382 $\pm$ 54 pmol/l in comparison to that of 135 $\pm$ 10 pmol/l during short days.

Kennaway, Sanford, Godfrey and Friesen (1983) found that in ewes night-time levels of melatonin varied markedly from hour to hour and between ewes. They found no consistent seasonal change in absolute levels of melatonin in the ewe or any significant changes in melatonin levels or pattern of melatonin secretion during the oestrous cycle. This is in contrast to work done by Arendt, Symons and Marston (1979) where they found that in ewes in mid-anoestrus (long days) a simple homogenous peak of melatonin was present at approximately 03.00 hours, i.e. during darkness, whereas in the breeding season (short days) two dark phase peaks were often found.

Kennaway, Gilmore and Seamark (1982) and Arendt, Symons, Laud and Pryde (1983) are agreed, however, that the daily administration of melatonin to ewes, via feedstuff, does advance the onset of the breeding season in this species.

Kennaway et al (1983) also reported that melatonin implants in ewes, resulting in sustained high plasma melatonin levels, had the same influence as short days, i.e. the breeding season was advanced.

Therefore sheep (short-day animals) and hamsters (long-day animals) appear to differ in their response to daily melatonin administration in that in sheep it appears to advance the breeding season, but in hamsters it appears to cause cessation of the breeding season. However, both sheep and hamsters appear to respond to the constant release of melatonin from a subcutaneous implant in the same way, i.e. with a continuation of the breeding season.

Therefore, as with the studies on pinealectomy, investigations into the role of melatonin, as the possible mediator of the effect of daylight length, while providing interesting results, has not clarified the part played by the pineal gland in the regulation of the breeding season in certain animals.

Melatonin, is not, however, the only substance produced by the pineal, which has been suggested as having antigonadotrophic effects.

In a review on pineal function, Reiter (1980) lists other indoles which have been implicated in the antigonadotrophic process. These include 5-methoxytryptophal, another metabolite of serotonin requiring the enzyme H.I.O.M.T. for its synthesis, 5-hydroxtryptophal and 6-hydroxymelatonin. In contrast to the others, 6-hydroxymelatonin is produced not in the pineal but in the liver, as a metabolite of melatonin.

In this review by Reiter (1980) and another by Benson (1977) pineal peptides with claims to antigonadotrophic effects are also discussed. These include pineal antigonadotrophin (P.A.G.), arginine vasotocin (A.V.T.) and other, as yet, un-named peptides.

Both the indoles and the peptides are reported as having

varying effects on one or more of the following – the inhibition

of pregnant mare serum and/or human chorionic gonadotrophin

induced hypertrophy of the reproductive organs in female rodents;

the reduction of accessory organ weights in immature domestic mice,

house mice and hamsters; and the inhibition of compensatory ovarian

enlargement in mice after unilateral ovariectomy.

Where, then, does the mare fit into this picture?

Mares are generally regarded as being seasonal breeders and

long-day animals.

Investigations into the regulation of seasonal breeding patterns,

involving photoperiod and the pineal gland, have included the horse,

although not to such an extent as, for example, the hamster or the

sheep. The results are, however, still confused.

Various lighting regimes have been used to try to advance the

onset of the oestrous season in anoestrous mares.

Loy (1968) suggested that a fixed daylight length of 16 hours

duration was optimum for bringing anoestrous mares into season.

He reported that this regime advanced the onset of the breeding

season in anoestrous mares by approximately two months, in

comparison to those receiving no additional lighting.

Sharp, Kooistra and Ginther (1975), using pony mares,

substituted the conditions of temperature and daylight length found

during the period 1st March to 1st July, i.e. the normal breeding

season, during the period 17th October to 15th February. Under

these artificial conditions it was found that the number of follicles with a diameter greater than 10 mm, the number of follicles with a diameter greater than 20 mm, the average follicular diameter and the greatest follicular diameter were all increased in comparison with the control group.

In this work, too, all of the treated group demonstrated positive oestrous behaviour. No oestrous behaviour was demonstrated in the control group. However, only two of the seven treated mares ovulated within the experimental period. This is unusual, as most pony mares would be expected to be cycling regularly by the 1st of July under normal conditions. He suggested that this might have been due to the mares being refractory to light stimulation at the beginning of the experimental period.

Sharp (1980) found that, using supplementary lighting, mares receiving 2.5 hours after sunset and mares receiving 2.5 hours after sunset plus 2.5 hours before sunrise, cycled earlier than those receiving only 2.5 hours before sunrise, or than a control group receiving no additional lighting. Extra light in the evening, therefore, seemed to be most effective.

Palmer, Driancourt and Ortavant (1982) found that when light treatment was begun during the previous November and December, the first ovulation occurred earliest in the following year in those mares exposed to lighting regimes in which light was present 9.5 – 10.5 hours after the onset of night, the total number of hours of light being of little importance.

As pinealectomy in the mare was initially deemed impossible (Sharp, Grubaugh, Berglund, Seamans, McDowell, Kilmer and Peck, 1981) superior cervical ganglionectomy was carried out in an attempt to determine the effect of the pineal in seasonal breeding.

Sharp (1980) found that mares with bilateral superior cervical ganglionectomy, carried out during winter anoestrus, showed no difference in the timing of the onset of the subsequent breeding season, when compared with pineal intact mares. However, the onset of the breeding season in the following year was markedly delayed with the first ovulation in ganglionectomised mares being, on average, 66 days later than that of pineal intact mares.

Later in pinealectomised mares, Sharp et al (1981) found that the timing of surgery appeared to affect results. In mares pinealectomised during the winter months, the onset of the second breeding season after surgery was delayed. This was not so in pineal intact mares or in those mares pinealectomised during the summer months. The onset of the first breeding season after surgery was not altered in mares pinealectomised in summer or winter.

Sharp et al (1981) also found that, whereas an additional 2.5 hours of light at sunset resulted in pineal intact anoestrous mares coming into season earlier than control mares, supplementary light had no effect in pinealectomised anoestrous mares. This suggests that extra light exerts its effect via the pineal gland in the mare.

The apparent involvement of light and the pineal gland in the regulation of the breeding season in the mare, as in other seasonal breeders, led to the investigation of the role of melatonin.

So far, these investigations have been few in number.

In 1980, Sharp reported that in bilateral superior cervical ganglionectomised mares, in comparison with intact and sham-ganglionectomised mares, the pattern of melatonin secretion showed no clear rhythm and suggested that they do not secrete significantly increased amounts of melatonin in darkness. Kilmer et al (1982) also found that greater night-time versus day-time melatonin levels were present in sham-operated and pineal-intact pony mares but not in ganglionectomised pony mares.

Sharp (1980) also reported that mean melatonin levels were higher in mares during anoestrus.

As with other species, the extent of the effect of light and melatonin secretion on the breeding patterns of the mare have still, as yet, not been adequately determined. Many other factors remain to be considered in this regulatory process. These include temperature and nutrition.

Sharp (1980) reports that, in his experience, there have been no critical studies in mares which demonstrate that temperature plays a major role in the regulation of the breeding season. However, there have been several studies which link nutrition with sexual status in laboratory animals.

Herbert (1980) in evaluating the endocrine profile of the protein-calorie malnourished rat, found that serum and pituitary gonadotrophins, serum steroid levels and hypothalamic L.H.R.H. were markedly reduced in malnourished rats when compared to control animals.

Herbert and Reiter (1981) went on to find that pineal protein

and melatonin levels were markedly depressed in the malnourished rat. The melatonin rhythm was not affected, although maximum levels in malnourished rats were observed 2 hours later than controls. The significance of these latter findings is not clear, as the pineal is thought to play only a minor role in the regulation of the reproductive system in the rat.

Ginther (1979) reports that anoestrous mares gaining weight as they entered the transitional period prior to the breeding season, had their first ovulation earlier than anoestrous mares losing weight during the same period.

Belonje and van Niekerk (1975) in reviewing the early literature, report that many authors observe that mares kept on grass tend to be seasonal breeders, whereas mares stabled, and well fed, tend to cycle all year round.

Allen (1978), however, reports that 80% of yarded mares when turned out on to spring grass, demonstrated oestrus and ovulation within fourteen days and that barren and maiden mares maintained on adequate but mainly dried feedstuffs, remain in anoestrus longer than those kept at grass.

Although Sharp (1980) believes that daylight length is the major "impeller" of reproductive rhythms, he suggests that " ------ nutrition must surely be the most likely environmental factor to counter the light-modulated rhythms since there presumably would be little need for annual rhythms in the presence of a guaranteed food supply".

The still unexplained regulatory mechanisms of seasonal breeders and the lack of knowledge as to the interaction of the roles of

daylight length, melatonin levels, nutritional status and ambient
temperature in breeding patterns in mares leave many questions
to be answered.

It is hoped that the following study of pony and thoroughbred-
type mares under natural and artificial lighting, while monitoring
clinical and hormonal changes together with melatonin levels, may
bring us somewhat closer to the answers to some of these questions.

CHAPTER II

2.1 - INTRODUCTION

The aim of this initial study was to try to establish what links, if any, exist between breeding patterns and melatonin levels in the mare.

Mares were monitored for behavioural, ovarian and hormonal changes throughout the months of October to May. They were expected to progress from cyclical activity to anoestrus and then return to cyclical activity.

After establishment of the expected anoestrus, it was planned to attempt advancement of the onset of oestrus in half of the mares with a specific lighting regime.

In addition, melatonin profiles were to be investigated by the detailed examination of melatonin concentrations in circulating plasma obtained on four occasions, over 24 hour periods, during the study. The resulting profiles would be compared with the state of reproductive function at the time of sample collection in each individual.

2.2 - MATERIALS AND METHODS

1. MARES

The available population of eight mares were divided randomly into two groups of four (Table 2.1). Mares ranged in size from 14.2 h.h. to 17 h.h. and were of varying ages and breeds. All of the mares were non-pregnant.

2. HOUSING AND MAINTENANCE

The mares were brought in from grass during late September, 1981 and housed as shown in Diag. 2.1. Three mares were housed simply in loose boxes, two mares shared a small barn and three mares a larger barn. All accommodation led into an uncovered courtyard. All mares were bedded on wheat straw. They were maintained on complete horse cubes (Horse and Pony Cubes, Spillers) and hay. Water was available ad lib.

3. BEHAVIOURAL STUDIES

Teasing was carried out approximately three times per week from 7th October, 1981 and daily from 23rd November, 1981. Each mare was teased individually for a duration of at least five minutes, using a teasing gate. During the winter, when inclement weather existed, the stallion was taken to each mare in turn and teasing carried out there, using the box or barn door as a teasing board.

Two stallions were used - stallion 1 was a 5 year old 3/4 T.B. and stallion 2 a 5 year old Welsh pony.

TABLE 2.1 – MARES USED DURING 1981/82

Group	Mare	Age (yrs)	Breed	Reproductive Status
1	Corrie	4	T.B. type	Maiden
	Melody	13	T.B. type	Barren
	Misty	1.1/2	Highland X.T.B.	Maiden
	Sacha	13	Belgian	Barren
2	Kirsty	4	Highland	Maiden
	Sunny	26	T.B. type	Barren
	Summer	12	T.B.X.	Barren
	Poppy	9	Anglo-Arab	Maiden

DIAG. 2.1 — PLAN OF ACCOMMODATION
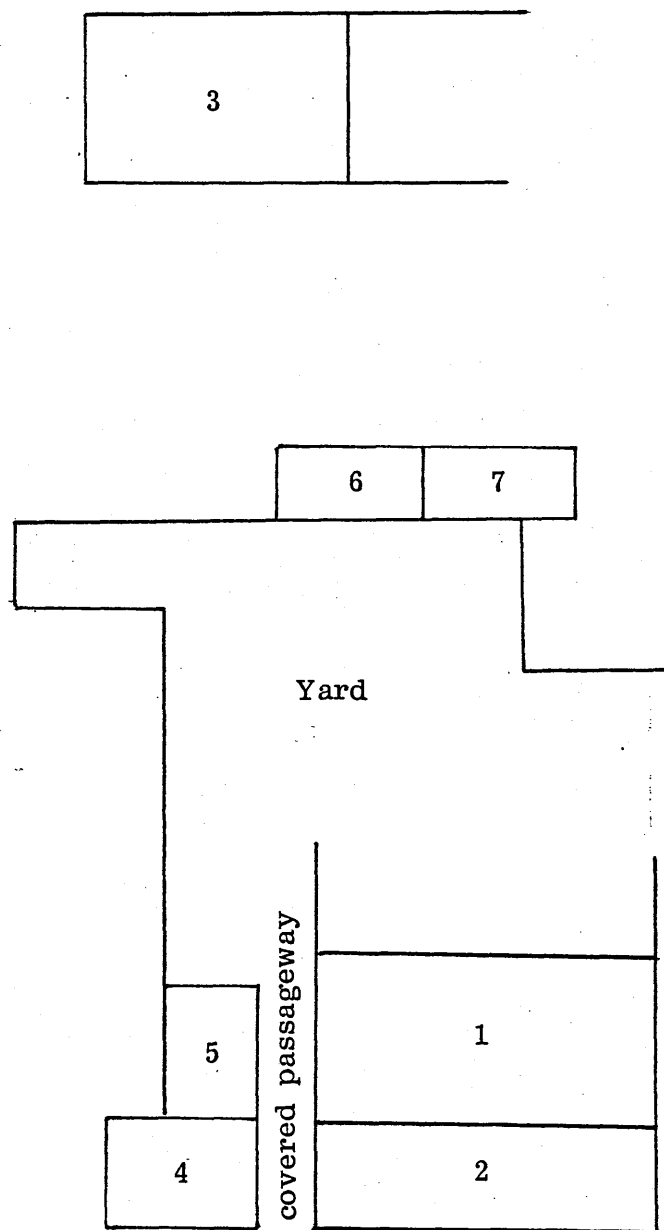
1. Corrie   – Group 1   5. Sacha   – Group 1
   Melody   – Group 1   6. Sunny   – Group 2
   Misty   – Group 1   7. Poppy   – Group 2
4. Kirsty   – Group 2
   Summer   – Group 2

Results were recorded according to the method described by Munro et al (1979).

4. RECTAL PALPATION

Rectal palpation was carried out twice weekly. Mares were put in stocks for adequate restraint. Ovaries, uterus and cervix were palpated. Parameters recorded were ovarian size and consistency; size, position and consistency of structures palpable on the ovaries; uterine size and tone; consistency of the cervix.

5. CERVICAL EXAMINATION

Cervical examination was carried out, on specific occasions, using a mare vaginascope and light source (Portex)
Before vaginascoping took place, the perineum was washed with a povidone iodine solution (Pevidine Surgical Scrub, Berk Pharmaceuticals). Colour, shape, position and wetness of the cervix were recorded.

6. BLOOD SAMPLING FOR PROGESTERONE AND OESTROGEN ESTIMATION

Blood samples for the estimation of progesterone and oestrogen concentrations were collected twice weekly from the jugular vein, initially using 20G $1\frac{1}{2}$" vacutainer-type needles (Becton-Dickinson) and heparinised vacutainers and then using 19G $1\frac{1}{2}$" needles (Terumo) and heparinised Monovets (Sarstedt). Immediately after collection, the samples were centrifuged (10 minutes, 4° C, 1500 g), decanted into labelled glass bottles and stored at -20° C until assayed.

7. ESTIMATION OF PLASMA PROGESTERONE

Plasma progesterone concentrations were estimated using an established radioimmunoassay procedure, as described by Munro et al (1979). In this study, coefficients of variance for high, medium and low plasma progesterone control samples were 14.7, 15.2 and 21.1% respectively. Extraction values averaged 89.3 $\pm$ 11.6%.

8. ESTIMATION OF PLASMA OESTROGENS

Plasma oestrogens were estimated using an established radioimmunoassay procedure as described by Munro et al (1979). In this study, coefficients of variance for plasma oestrogen control samples of high, medium and low values were 12.6, 13.7 and 18.5% respectively. Extraction values averaged 90.5 $\pm$ 7.9%.

2.3 - RESULTS

Fig. 2.1

GROUP 1 - CORRIE

After a prolonged oestrous period in October, this mare continued to cycle regularly throughout the period of study. Progesterone and oestrogen patterns correlate with behavioural changes. Note, however, the low progesterone levels recorded during late February.

Fig. 2.1

GROUP 1 – CORRIE
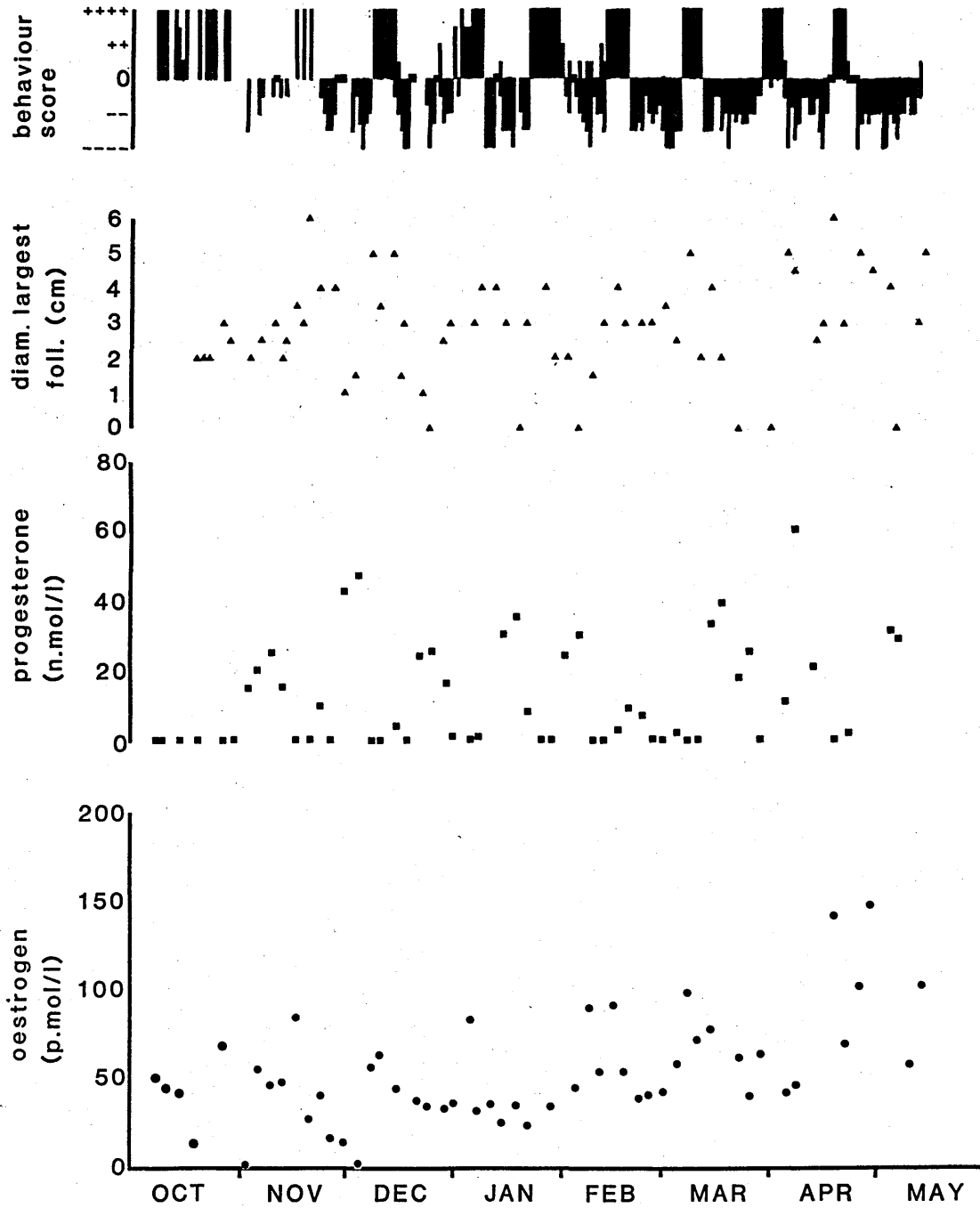
RESULTS OF BEHAVIOURAL STUDIES, RECTAL PALPATION,
PLASMA PROGESTERONE AND OESTROGEN ESTIMATIONS
CARRIED OUT DURING 1981/82

Fig. 2.2

GROUP 1   - MELODY

Two normal oestrous periods occurred during October and November. The next oestrous period in December was interrupted and many negative as well as positive behavioural signs were recorded.   Follicles of only 1 cm in diameter were palpated.   There was no corresponding increase in circulating oestrogens and progesterone levels did not rise subsequently.

Progesterone and oestrogen levels remained basal throughout January and February.   However, positive oestrous behaviour was expressed for prolonged periods during this time.   Follicles ranging from 1 to 4 cm in diameter were present in the ovaries, the larger structures being recorded 5 - 6 days before the cessation of oestrous behaviour in early March. Raised plasma progesterone levels followed.

Fig. 2.2

GROUP 1   –   MELODY
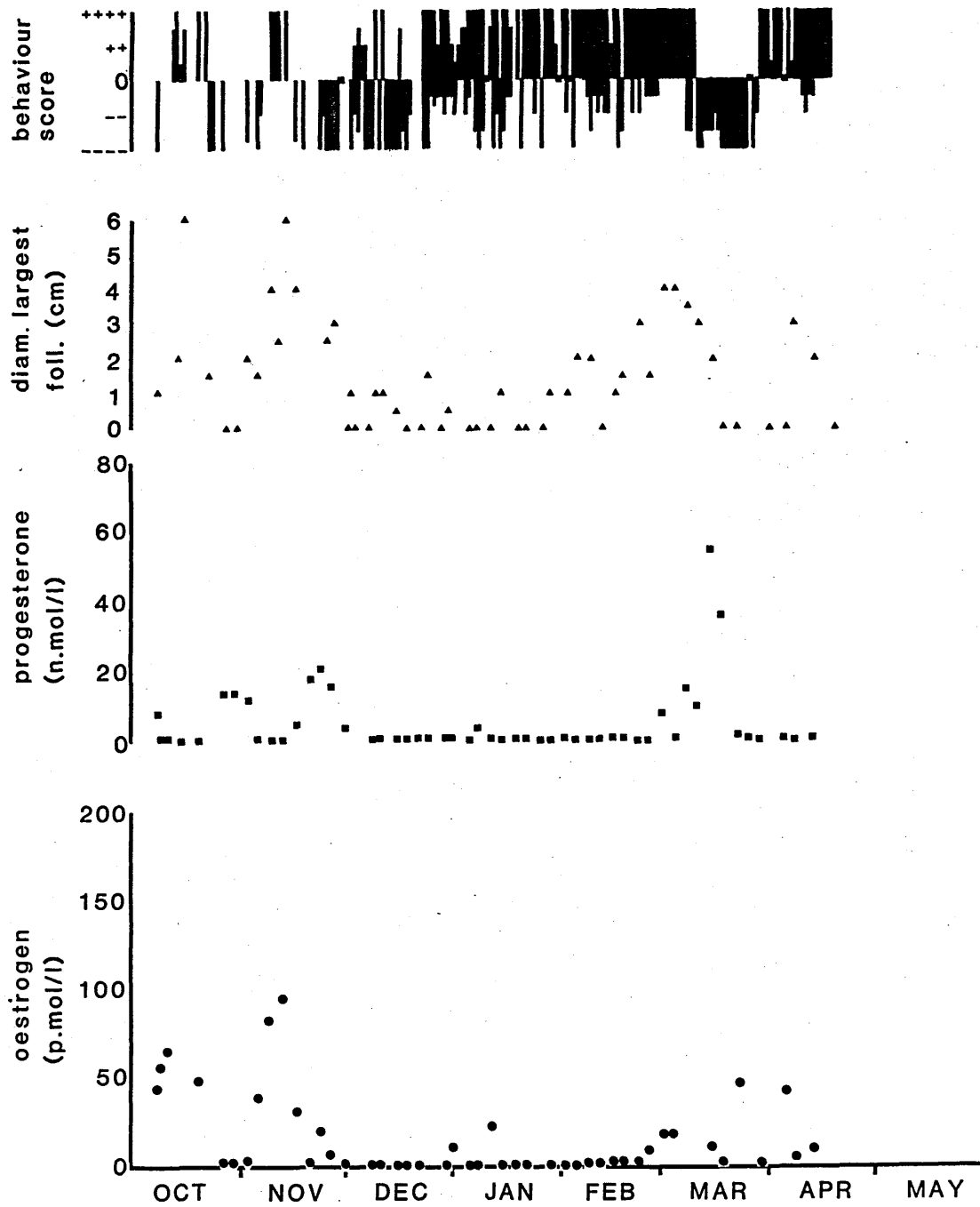
RESULTS OF BEHAVIOURAL STUDIES, RECTAL PALPATION,
PLASMA PROGESTERONE AND OESTROGEN ESTIMATIONS
CARRIED OUT DURING 1981/82

Fig. 2.3

GROUP 1   -   MISTY

Initially, on teasing, this mare was mainly indifferent to the stallion with occasional demonstration of positive oestrous signs. However, she then showed mainly negative signs during December. Throughout this three-month period, progesterone levels remained basal and follicles ranged from 0 to 5 cm in diameter.

A normal oestrus occurred in early January, with a follicle reaching 2.5 cm in diameter, followed by a short period of increased circulating progesterone.

Subsequently, progesterone levels remained basal, follicles ranged from 0 - 4 cm in diameter and mainly positive oestrous behaviour was recorded. This continued until the beginning of April, whan a 5 cm follicle was recorded. Positive oestrous behaviour ceased 7 days later and was followed by a rise in progesterone levels.

Fig. 2.3

GROUP 1 - MISTY
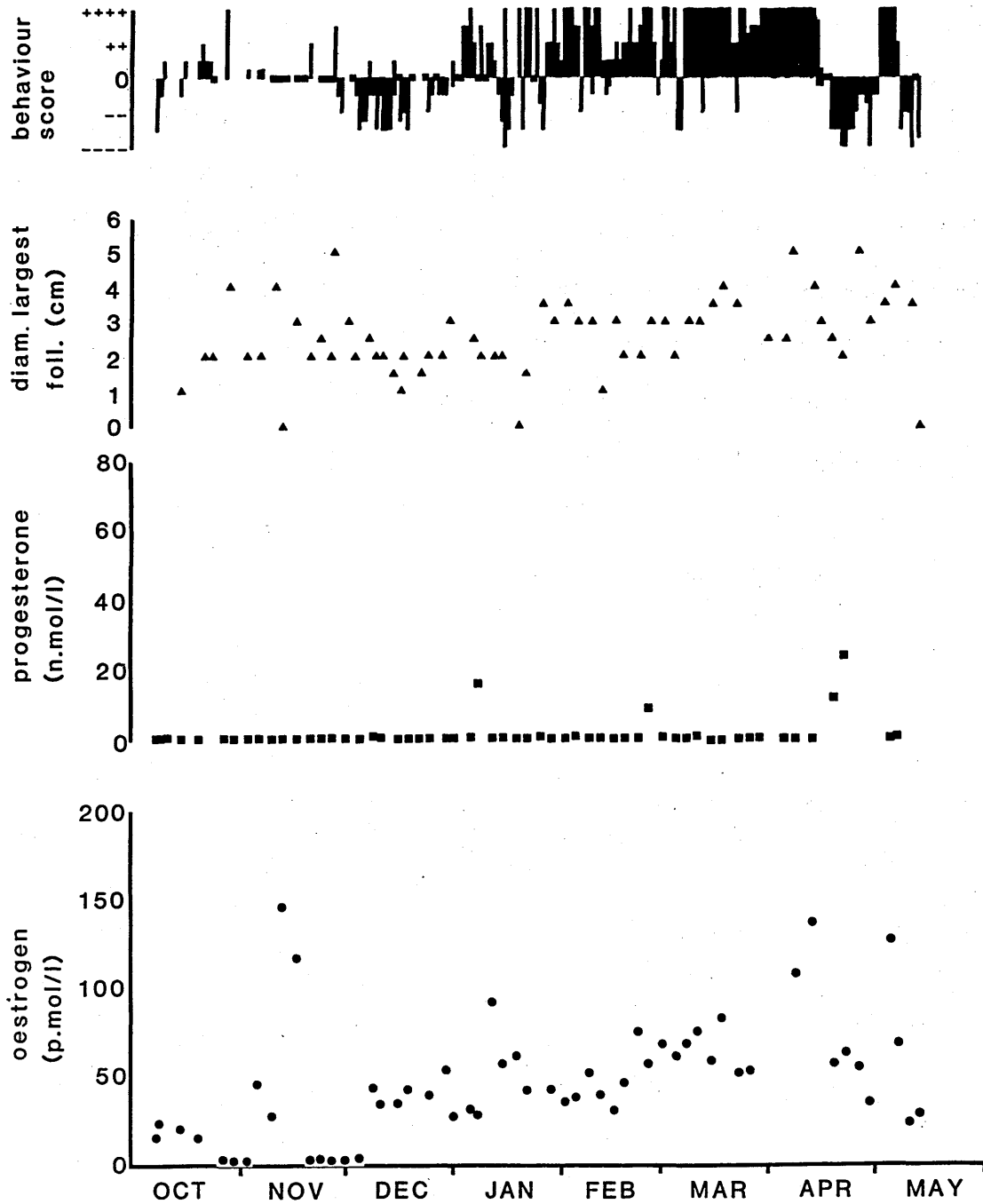
RESULTS OF BEHAVIOURAL STUDIES, RECTAL PALPATION,
PLASMA PROGESTERONE AND OESTROGEN ESTIMATIONS
CARRIED OUT DURING 1981/82

Fig. 2.4

GROUP 1   -   SACHA

After a normal oestrous cycle in October, Sacha's next period of oestrous behaviour occurred in November, when a 6 cm follicle was palpated.   However, although positive oestrous behaviour ceased, progesterone levels subsequently remained basal throughout the months of December to April.   Negative behaviour on teasing was demonstrated from December to March, interrupted only by one period of oestrous behaviour during early February, with a subsequent small rise in progesterone levels, of short duration.   Maximum follicular diameter at this time was 2 cm.

Fig. 2.4

GROUP 1 — SACHA

RESULTS OF BEHAVIOURAL STUDIES, RECTAL PALPATION,
PLASMA PROGESTERONE AND OESTROGEN ESTIMATIONS
CARRIED OUT DURING 1981/82

Fig. 2.5

GROUP 2   -   KIRSTY

Note in this mare, during mid-December, the delay in demonstrating positive oestrous behaviour after the return of plasma progesterone concentrations to basal values.   Plasma progesterone concentrations did rise subsequent to this oestrus, but for a shorter period than expected. Following this, Kirsty's plasma progesterone concentrations remained basal until late April.   Throughout this time she demonstrated intermittent positive oestrous behaviour, follicles of up to 4 cm were palpable in the ovaries and plasma oestrogen concentrations fluctuated accordingly.

Fig. 2.5

GROUP 2 - KIRSTY

RESULTS OF BEHAVIOURAL STUDIES, RECTAL PALPATION, PLASMA PROGESTERONE AND OESTROGEN ESTIMATIONS CARRIED OUT DURING 1981/82

Fig. 2.6

GROUP 2 - SUNNY

Note the prolonged period of elevated progesterone which occurred during November, December and part of January.   These high levels of progesterone were accompanied by negative behaviour on teasing, follicles ranging from 0 to 3.5 cm in diameter and low oestrogen levels.

Progesterone levels fell during mid-January, oestrogen levels rose and a prolonged oestrous period ensued.   In late February a follicle of 5 cm in diameter was palpated.   This was followed by an increase in plasma progesterone levels.

Fig. 2.6

GROUP 2 — SUNNY

RESULTS OF BEHAVIOURAL STUDIES, RECTAL PALPATION,
PLASMA PROGESTERONE AND OESTROGEN ESTIMATIONS
CARRIED OUT DURING 1981/82

Fig. 2.7

GROUP 2 - SUMMER

This mare demonstrated negative or indifferent behaviour on teasing during the first three months of the study. However, during this period, follicular growth and progesterone levels continued to show a normal cyclical variation.

At the end of December, one day of positive oestrous behaviour occurred. Although no follicles were palpable per rectum, a subsequent rise in progesterone levels was demonstrated.

Fig. 2.7

GROUP 2 — SUMMER
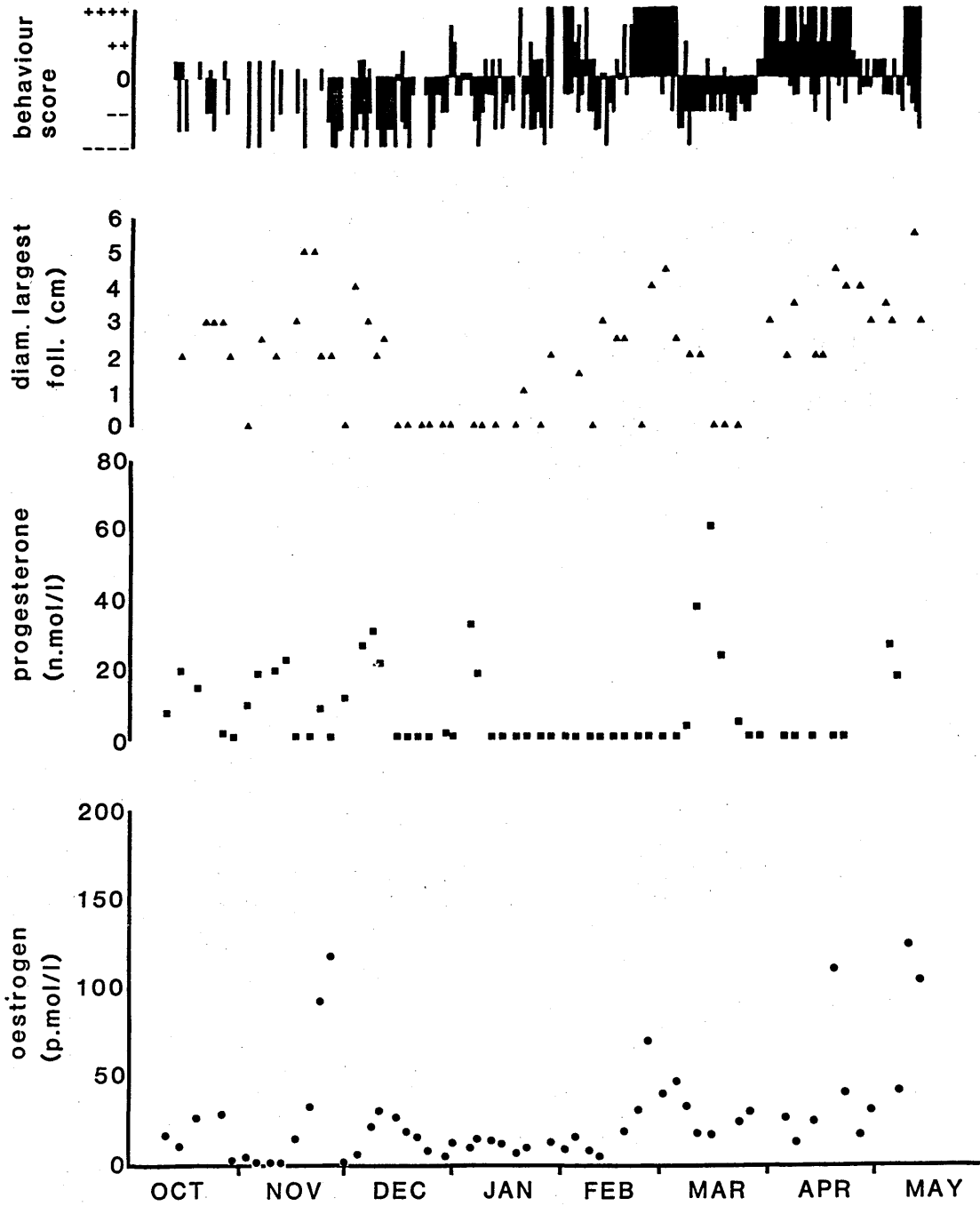
RESULTS OF BEHAVIOURAL STUDIES, RECTAL PALPATION,
PLASMA PROGESTERONE AND OESTROGEN ESTIMATIONS
CARRIED OUT DURING 1981/82

Fig. 2.8

GROUP 2 - POPPY

Note in this mare, during December and January/February, the
occurrence of increased plasma progesterone levels related to the
presence of follicles of 6 cm in diameter, 4 - 5 days previously.
However, no positive oestrous behaviour was associated with these
findings.

Normal oestrous periods with basal progesterone levels, positive
oestrous behaviour, maximum follicular diameters of 4 cm and
subsequent raised progesterone levels, occurred during mid-February
and early May.

Fig. 2.8

GROUP 2 – POPPY
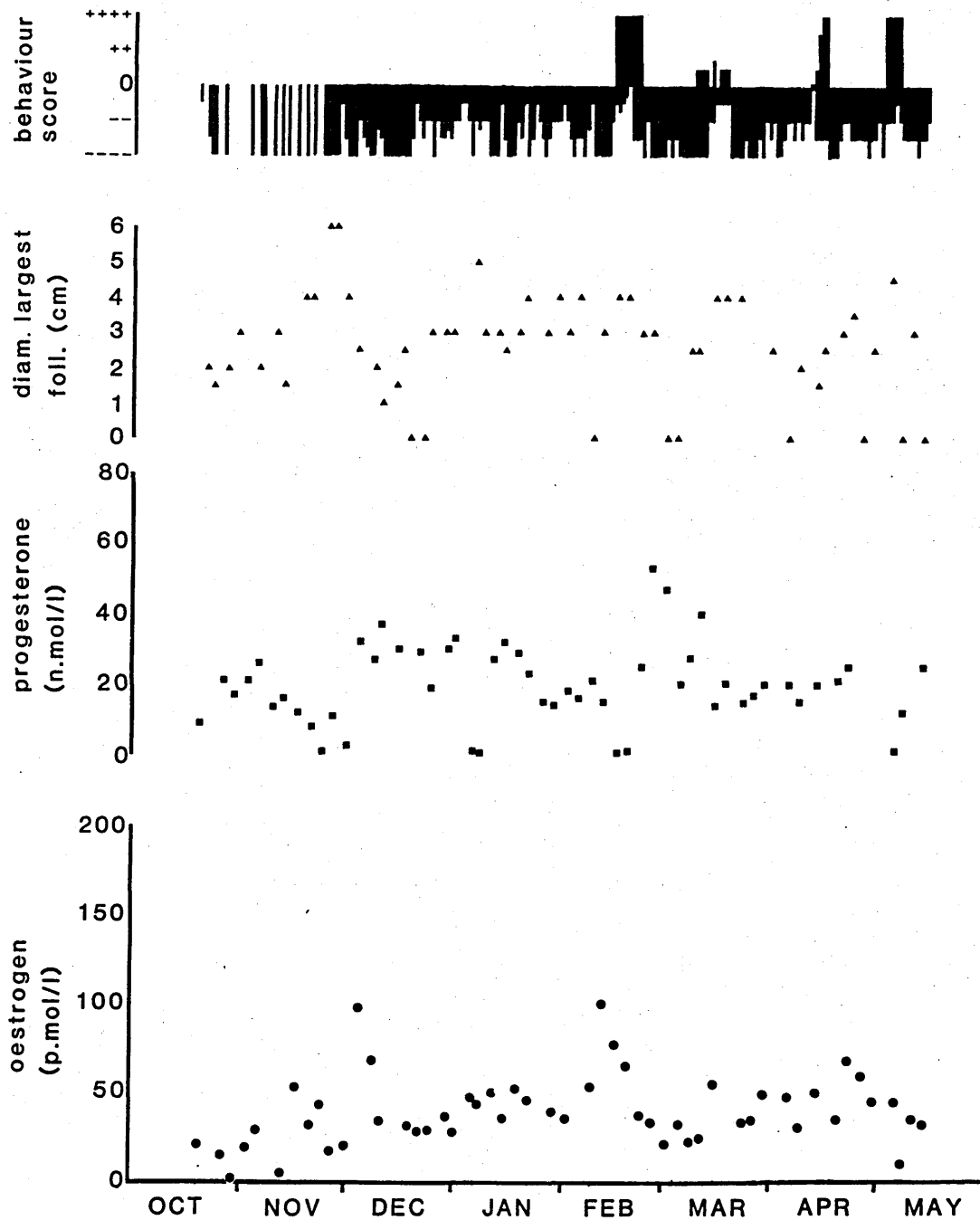
RESULTS OF BEHAVIOURAL STUDIES, RECTAL PALPATION,
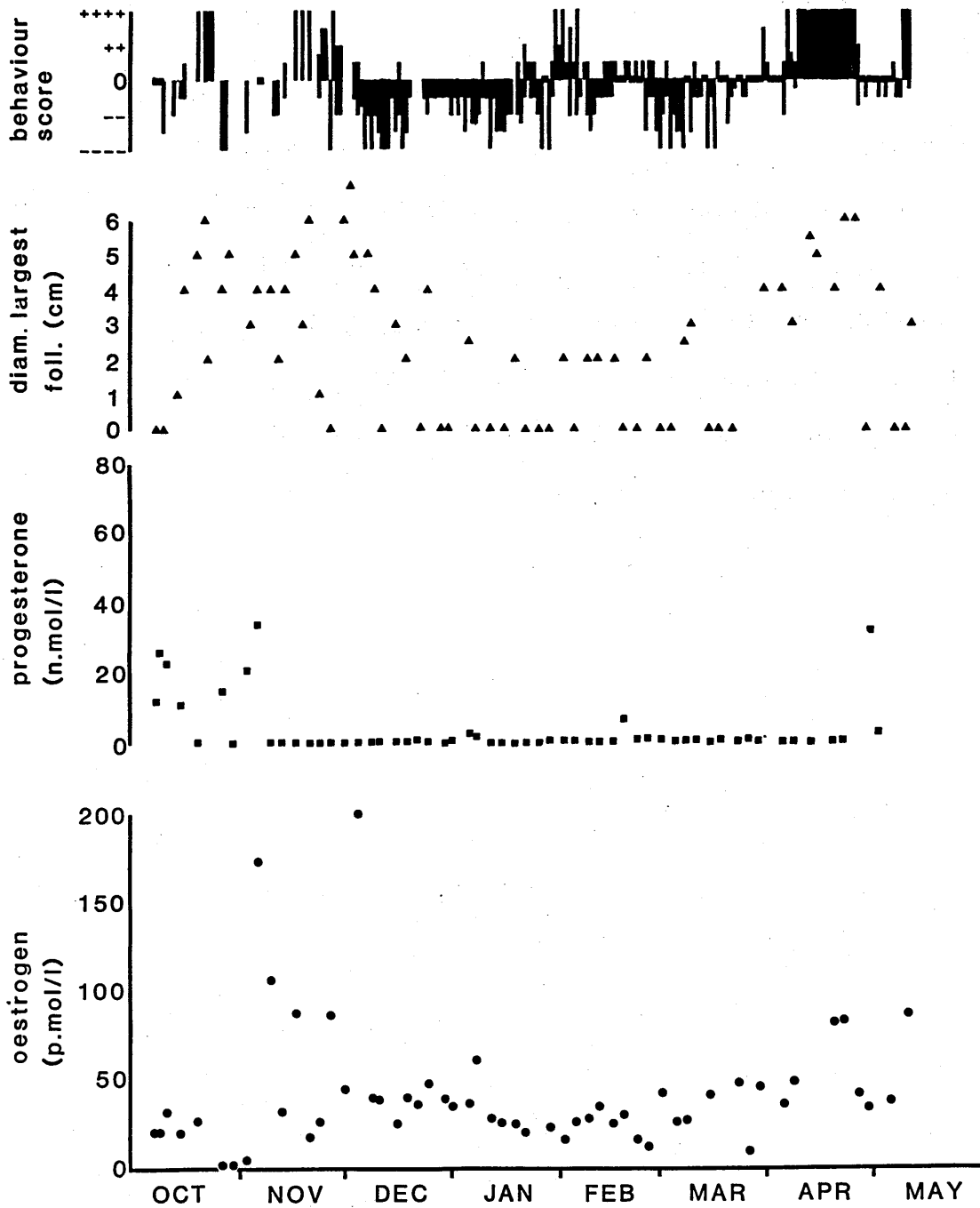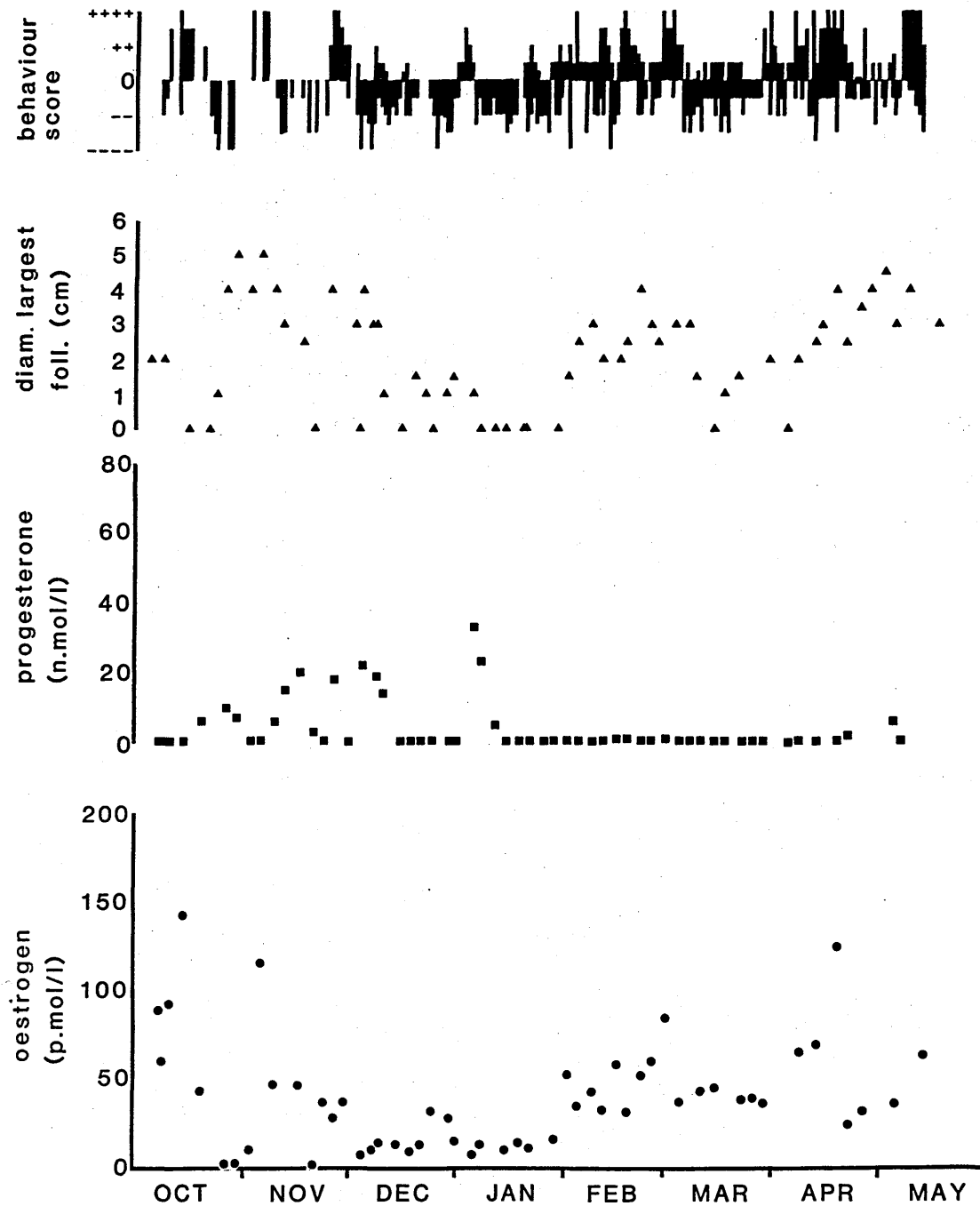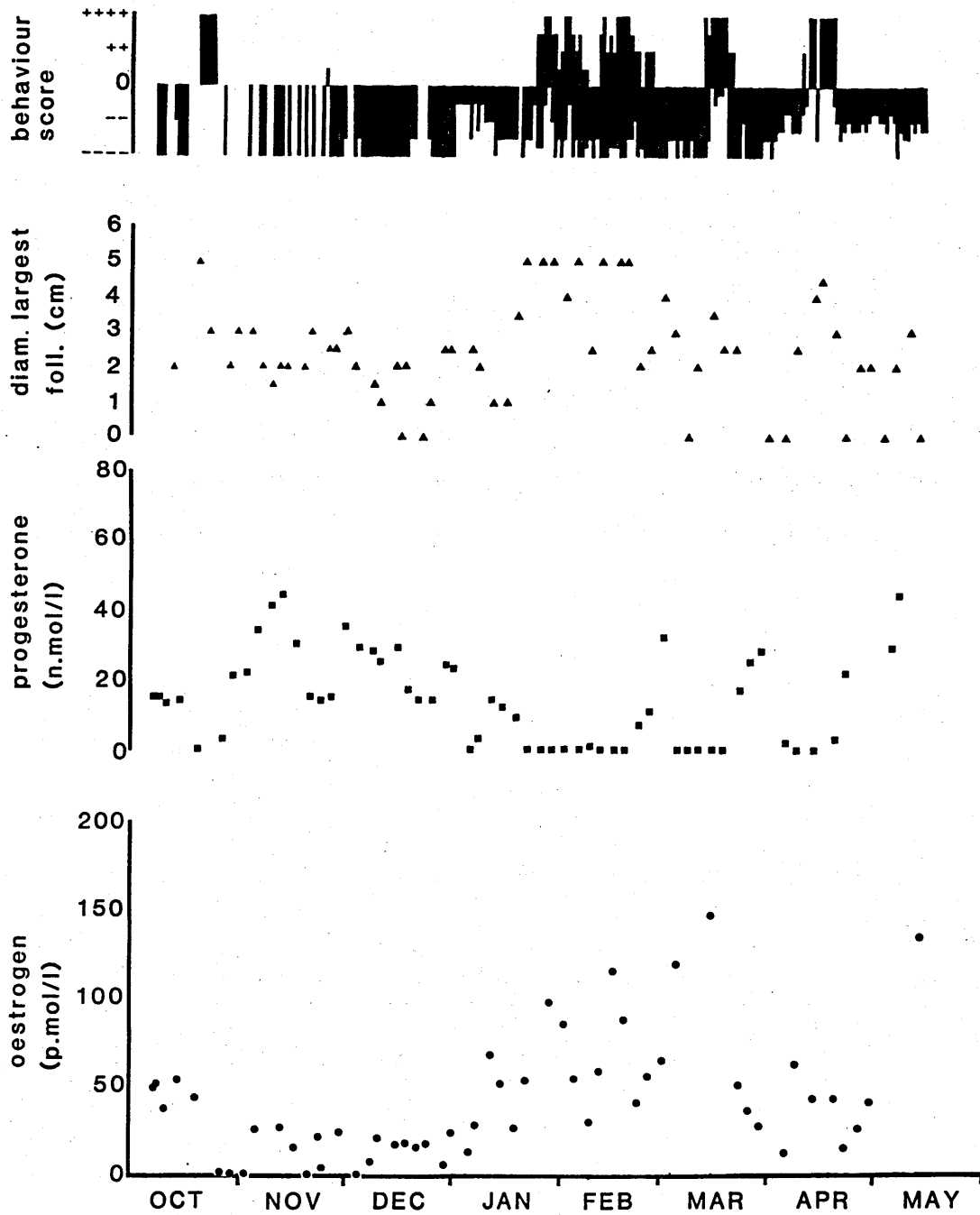PLASMA PROGESTERONE AND OESTROGEN ESTIMATIONS
CARRIED OUT DURING 1981/82

2.4 - DISCUSSION

The most notable feature of the preceding results is the variation in sexual status of this group of mares during the winter months, with only three out of eight making the transition into anoestrus.

October is traditionally thought of as the "last" month of the breeding season (Hughes et al, 1980; Arthur et al, 1982). In Central Scotland experience has shown that a mare which is in good condition after a summer at grass could still be cycling at this time. Other mares at lesser pasture, or with a foal at foot, might be expected to be winding down, i.e. beginning to make the transition from regular cyclical behaviour to anoestrus.

The eight mares discussed in this thesis were barren, had spent the summer at grass and were in good condition. During the month of October, five of the mares exhibited a normal oestrus (Corrie, Fig. 2.1; Kirsty, Fig. 2.5; Sunny, Fig. 2.6; Sacha, Fig. 2.4 and Melody, Fig. 2.2). One mare exhibited occasional days of positive oestrous behaviour (Misty, Fig. 2.3) and two mares did not exhibit positive oestrous behaviour at all (Summer, Fig. 2.7; Poppy, Fig. 2.8).

Of the five mares which demonstrated a normal oestrus during October, one, Corrie, a 3/4 T.B., continued to cycle throughout the study period with, however, some deviations from a normal pattern.

In Corrie (Fig. 2.1) the first standing oestrus in October was prolonged.

Prolonged oestrus is a relatively common feature in the mare but is said to occur at the beginning of the breeding season in the spring, during the transition from winter anoestrus to cyclical behaviour, (Ginther, 1979; Noakes, 1979; Hughes et al, 1980; Rossdale and Ricketts, 1980; Arthur et al, 1982) rather than as in Corrie, during October.

At the beginning of the breeding season, prolonged oestrus is associated with an increase in both numbers and size of follicles together with a high level of circulating FSH. These follicles grow and regress but do not ovulate. However, oestrogen secretion appears to lag behind this follicular development (Sharp, 1980). This may be due to the fact that although these follicles develop, they rarely reach the diameter associated with maturity. Attainment of a certain size appears to represent an important stage in the maturation of a follicle, which relates to a three-fold increase in circulating oestrogens (Kenney, Condon, Ganjam and Channing, 1979). Thus plasma oestrogen levels do not rise significantly until follicles reach this certain size which happens in mares towards the end of the transitional period prior to the beginning of the breeding season.

During this time oestrous behaviour does not appear to be dependent on markedly increased levels of circulating oestrogens (Sharp, 1980) as positive oestrous behaviour may be demonstrated even when only slightly increased levels of plasma oestrogens are present. It would appear that the behavioural centre of the

hypothalamus is, at this time, more sensitive to any increase in plasma oestrogens in the presence of basal plasma progesterone.

Corrie's findings in October tend to agree with those discussed above and were a prelude to the continuation of cyclical activity.

Corrie may well have been about to progress into anoestrus, where the characteristic feature of this transition is reported as being anovulatory oestrus (Ginther, 1979; Hughes et al, 1980; Sharp, 1980) and was in some way, perhaps by housing, feeding and/or daily teasing with a stallion stimulated to continue with cyclical behaviour.

After this first prolonged oestrus, Corrie continued to cycle regularly throughout the remainder of the study period with average oestrous and interoestrous lengths of 6.9 and 14.8 days respectively. Ginther (1979), reviewing the literature, suggests that the average oestrous and dioestrous lengths for larger breeds of equines are 7 and 15 days respectively. Therefore, in this respect, Corrie would appear to be normal.

However, Corrie did have two shorter dioestrous periods during January and early February. Alteration in the length of the oestrous cycle in the mare is usually due to variation in the length of the oestrous period rather than the dioestrous length which usually remains fairly constant (Hughes et al, 1980). Shortened dioestrus, due to premature release of prostaglandins by the uterus, can occur in mares with endometritis (Hughes, Stabenfeldt, Kindahl, Kennedy, Edqvist, Neely and Schalm, 1979;

Neely, Kindahl, Stabenfeldt, Edqvist and Hughes, 1979).
Although this mare had no evidence of such a condition on
rectal palpation or on clinical examination, a sub-clinical
infection may have been present.   If this was indeed so, there
was no long-term effect as this mare conceived in the following
October.

After each oestrus in Corrie, plasma progesterone concen-
trations rose, indicating the production of luteal tissue in the
ovaries at these times.   The only exception to this occurred in
mid-February where progesterone levels began to rise, and in
fact peaked, during the oestrous period.   At this time the
maximum recorded plasma progesterone concentration was
considerably lower than those in previous and subsequent
dioestrous periods.

This pattern, where a rise in plasma progesterone concentration
occurs during the oestrus, can be seen in the bitch where pre-
luteinisation of ovulatory follicles takes place 60 - 70 hours prior
to ovulation (Concannon, Hansel and McEntee, 1977).   This might
suggest that in Corrie, ovulation did not occur, the small rise in
plasma progesterone concentrations being due to luteinisation of the
intact follicle.

Ginther (1979) suggests that anovulation is a rare occurrence
in mares, during the breeding season, referring to one study where
a 3.1% incidence was recorded (Hughes, Stabenfeldt and Evans, 1972).
He suggests that this low incidence is due to the considerable
variation in the diameter of a mature follicle in the mare and to the

greater length of time over which increased concentrations of LH are present in comparison to the other domestic species. However, van Rensburg and van Heerden (1953) suggested that ovulatory failure in the cycling mare was one of the most serious types of infertility but gave no information as to whether the follicles underwent atresia or luteinisation.

In the cow, when anovulation occurs, the follicle either regresses or becomes luteinised. These luteinised follicles are known to secrete progesterone and are lysed by prostaglandins in the same way as a corpus luteum, with the cow returning to oestrus at the expected time (Arthur et al, 1982).

Evidence for luteinisation of follicles occurring in the mare has been obtained from post-mortem studies. Luteal structures were found in the ovary which had no connection with the ovulation fossa (Ginther, 1979). This suggests that ovulation did not occur although luteal tissue had been produced. Therefore, luteinisation rather than ovulation could have occurred in Corrie at this time.

It would appear that, in Corrie, in general, hormonal, ovarian and behavioural findings during the winter months were as would be expected during the breeding season. Therefore, it can be said that this mare did continue to cycle throughout the winter months.

Kirsty (Fig. 2.5), a Highland Pony mare, continued to cycle throughout October, November and December. During the first oestrous and dioestrous period respectively, there was a lack of recorded follicular growth and increased plasma progesterone concentration. The rise in plasma oestrogens associated with this

first oestrous period would tend to indicate the presence of a follicle of greater than the 2 cm recorded. One explanation for this could be the irregularity with which rectal palpation and blood sampling were carried out at this time.

After the third dioestrous period, in December, regular cycling ceased and plasma progesterone concentrations returned to basal values. A period of sexual quiescence followed which lasted for approximately 18 days. Then, although no palpable follicular growth took place, plasma oestrogen concentrations rose slightly. This was apparently enough to trigger a behavioural response and positive oestrous behaviour was demonstrated over two days. A similar pattern of quiescence has been recorded in the mare (Allen, 1974) during the breeding season.

In Kirsty, following the demonstration of this positive oestrous behaviour, plasma progesterone concentrations rose, indicating the presence of luteal tissue in the ovaries. On this occasion a follicle may have been luteinised without ovulation as the plasma progesterone concentrations began to rise, as in Corrie, as oestrous behaviour was demonstrated and remained elevated for a shorter time than expected.

After this abrupt oestrus and dioestrus, reproductive function again ceased and Kirsty entered anoestrus.

Anoestrus was short-lived in Kirsty, with follicular development, increasing plasma oestrogen concentration and positive oestrous behaviour re-commencing in early February. However, the transitionary period was lengthy with the first recorded rise in plasma

progesterone concentrations occurring in late April.

Kirsty, in continuing to cycle until late November / early December, cycled considerably longer than expected of a native pony mare. These findings, together with the oestrous period in December, the short anoestrus and the prolonged transition to regular cyclical activity, may suggest that Kirsty was endeavouring to maintain reproductive function throughout the winter months. However, cyclicity failed.

Another mare in which oestrus was observed during October was Sunny (Fig. 2.6), again a 3/4 T.B.

After a normal oestrous period in October, when Sunny entered a phase of prolonged dioestrus, circulating plasma progesterone concentrations rose and remained elevated until early January.

In the mare, prolonged dioestrus, due to the spontaneous prolongation of a C.L., can occur at any time during the breeding season (Allen, Stewart, Cooper, Crowhurst, Simpson, McEneny, Greenwood, Rossdale, Ricketts, 1974; Stabenfeldt, Hughes, Evans and Neely, 1974) and it is one of the unusual facets of reproduction in this species.

Prolongation of the life of a C.L. does occur in other domestic animals (Coudert and Short, 1966; Ginther, 1968; Hansel, Concannon and Lukaszewska, 1973). However, the mare is the only one in which it can occur in the absence of any uterine anomaly. Under normal conditions, at the end of dioestrus, the uterus produces sufficient prostaglandin to lyse the reigning C.L. In mares exhibiting prolonged dioestrus, this mechanism goes wrong.

One of the reasons for this failure to lyse the C.L. may be that the mare does not have such an efficient mechanism for transporting prostaglandin from the uterus to the ovary as do some of the other domestic animals. In the ewe and the cow, the ovarian artery and the utero-ovarian vein lie in close proximity. This allows for the transfer of the luteolytic substance from the uterine vein into the ovarian artery, and so to the ovary, by a counter-current exchange mechanism (Barret, de Blockey, Brown, Cumming, Goding, Mole and Obst, 1971; Hansel et al, 1973).

No such local effect can be demonstrated in the mare (Ginther and First, 1971) and it is generally accepted that prostaglandin is transported via the systemic circulation. Therefore, a much less concentrated supply of prostaglandin may reach the luteal tissue within the ovary in the mare in comparison with that in the ewe and the cow.

Throughout the period of prolonged dioestrus, Sunny's plasma progesterone concentrations, although always high, did fluctuate. This fluctuation may be a normal variation in progesterone production by a corpus luteum which exists for a greater length of time than is usual, or it may be due to partial lysis of the C.L. by insufficient production of prostaglandin by the uterus, after which the luteal structure resumes full function. On the other hand, the continued progesterone production may be due to further luteinisation or ovulation of follicles. Ovulation or luteinisation of follicles can occur during any normal dioestrous period (Stabenfeldt et al, 1975).

In Sunny's case, it must be questioned whether follicles reached a large enough size, and oestrogens a high enough value for this latter suggestion to ensue. Since the largest follicle present at this time was only 2.5 cm, it is unlikely that this sequence of events took place.

After this period of prolonged dioestrus, Sunny's plasma progesterone concentration returned to a basal value in early January, but almost immediately rose again and remained elevated for a further ten to twelve days. At this time there was apparently no mature follicle present in the ovaries and no positive oestrous behaviour was demonstrated. Therefore, the source of this progesterone is not evident.

Progesterone can also be produced by the adrenals (White, Handler, Smith, Hill and Lehman, 1978). However, in mares and women, it is thought that most of the plasma progesterone during dioestrus is derived from the C.L. (White et al, 1978; Asa and Ginther, 1982). Therefore, the rise in plasma progesterone concentrations in Sunny during early January may indicate that further luteinisation of follicles that were not palpable occurred.

During late January and February, Sunny, like Kirsty (Fig. 2.5), demonstrated a period of prolonged positive oestrous behaviour. However, in contrast to Kirsty, follicular development and the associated rise in plasma oestrogen concentrations followed immediately the return of plasma progesterone concentrations to basal values. Follicle size fluctuated but, in contrast to both Kirsty and Corrie during prolonged oestrus, there was, in general,

a fairly large follicle present throughout. The fluctuation in follicular size correlated well with variations in circulating plasma oestrogens.

Plasma oestrogens appeared to peak first in late January but it would seem that no ovulation took place at this time.

The presence of a large follicle, and an associated substantial elevation of plasma oestrogens, which does not result in ovulation or luteinisation of that follicle with the concomitant increase in plasma progesterone concentration, would suggest that there may be some deficiency in LH production. This may be due to insufficient stimulation of the hypothalamus by circulating oestrogens either in quantity or duration. It may also be due to a failure of the pituitary to be stimulated or to respond to stimulation with the production of adequate amounts of LH.

Plasma oestrogens peaked again in mid-February and after this second rise, ovulation or luteinisation did occur.

Therefore, Sunny's main deviation from normal regular cycling was prolonged dioestrus which lasted through November, December and the first half of January and led into a period of prolonged oestrus. These are happenings associated with the breeding season. It can be hypothesised that this mare would have returned to oestrous at any time during this prolonged dioestrus period with the administration of prostaglandin.

And so to the remaining two mares who demonstrated oestrus cycles during October. These two mares, Sacha (Fig. 2.4) and Melody (Fig. 2.2) progressed from cyclical behaviour into anoestrus

but, prior to anoestrus, they adopted different reproductive patterns during the transitional stages.

In Sacha, after one normal oestrus, the following oestrus was prolonged and associated with anovulation. During this oestrous period two large follicles developed, in turn, in opposite ovaries. Although the first appeared to ovulate on clinical examination, the second did not and there was no resulting increase in plasma progesterone concentrations. This failure of ovulation and lutein-isation could have been caused by an insufficient supply of LH. Ginther (1974) suggests that anovulation occurs commonly in mares making the transition into anoestrus, and is due not to a deficiency in the number of follicles or available FSH, but to a deficiency in LH which is said to bring about the final growth spurt of a large follicle.

In Sacha's case, the final growth spurt appeared to have occurred, as may also have ovulation. However, LH is thought to be required not only to initiate ovulation but also for establishment and maintenance of the CL, probably in conjunction with prolactin (Arthur et al, 1982). The mechanism for maintenance of the CL apparently varies with the species. According to Evans and Irvine (1977), LH is involved in the establishment of the CL in the mare but so far little information is available in the literature as to its maintenance in this species. It has been established that LH maintains the CL in the cow (Hansel et al, 1973) and that administration of LH will prolong luteal function in the cow and sow. However, prolactin appears to be more important in this role in the

ewe (Arthur et al, 1982). Perhaps in Sacha, after initiation of
the one ovulation, pituitary LH stocks were exhausted and lack
of luteinisation and ovulation of the second follicle were the result.

In Sacha, the transition from cycling to anoestrus did not
happen abruptly as ovarian activity slowed down over a period of
weeks. Anoestrus lasted in Sacha from December to March.
One aberration occurred during this anoestrous period, in Sacha,
namely the demonstration of positive oestrous behaviour over seven
days during late January / early February. This coincided with
a small rise in plasma oestrogens which may have been enough to
stimulate the behavioural centre of the hypothalamus. However,
follicular diameter did not exceed 2 cm and there was no subsequent
rise in plasma progesterone concentration.

Sacha's return to cyclical behaviour was accomplished in mid /
late April with a prolonged oestrous period of seventeen days.
During this time, twin ovulations apparently occurred, with one
follicle ovulating five to six days before the end of positive oestrous
behaviour and a second ovulating during early dioestrus. A normal
dioestrous period of fourteen days followed.

Twin ovulations are relatively common in the larger breeds of
mare (Burkhardt, 1948; Arthur, 1958; Osborne, 1966). With the
use of the ultra sound scanner in the mare, some twin ovulations
are being recognised even where only one follicle was palpable
during the preceding oestrus. This is probably the result of
further follicular growth and ovulation after the end of positive
oestrous behaviour which, in turn, is probably due to the prolonged

period of elevated plasma LH concentration found in the mare
(Geschwind, Dewey, Hughes, Evans and Stabenfeldt, 1975).

Therefore, Sacha, although cycling at the beginning of the
investigation, did enter anoestrus with lengthy transitional
periods before and after.

Sacha had no obvious reasons for making the transition into
anoestrus.   She had no vices and no health problems.   However,
she was a very large mare, approximately 17 h.h., with an appetite
to match, and may not have been receiving sufficient total energy
input.   Although in good condition, there was perhaps no extra
energy to maintain reproductive function as well.   However, she
was already receiving more food than any of the other mares, and
to increase this further would have been disastrous to our economy.

In Melody (Fig. 2.2), two normal oestrous periods were followed
by an anovulatory oestrus after which she progressed to anoestrus.

In contrast to Sacha, the transitionary oestrous period before
anoestrus was associated with the development of only small follicles.
This was most probably due to the lack of a final growth spurt of
a follicle because of insufficient LH, (Ginther, 1974).

Anoestrus did not last as long in Melody, with the first
ovulation of the breeding season taking place in early March.

The main deviation from the normal pattern in Melody was the
almost constant demonstration of positive oestrous behaviour during
late December, January and February.   Plasma oestrogen
concentrations, as well as plasma progesterone concentrations,

remained basal during these months. The ovaries were small and firm throughout.

From the hormonal patterns and ovarian findings, Melody would be classed as being in deep anoestrus. However, these findings are in stark contrast to the behavioural records.

One factor which must be taken into account in this case is that Melody was loose-housed in a barn with the mares Corrie (Fig. 2.1) who continued to demonstrate cyclical behavioural changes, and Misty (Fig. 2.3) who exhibited intermittent positive oestrous behaviour from the beginning of January. During the winter of 1981/82 the weather was particularly severe, with the yard where teasing was carried out being just rutted ice for quite some time. For the safety of the mares, instead of teasing at the outside teasing gate, the small pony stallion was taken round the barns and boxes. Therefore, mimicry of the other mares in the barn may have played a part in Melody demonstrating this constant positive oestrous behaviour.

On the other hand, Ginther (1979) reported that the demonstration of positive oestrous behaviour is normal in anoestrous mares and suggested that it reflects the balance between circulating concentrations of oestrogens and progestagens, whether of ovarian or extra-ovarian origin. Unseasonal oestrous behaviour is also demonstrated by ovariectomised mares and can be suppressed in them and in intact anoestrous mares by the administration of dexamethasone (Asa, 1982). This would suggest an adrenal source for the hormones. If this was the case in Melody, either some hormone capable of switching on oestrous behaviour other than oestrogen or progesterone was being

produced or the plasma concentrations of these two hormones were so low as to be undetectable with our assay.

Perhaps a reason for Melody becoming anoestrus was that she is an habitual weaver and uses up a great deal of energy in this way. Weaving is considered an unsoundness in a working horse (Adams, 1974) where feeding, which should be producing condition and energy for competition, is being expended uselessly in this continuous movement. The wasted energy, in this case, would perhaps have enabled reproductive function to continue.

This mare was also prone to colic, during which times her biochemical liver enzyme picture, always slightly abnormal, became even more so. For this reason, she may well have not been utilising her feeding as efficiently as the other mares, resulting in a loss of condition and cessation of reproductive function.

At the beginning of this investigation in October, 1981 the mare, Misty, a half T.B. (Fig.2.3) was only 18 months old.

During October, reproductive function in this mare appeared to be abnormal in that follicles of a large diameter palpable in the ovaries were not, as would be expected, associated with any rise in plasma oestrogen concentrations or subsequent increases in plasma progesterone concentrations. Subsequently, during November to early April, the same degree of follicular development was associated with elevated plasma oestrogen concentrations and, from early January, with almost constant positive oestrous behaviour.

The explanation for these results could be that this mare was peripubertal during October and was stimulated in some way –

perhaps by supplying extra energy through housing and feeding or perhaps by supplying stallion presence through daily teasing - to undergo the various changes resulting in puberty being achieved.

Although little investigation has been carried out into changes in the reproductive tract and hormonal profiles during puberty in the mare, the age of puberty is generally given as 18 months (Ginther, 1979; Arthur et al, 1982).

Cervical changes seen in this mare during October and November are of interest. In October, the cervix was pale, dry and wide open with a direct view into the uterus. During November, as circulating oestrogen concentrations increased, the cervix became pinker, moist and began to close down as the folds became more oedematous. This complements the changes in the rest of the reproductive tract, suggesting that this mare became mature during the winter months. These changes would probably not have occurred until the following spring had Misty been left to winter out.

The final two mares in the group, Summer (Fig.2.7) and Poppy (Fig.2.8) did not demonstrate positive oestrous behaviour during the month of October. However, their ovarian findings and plasma oestrogen and progesterone concentrations indicate that, rather than being in anoestrus during the period of investigation, one mare demonstrated prolonged dioestrus (Poppy, Fig.2.8) while the other demonstrated silent oestrus (Summer, Fig.2.7).

Silent oestrus occurs in many of the domestic species but in the majority the causal factors have not yet been established. Ovarian and hormonal findings are suggestive of oestrus but positive

oestrous behaviour is lacking. As has already been discussed earlier in this thesis, demonstration of positive oestrous behaviour does not appear to be linked to absolute levels of circulating plasma oestrogens.

Oestrogens exert their effect on the hypothalamus in two ways. They act to regulate the production of gonadotrophin releasing hormone and to regulate the demonstration of positive oestrous behaviour (Austin and Short, 1979).

In some mares, although normal follicular development, with the concurrent rise in circulating plasma oestrogens, followed by ovulation, proceeds as normal, the area of the hypothalamus which regulates oestrous behaviour appears not to be stimulated and positive oestrous behaviour is not demonstrated.

This would tend to suggest that there is a more delicate and complicated mechanism of action, other than just increased concentrations of oestrogens in the plasma, required to trigger the demonstration of positive oestrous behaviour.

In the ewe, behavioural oestrus is not demonstrated until there has been a previous rise in plasma progesterone concentration (Schinkel, 1954). In the bitch, accpetance of the male does not occur until progesterone levels begin to rise (Concannon et al, 1977). To date, there are no reports which indicate that progesterone priming appears to be necessary for the demonstration of positive oestrous behaviour in the mare.

In the cases of silent oestrus in the mare, the sensitivity of the hypothalamus to circulating plasma oestrogens may be affected

by physical or psychological factors.

Arthur et al (1982) report that silent oestrus, or sub-oestrus as it is sometimes called, is common in mares in poor body condition or those exposed to an adverse environment. Both of these can be ruled out in this case as Summer was in good condition and was housed and fed an adequate diet.

However, during the previous summer, she had suffered from a badly infected foot and, in October 1981, was still being treated, although not lame at this time. Rossdale and Ricketts (1980) report that painful conditions may cause irregularities of the oestrous cycle and this could have played a part in the lack of demonstrable positive oestrous behaviour in this mare.

One factor which must be considered when positive oestrous behaviour fails to be exhibited is the time taken for teasing. Each mare in this study was teased for five minutes. This appeared to be adequate in the majority of cases but Summer may have required more time to respond. However, she did demonstrate positive oestrous behaviour later in the study with the teasing regime being unchanged.

There may well have been psychological reasons for Summer's lack of positive oestrous behaviour as this mare was new to the unit and to the daily routine of teasing with a stallion.

In addition, it was recognised that, as well as being unused to stallion attention, Summer exhibited a preference for one particular stallion. When in oestrus, she demonstrated negative oestrous behaviour when teased with the larger, more aggressive

3/4 T.B. stallion but positive oestrous behaviour when teased with the small pony stallion. It was the former which was used most extensively in the early part of the study. Therefore, this could be the explanation of Summer's lack of positive oestrous behaviour.

When Summer apparently first exhibited positive oestrous behaviour, towards the end of December, as with Kirsty, there was some lag between the fall of plasma progesterone concentrations to basal values and the demonstration of this behaviour. During this oestrous period, Summer had no palpable follicles and plasma oestrogen concentrations remained low. A subsequent increase in plasma progesterone concentration did occur, although of short duration.

This lack of palpable follicles could be explained by the growth of a follicle deep within the structure of the ovary. However, both ovaries remained small and no plasma oestrogen rise associated with the presence of a mature follicle was found. Therefore, the origin of the elevated plasma progesterone concentration is unknown. Perhaps a steady tonic release of LH in the absence of progesterone caused a small follicle or follicles, always present in the ovaries of the mare, to become luteinised; or, perhaps this progesterone originated from the adrenals.

Following this sequence of events, Summer exhibited a period of prolonged positive oestrous behaviour of 50 days. As with Misty and Kirsty, positive behavioural signs were at first combined with, and split by, negative behavioural signs. Maximum follicular diameter increased gradually, as did plasma oestrogen concentration, although these appeared to lag behind the follicular development to

some degree. Positive oestrous behaviour at the beginning of the period was not associated with elevated plasma oestrogens. Plasma progesterone concentrations rose subsequently and a slightly lengthened dioestrous period of 26 days followed. This was in turn followed by a further prolonged oestrous period of 24 days and a normal dioestrous period of 16 days. It therefore took some time before a regular rhythm was re-established.

In this mare, there appears to be some deviation from normal patterns around the months of January to March; months when the transition to regular cycling would be expected in anoestrous mares. Although there was not a total run-down of reproductive function into anoestrus, Summer experienced the same problems as are reported in the anoestrous mare in re-establishing cyclicity in the following spring (Osborne, 1966; Hughes et al, 1980; Sharp, 1980).

Poppy (Fig. 2.8) also exhibited two periods of silent oestrus at the beginning of the study. However, in contrast to Summer, these were interspersed, as were later behavioural oestrous periods, by periods of prolonged dioestrus.

This mare was in excellent condition at the beginning of the study and so poor body condition and adverse environment can be ruled out here as causes of silent oestrus.

However, again this mare was new to the unit and to the daily teasing with a stallion and, as with Summer, these silent oestrous periods occurred at the beginning of the study and could have had a phychological cause.

In the main, Poppy exhibited periods of prolonged dioestrus

throughout the study. She was a maiden mare and had no record of any uterine abnormality. In addition, plasma progesterone concentrations when they fell, did so sharply, not in a steady decline over a period of days. This would tend to indicate lysis of the corpus luteum rather than a gradual decrease in progesterone production by an ageing luteal structure. Why this mare should only produce sufficient prostaglandins to lyse a corpus luteum at such prolonged intervals is unknown. She has continued to demonstrate prolonged negative behaviour under the influence of high plasma progesterone concentrations. This has made it difficult for her owners to know when she is in season and to judge if she is in-foal without veterinary assistance.

The continuing reproductive activity of this group of mares during the winter months was not as expected. For some reason, or reasons, the majority of mares, instead of progressing into anoestrus, continued with reproductive activity. However, this appeared to be a graded response.

Corrie (Fig. 2.1) cycled regularly throughout; Sunny (Fig. 2.6) and Poppy (Fig. 2.8) also carried on as during the breeding season; Misty (Fig. 2.3) appeared to undergo the transition to puberty some six months earlier than probably would have been the case if out-wintered; Summer (Fig. 2.7) did not exhibit anoestrus but experienced the prolonged oestrus associated with the transition to the breeding season in anoestrus mares; Melody (Fig. 2.2), Sacha (Fig. 2.4) and Kirsty did progress into anoestrus.

Daylight length has been implicated as a factor in the control of reproductive function in the mare (Burkhardt, 1947; Loy, 1968; Arthur, 1969; Kooistra and Ginther, 1975; Palmer, 1978).

After carrying out the first overnight bleed for the collection of samples for melatonin estimation in early December, it was recognised that throughout this period of investigation the mares were receiving an abnormal light pattern. Our buildings, situated in a built-up area, receive additional lighting throughout the night. It is recognised that this environmental lighting could be a reason for the variety of sexual states demonstrated by these mares during the winter months. However, since the ensuing chapters of this thesis undertake a detailed investigation of light and melatonin as factors in the regulation of breeding patterns in the mare, it was decided not to introduce the increased lighting as part of the discussion in this first section.

CHAPTER III

PART I - CLINICAL STUDIES

3.1 - INTRODUCTION

From the investigations carried out during 1981 - 82, it became obvious that alterations required to be made to the experimental programme.

One problem was that of environmental lighting. In 1981 - 82 the amount of light present during the hours of darkness was much greater than normal. Therefore, to rule out any influence of this on the reproductive function of the mares, some of the housing was made totally lightproof.

Another modification was that, instead of the larger T.B.-type mares, pony mares were studied. The larger breeds, especially the T.B., are more domesticated and therefore may tend to cycle throughout the winter months anyway. The more "primitive" pony breeds are more likely to make the transition into anoestrus during the winter months (Ginther, 1979). In addition, pony mares are easier to house and handle.

Therefore, during 1982 - 83, three groups of three pony mares were housed in different environments with respect to the type and amount of lighting received.

Behavioural, ovarian and hormonal changes were recorded throughout. In addition, melatonin concentrations in blood samples collected over 24-hour periods on specific occasions were estimated.

In the following year, 1983 - 84, these same groups of pony mares were subjected to an identical programme of investigation. However, in addition, those mares exhibiting positive oestrous behaviour during December, 1983 and January, 1984 were mated.

3.2 - MATERIALS AND METHODS

1. ANIMALS USED

a. Mares

Nine pony mares of varying ages and breeds were divided into three groups of three, as shown in Table 3.1. Three mares investigated in 1982 - 83 were replaced during 1983 - 84. The replacement mares were allocated, one to each group, at random. All mares were housed from the end of September, 1982 and 1983 and routine studies carried out until May, 1983 and March, 1984. All mares were fed a ration of concentrates (Horse and Pony Cubes; Spillers) and hay. Water was available ad lib.

b. Stallions

During 1982 - 83 the following stallions were used for teasing - 1 Welsh Pony stallion and 1 3/4 T.B. stallion, as described in Chapter 2.

During 1982 - 83 the following stallions were used for teasing and mating. A Welsh Pony stallion, as described previously, who was a proven sire, an aged Welsh Pony stallion and a 3 y.o. Fell Pony stallion.

2. ACCOMMODATION

a. Group 1 (Control Group)

During 1982 - 83 these mares were housed in two boxes and Barn 1 (see Diag. 2.1). These locations were exposed to the same lighting patterns as all the mares during 1981 - 82.

During 1983 - 84 all mares in the control group were housed in Barn 1.

TABLE 3.1

MARES USED DURING 1982/83 AND 1983/84

Group	Mare	Age (yrs.)	Height (h.h.)	Reproductive Status	Accommodation 1982/83	1983/84
1	Sylvia	20	13.0	Barren	Box 1	Barn 1
	Misty	3	15.0	Maiden	Barn 1	Barn 1
	Maytime *	8	13.3	Maiden	Box 2	–
	Dotty **	15	13.0	Barren		Barn 1
2	Filly	5	13.2	Barren	Barn 2	Barn 2
	Polly *	9	12.0	Barren	Barn 2	–
	Hot Stuff	3	13.2	Maiden	Barn 2	Barn 2
	N.T. **	8	13.2	Barren	–	Barn 2
3	Susie *	10	12.2	Barren	Barn 3	–
	Wellie	12	13.2	Barren	Barn 3	Barn 3
	Tula	8	13.2	Maiden	Barn 3	Barn 3
	Snowdrop **	15	12.2	Barren	–	Barn 3

    *   –   included during 1982-83 only

    **   –   included during 1983-84 only

b. Group 2

Throughout the experimental period the mares in this group
were housed in Barn 2. This barn was made as lightproof as
was economically possible with roof windows blacked out and the
door into the yard kept closed during the hours of darkness.
The only connection with the outside environment was via a
half-door leading into a covered passageway (see Diag. 2.1).
Lighting was supplied by 4 x 2 fluorescent tubes (80 W.,
Philips) and these were switched on and off automatically, using
time clocks.

c. Group 3

Throughout the period of study, the mares in this group were
housed in Barn 3. This barn was totally lightproof. The main
exit was sealed with brushes and rubber flaps. Ventilation was
by means of air inlets and extractor fans. Both inlets and fans
were baffled. A double-doored chamber was constructed in the
passageway to allow entry into the barn during the hours of
darkness. Light was supplied by two mercury vapour wall lights
and 8 x 2 fluorescent tubes (80 W., Philips). The wall lights
were timed to come on 30 minutes before, and switched off 30
minutes after, the fluorescent tube lights to simulate dawn and
dusk.

3. LIGHTING PATTERNS

a. 1982 - 83

On the 4th October, 1982 mares in Barns 2 and 3 received
16 hours of light (16L) per day. This was reduced to 14L on

18th October, to 12L on 25th October, to 9L on 1st November, to 8½L on 29th November and to 8L on 20th December, 1982. This pattern of eight hours light, sixteen hours dark (8L : 16D) was continued until 17th January, 1983 when the light received was increased by thirty minutes per week until the 21st May, when 16 hours of light per day were present.

b. <u>1983 - 84</u>

On the 3rd October, 1983 mares in Barns 2 and 3 received 12½ hours of light per day.   This was reduced by thirty minutes each week until the 28th November, when they were receiving only eight hours of light per day.   The light pattern remained as 8L : 16D until 6th February, 1984 when the duration of light was increased by thirty minutes per week until the end of the study.

4. <u>TEASING</u>

Teasing was carried out daily and results recorded as described in Chapter 2.

5. <u>COVERING</u>

Mares demonstrating positive oestrus behaviour during December, 1983 and January, 1984 were covered daily, where possible. If more than one mare was being covered by the same stallion, covering took place every second day.

6. <u>RECTAL PALPATION</u>

Rectal palpation was carried out three times per week.   This continued until the mares were mated.   After mating, rectal palpation ceased until 19 days post-covering.   If the mare was not pregnant at this stage, rectal palpation was resumed.

7. PREGNANCY DIAGNOSIS

a. Rectal Palpation

Palpation of the uterus per rectum was carried out at 19 days post-covering.

Subsequent rectal palpation of pregnant mares was carried out weekly until the end of the study.

b. Blood Sampling

Blood samples were obtained at 42 days post-covering and the presence or absence of eCG tested for, using haemoglutination-inhibition test (M.I.P. Test, Carter-Wallace Inc., Cranbury, New Jersey).

8. BLOOD SAMPLING FOR PROGESTERONE ESTIMATION

Blood samples were taken twice weekly, as described in Chapter 2.

9. PROGESTERONE ESTIMATION

As described in Chapter 2.

3.3 - RESULTS

Fig. 3.1

GROUP 1 - SYLVIA

The first oestrous period, recorded in October, was associated with the absence of a palpable follicle in the ovaries. However, plasma progesterone concentrations rose subsequently.

Apparently normal cyclical activity continued until mid-January when an anovulatory oestrus occurred.

Although positive oestrous behaviour was demonstrated in mid-February, maximum follicular diameter was only 1.5 cm and no rise in plasma progesterone concentration was recorded. The next production of luteal tissue occurred after a prolonged oestrus in early March. A normal dioestrus was followed by a further anovulatory oestrus in early April.

Fig. 3.1

GROUP 1   -   SYLVIA
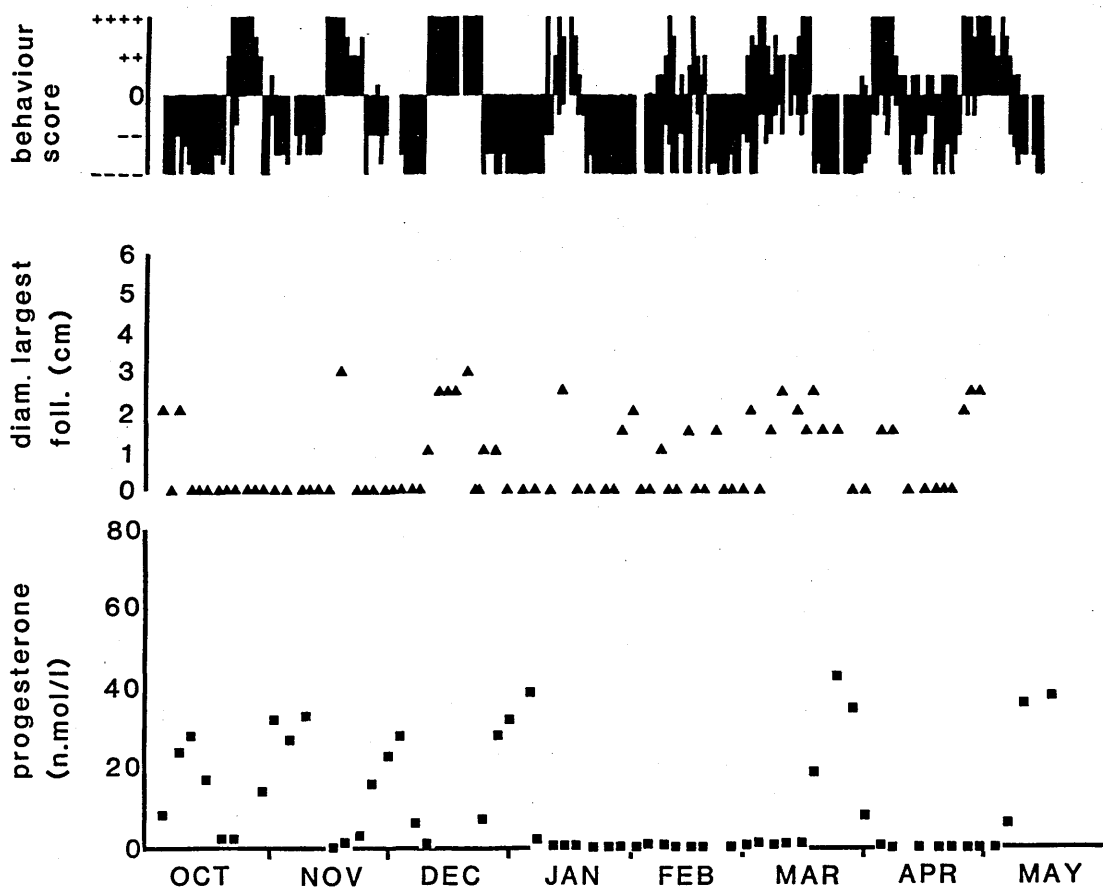
RESULTS OF BEHAVIOURAL STUDIES, RECTAL PALPATION
AND PLASMA PROGESTERONE ESTIMATIONS CARRIED OUT
DURING 1982/83

Fig. 3.2

GROUP 1   -   MAYTIME

Investigations in this mare did not begin until 20th December, 1982.

Both positive and negative behavioural signs were exhibited in varying combinations throughout the period of study.   Follicular and plasma progesterone patterns indicated regular cycling.

Basal plasma progesterone concentrations in early March were associated with no palpable follicles, although plasma progesterone concentrations rose subsequently.

Prolonged dioestrus was recorded during April and May.

Fig. 3.2

GROUP 1 - MAYTIME
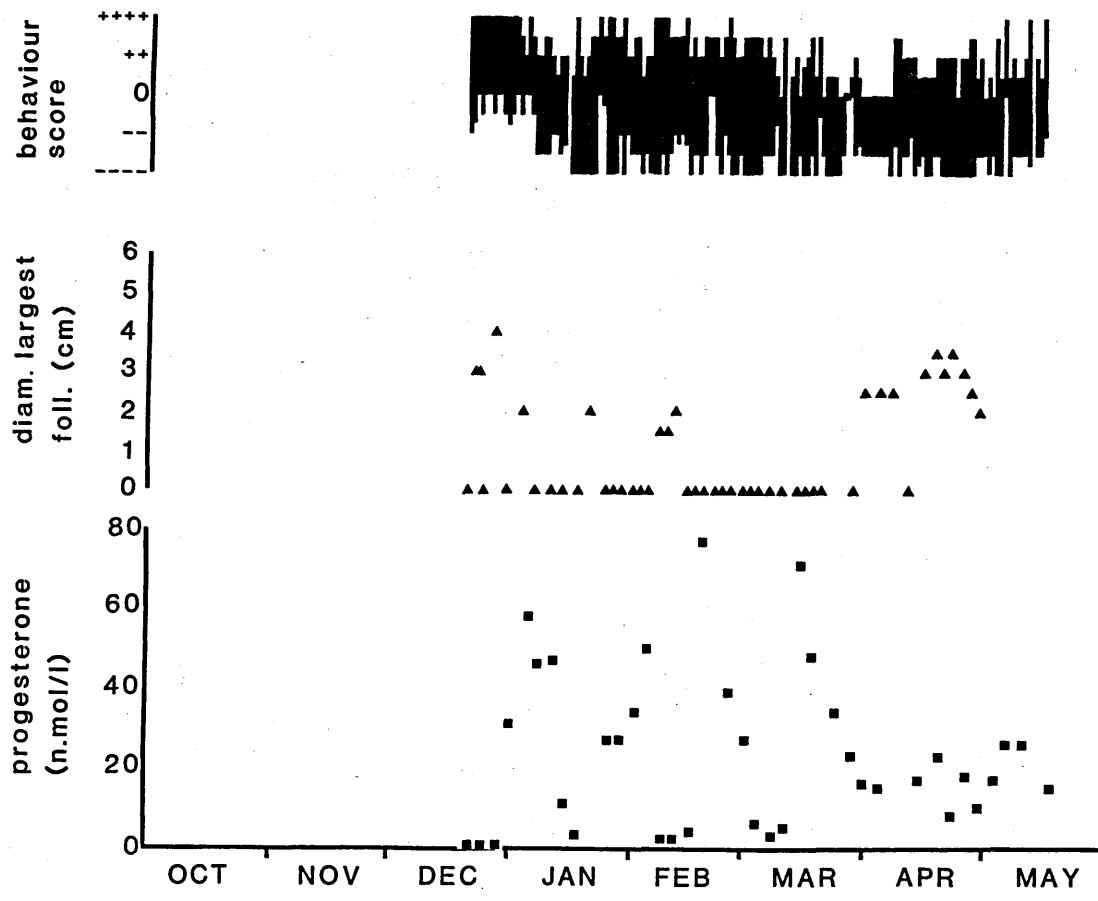
RESULTS OF BEHAVIOURAL STUDIES, RECTAL PALPATION
AND PLASMA PROGESTERONE ESTIMATIONS CARRIED OUT
DURING 1982/83

GROUP 1 - MISTY

Two normal oestrous and interoestrous periods in October and November were followed by a period of anoestrus with plasma progesterone concentrations remaining basal from early December to mid-February and oestrous behaviour remaining negative until early February. During this period of anoestrus, there were only a few days in late December/ early January where no follicles were palpable.

Misty's next oestrous period occurred in February and regular cycling followed.

Fig. 3.3

GROUP 2   -   POLLY

After a normal oestrus and dioestrus in October, this mare became anoestrous. Plasma progesterone concentrations remained basal and oestrous behaviour negative until the end of the study.   No follicles were palpable in the ovaries from mid-November until mid-January. From mid-January, follicular size varied but did not exceed 2 cm in diameter.

Fig. 3.3

GROUP 2 — POLLY
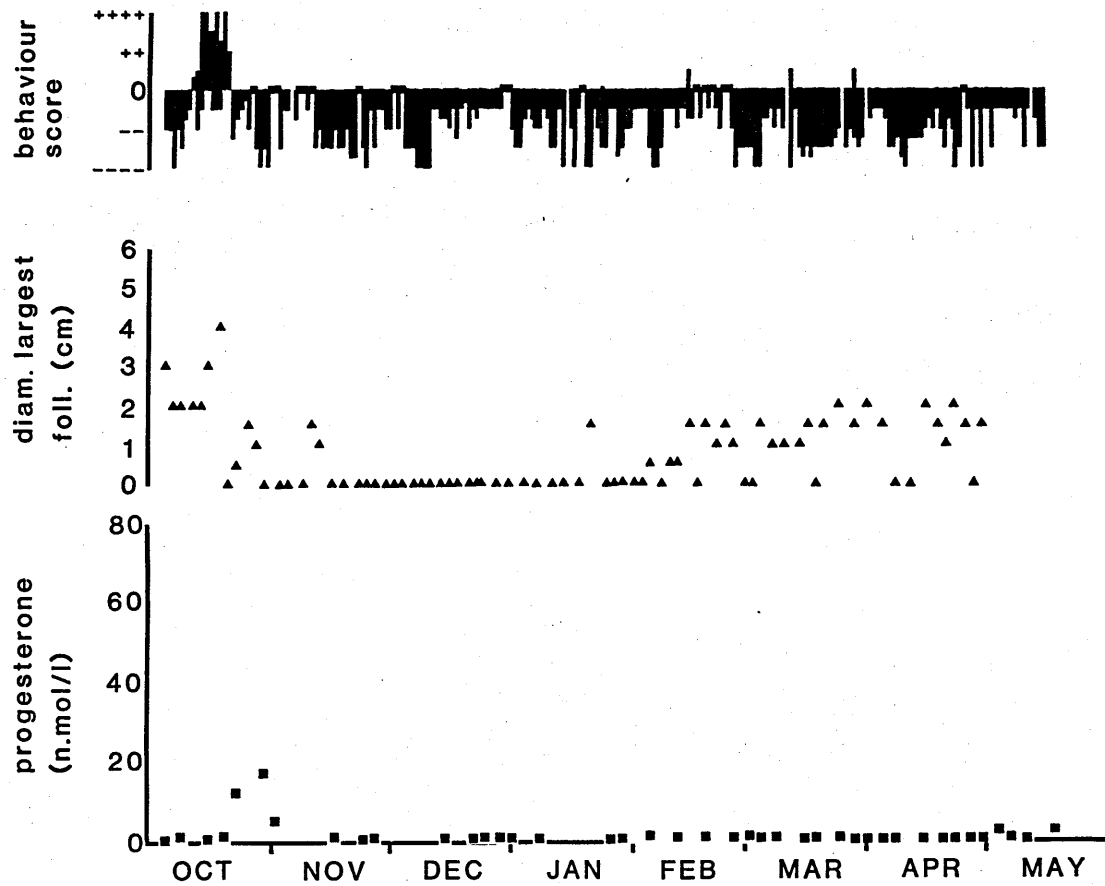
RESULTS OF BEHAVIOURAL STUDIES, RECTAL PALPATION
AND PLASMA PROGESTERONE ESTIMATIONS CARRIED OUT
DURING 1982/83

Fig. 3.4

GROUP 2   -   HOT STUFF

Hot Stuff cycled throughout the period of study.   A prolonged dioestrous period was recorded during December, January and February. This was followed by a silent oestrus in early March associated with a 6 cm follicle and a subsequent rise in plasma progesterone concentrations.

All periods of positive oestrous behaviour were associated with the demonstration of many negative behavioural signs.

Fig. 3.4

GROUP 2 - HOT STUFF

RESULTS OF BEHAVIOURAL STUDIES, RECTAL PALPATION
AND PLASMA PROGESTERONE ESTIMATIONS CARRIED OUT
DURING 1982/83

GROUP 2   -   FILLY

This mare cycled throughout the period of investigation.
A prolonged dioestrous period was recorded during November
and December and a prolonged interoestrous period recorded
during April and early May.   The latter was associated with
an anovulatory oestrus in early April.

Fig. 3.5

GROUP 3   -   SUSIE

This mare cycled regularly throughout the period of investigation.
Oestrous and interoestrous lengths averaged 4 and 23 days respectively.
In May, an interoestrous period of 26 days was associated with a delay
between the return of plasma progesterone concentrations to basal levels
and the demonstration of positive oestrous behaviour.

Fig. 3.5

GROUP 3   –   SUSIE
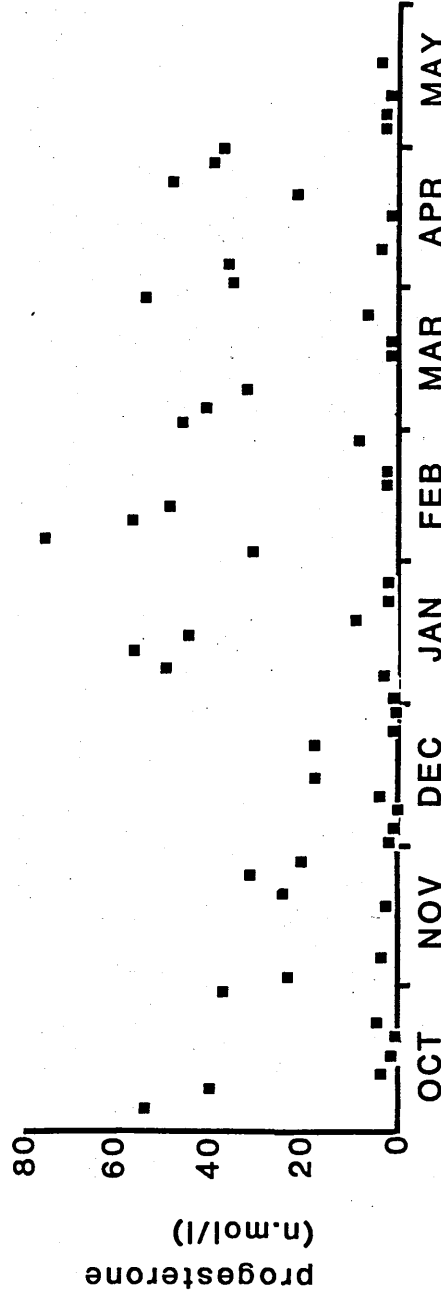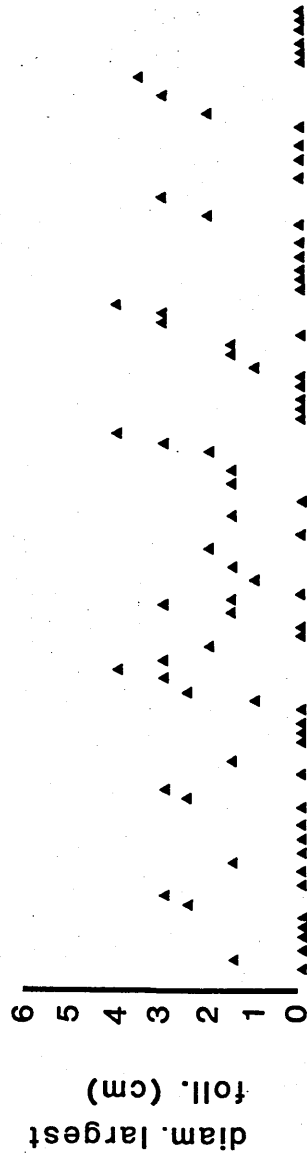
RESULTS OF BEHAVIOURAL STUDIES, RECTAL PALPATION AND PLASMA
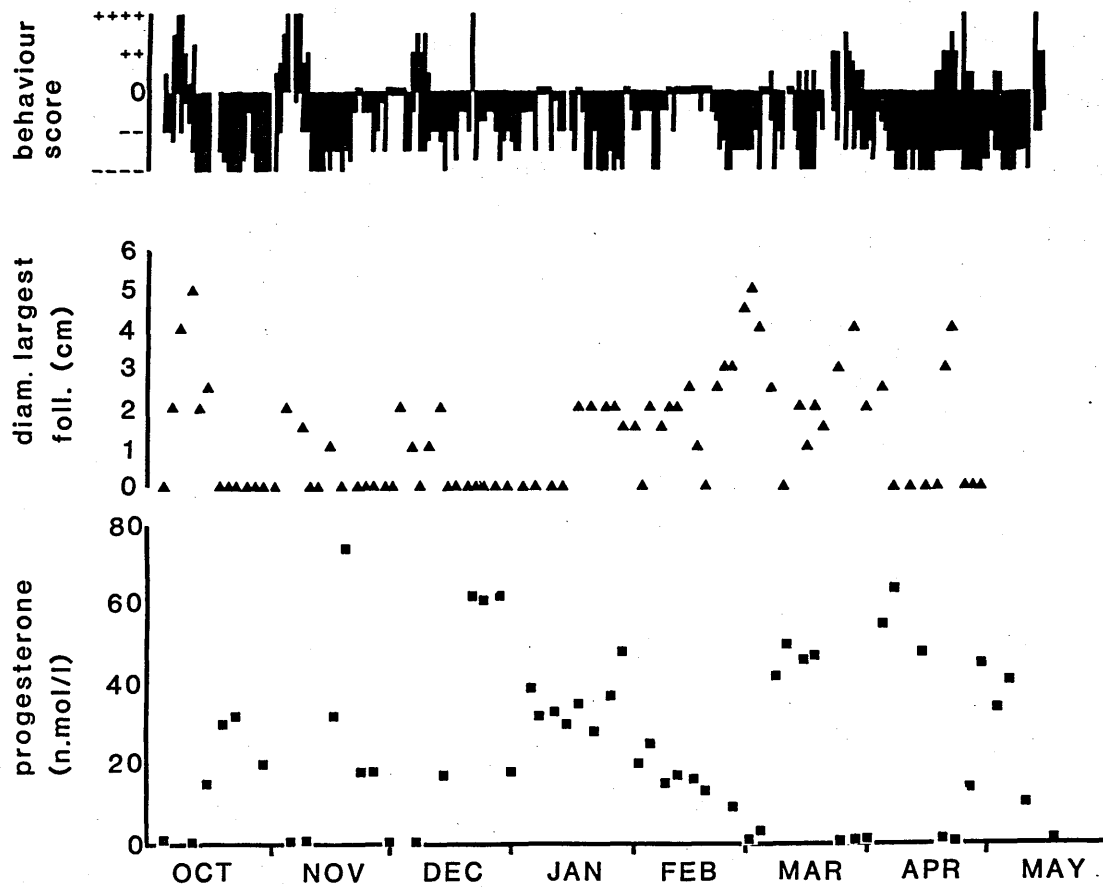PROGESTERONE ESTIMATIONS CARRIED OUT DURING 1982/83

Fig. 3.6

GROUP 3   -   WELLIE

Cycled regularly until the end of January with oestrous and inter-oestrous lengths averaging 6.25 and 20.8 days respectively.

In February, although standing oestrus lasted only 4 days, some positive oestrous behaviour was demonstrated over 12 days.   Maximum follicular diameter recorded at this time was 4 cm and plasma progesterone concentrations rose subsequently.

On the return of these plasma progesterone concentrations to basal values, after approximately 16 days, there was a lag of several days before positive oestrous behaviour was demonstrated, resulting in an interoestrous period of 40 days.   A maximum follicular diameter of 2 cm was recorded during the latter half of this period.   Oestrus and regular cycling ensued.

Fig. 3.6

GROUP 3 – WELLIE
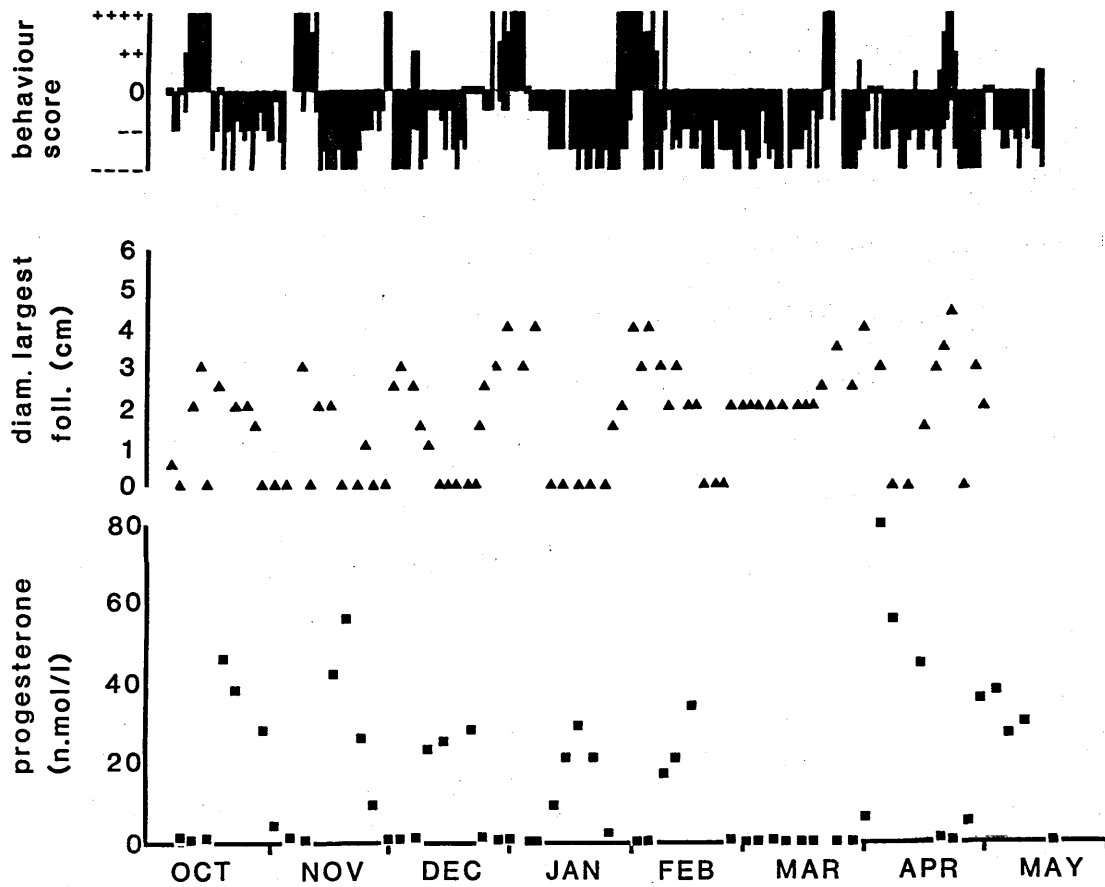
RESULTS OF BEHAVIOURAL STUDIES, RECTAL PALPATION
AND PLASMA PROGESTERONE ESTIMATIONS CARRIED OUT
DURING 1982/83

Fig. 3.7

GROUP 3 - TULA

Tula demonstrated prolonged dioestrus, with negative behaviour on teasing, variable maximum follicular diameter of up to 6 cm and an elevated but steadily decreasing plasma progesterone concentration, from October to early February. This was followed by an anovulatory oestrus in early February, after which this mare entered a period of anoestrus.

Further positive oestrous behaviour was not demonstrated until early May when a follicle of 5 cm in diameter was recorded in the ovaries. Plasma progesterone concentrations rose subsequently, indicating the production of luteal tissue and the resumption of normal cyclical activity.

Fig. 3.7

GROUP 3 — TULA
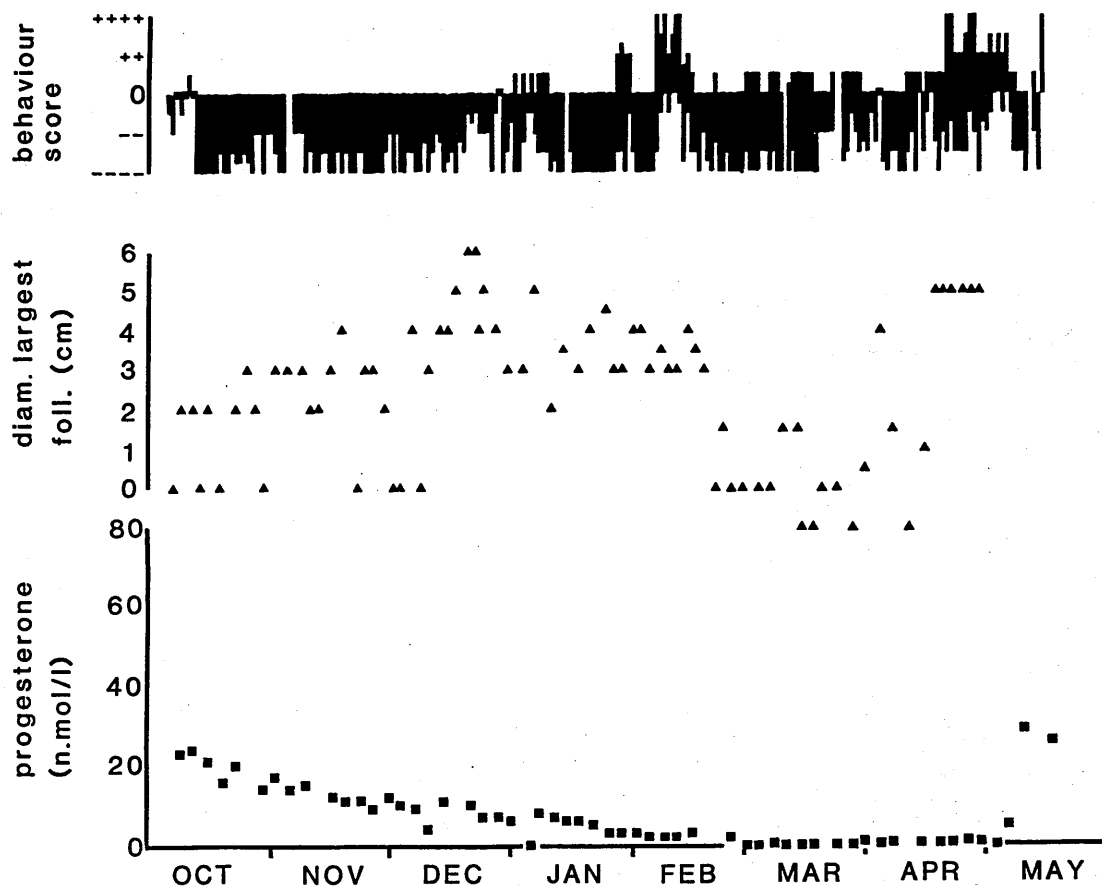
RESULTS OF BEHAVIOURAL STUDIES, RECTAL PALPATION
AND PLASMA PROGESTERONE ESTIMATIONS CARRIED OUT
DURING 1982/83

GROUP 1

SYLVIA

Cycled regularly until covered in early December. Covering took place on the 2nd, 3rd, 4th, 7th and 9th December.

MISTY

Cycled regularly until covered in mid-December. Covering took place daily from the 9th to the 14th of December.

DOTTY

Cycled regularly until covered in early December. She was covered on 5th, 6th and 7th December and then daily from the 9th to 15th December. During this oestrous period in early December, negative as well as positive behavioural signs were demonstrated on several occasions. However, the mare was still able to be covered on such days.

GROUP 2

FILLY

Cycled regularly until covered in mid-December, on 10th, 12th and 13th. One silent oestrous period was recorded during late October.

HOT STUFF

Cycled regularly until covered in mid-December - covering took place daily from 15th to 20th. Pregnancy was diagnosed by rectal palpation at 19 days post-covering but was not confirmed subsequently either by rectal palpation or by the presence of eCG in the mare's serum at 42 days. This mare was in prolonged dioestrus.

N.T.

Cycled regularly until covered on three consecutive days, from the 22nd - 24th November. Diagnosed pregnant on rectal palpation at 19 days post-covering; this was not confirmed at 42 days. Observed in oestrus at 55 days and covered at this oestrous period on the 22nd and 23rd of January, 1984.

GROUP 3

WELLIE

This mare cycled regularly throughout October, November and December. Positive behaviour was demonstrated during oestrous periods until December, when covering took place. Pregnancy did not ensue and subsequent oestrous periods were silent. Prostaglandin (1 ml Lutalyse, i.m., Upjohn) was administered during a dioestrous period at the end of January but, although plasma progesterone concentrations fell to basal values, again no positive oestrous behaviour was demonstrated.

TULA

Demonstrated one period of positive oestrous behaviour at the end of October. Plasma progesterone concentrations rose subsequently and a prolonged dioestrous period ensued. Prostaglandin (1 ml Lutalyse, i.m., Upjohn) was administered on 21st December, 1983 and positive oestrous behaviour demonstrated first on 24th December, 1983. She was covered at this oestrous period on 24th to 28th December.

SNOWDROP

This mare was very aggressive on teasing. During oestrus at the end of October, positive behavioural signs were recorded on only one day. A prolonged dioestrous period followed this oestrus.

After the administration of prostaglandin (1 ml Lutalyse, i.m.,
Upjohn) on 21st December, 1983, although plasma progesterone
concentrations fell to basal values and a 4 cm follicle was palpable,
no positive oestrous behaviour was demonstrated. Ovulation did
not occur and this mare progressed into anoestrus.

TABLE 3.2

SUMMARY OF RESULTS OF MARES COVERED DURING DECEMBER, 1983 AND JANUARY, 1984

Mare Identity	Date of last Covering	Pregnancy Diagnosis - 19 days	Pregnancy Diagnosis - 42 days	Subsequent Rectal Palpation - Approx. 5 - 6 months Gestation	Comments
Sylvia	9/12/83	+	+	-	-
Misty	14/12/83	+	+	+	-
Dotty	15/12/83	+	+	+	Aborted normal foetus May, 1984
Filly	13/12/83	+	+	+	
Hot Stuff	20/12/83	+	-	-	Prolonged dioestrus
N.T.	1. 24/11/83 2. 23/ 1/84	+	+	+	
Wellie	5/12/83	-	+	+	Mucometra
Tula	28/12/83	+	+	+	

3.4 - DISCUSSION

As with the mares in the previous year, the pony mares studied in 1982/83 demonstrated a variety of sexual states during the winter months. Anoestrus was, again, not the major feature.

One mare, Susie (Fig. 3.5) cycled regularly throughout the period of investigation.

Four mares, Wellie (Fig. 3.6), Hot Stuff (Fig. 3.4), Maytime (Fig. 3.2) and Filly, continued to cycle with minor variations, all of which were demonstrated by the mares in the previous year and have already been discussed in the preceding chapter.

The remaining four mares were in anoestrus for varying periods of time.

One mare, Polly (Fig. 3.3), had a period of prolonged anoestrus. In this mare, anoestrus was established in November and she had not returned to cyclical activity by the end of the period of investigation in May. Polly foaled in July, 1982 and the foal was not weaned until just before the start of the study at the end of September, 1982. The last third of gestation and, in particular, lactation place great demands on energy and energy reserves (Burkhardt, 1948; Cooper and Wert, 1975; Cuhna, 1980). This lack of available energy could have been the reason for the complete cessation of reproductive function in this mare.

The other three mares, Tula (Fig. 3.7), Sylvia (Fig. 3.1) and Misty were in anoestrus for much shorter periods. Pony mares are generally described as being in anoestrus from November to April (Hughes et al, 1980). However, Sylvia and Tula did not

progress into anoestrus until January and March respectively.
The duration of the anoestrous period in these three mares was
also shorter than expected. All three mares were in excellent
condition throughout the study and no real explanation can be
given for their transition into anoestrus.

Several variations relating to cyclicity not already discussed
did occur in some of the mares during 1982/83.

For example, Maytime (Fig. 3.2) persistently demonstrated
a combination of positive and negative behavioural signs, although
ovarian and hormonal patterns indicated regular cyclical activity.
Maytime was new to the unit and to daily teasing with a stallion.
She was "in season" at the beginning of her investigatory period
and so the behavioural responses of this mare to the stallion may
have been due to habit. Mimicking can be ruled out in this
mare as she was boxed on her own. It was noted that some
mares when teased routinely when not in season will "wink" and
void urine in anger. This could have been the case, and is
probably the most likely explanation, in this mare.

During early March, Maytime's plasma progesterone
concentrations dropped to basal values and then rose again,
suggesting that ovulation or luteinisation had occurred. However,
no follicles were palpable in either ovary during this period.
This could have been due to the fact that palpation of the left
ovary in this mare was very difficult as it was adhered to the
body wall, to the left horn and body of the uterus. Therefore,
a follicle may have been present on this ovary.

Another variation not already discussed was that in Tula
(Fig. 3.7) during a prolonged dioestrous period lasting from
October, 1982 to January, 1983, there was a continuous, steady
decrease in plasma progesterone concentrations.   In all the mares
in the previous year who demonstrated prolonged dioestrus, the
circulating plasma progesterone concentrations remained high until
the end of the period, at which time they dropped abruptly.
These latter findings agree with the results reported by Stabenfeldt
et al (1974).

The results found in Tula suggest ageing of the corpus luteum
rather than lysis.   One could suggest an abnormal prostaglandin
production by the uterine endometrium as a cause.   Although none
of the mares investigated, who demonstrated periods of prolonged
dioestrus, were known to have uterine damage, this does not exclude
it as a possible explanation.

Overall, the clinical results recorded during 1982/83 indicate
that, despite the different lighting regimes operating, regular
cyclical activity continued in the majority of the mares and
anoestrus when it occurred tended to be later and shorter than
normally associated with pony mares during the winter months.

Six of the nine mares used during 1982/83 were involved
in the investigations carried out the following year.   The other
three mares were excluded from the studies for various reasons.
Susie had to be put down after several bouts of laminitis, Polly
was excluded due to difficulties in cannulation for the 24-hour

bleeds and Maytime was replaced because of ovarian adhesions.

Three new mares, Dotty, N.T., and Snowdrop were assigned

one to each group at random.

From October to December, 1983 all nine mares continued to

cycle. In three mares minor variations from the oestrous cycle

occurred. These included prolonged oestrus, silent oestrus and

prolonged dioestrus. None of the mares had entered anoestrus

by December, 1983.

Reports in the literature suggest that at this time of year,

most pony mares are in anoestrus (Hughes et al, 1980). Therefore,

it was decided to cover those mares demonstrating positive oestrous

behaviour during December, 1983 and January, 1984 to determine

whether or not these oestrous periods were fertile.

Seven of the nine mares exhibited standing oestrus during

this period. One mare, N.T., was covered at the end of November

and the rest, Wellie, Filly, Hot Stuff, Sylvia, Misty and Dotty during December.

Of these seven mares, six were diagnosed as pregnant at

18/19 days after mating. At this time, pregnancy was diagnosed

by rectal palpation based on the presence of increased turgidity

of the uterus. This is the occasion when the greatest degree of

uterine tone is present in the mare (van Niekerk, 1965 a; Arthur

and Allen, 1972; Ginther, 1979; Rossdale and Ricketts, 1980).

This is at variance with the cow, in which greatest uterine tone is

normally palpable around standing oestrus when peak levels of

oestrogens are present (Munro, 1976) and illustrates a difference

in species response to circulating hormone concentrations.

Some workers suggest that in the mare at oestrus, the uterus is at its most flaccid with a greater degree of tone present during dioestrus. However, these changes are generally considered not sufficiently uniform to be of clinical significance (Hughes, Stabenfeldt and Evans, 1972, b). During dioestrus in the mare, although progesterone predominates, there would appear to be increased levels of circulating oestrogens as well since it is established that follicular development continues during dioestrus (Hughes et al, 1980).

In the pregnant mare at 19 days gestation progesterone also predominates, but again since it has been shown that recurring surges of FSH continue to occur at intervals throughout early pregnancy, similar to those occurring during the oestrous cycle (Evans and Irvine, 1975; Urwin and Allen, 1982) there could be follicular development and oestrogen secretion as well. Therefore, it may well be that greatest uterine tone in the mare occurs when increased levels of both hormones are present in the circulation.

In some mares, especially older multiparous mares and mares covered at the foal heat, uterine tone is not as obvious at this early stage of pregnancy. However, the mares involved in this investigation were either maiden mares or had been barren for at least two years previously. Therefore, if a positive diagnosis of pregnancy was made at this stage, it was felt to be correct.

With experience, the presence of the conceptual vesicle can often be palpated in the mare 18/19 days after covering. In the area where the conceptual vesicle lies, uterine tone is decreased

or absent and a small swelling can be palpated ventrally in either of the uterine horns usually close to the body (Van Niekerk, 1965, a; Allen, 1974).

In the mares diagnosed as pregnant in the studies described, all the conceptual swellings at day 19 of gestation were found in either horn close to the body of the uterus.

In the 19-day pregnant mare, the conceptus is not necessarily located in the horn adjacent to the ovary from which ovulation took place (Allen, 1974). Implantation in the equine does not take place until 45/50 days after conception (Allen, 1980). Till then the conceptus lies free in the uterine lumen and may migrate from one horn to the other.

Very recently, Leith and Ginther (1984), using a real-time ultrasound scanner, traced the conceptus in the uterus of mares from day 9 post-mating and reported that on days 9/11 the vesicle spent most of its time in the uterine body; was most mobile on days 12/14; and that cessation of mobility tended to occur on days 16/17. These results would tend to suggest a predetermined sequence of events with regard to the mobility of the conceptus. Previously the view generally held was that the conceptus in the mare is able to migrate freely from body to horns due to the position and shape of the uterus in this species (Ginther, 1979).

One might suggest that plasma progesterone estimation could have been used for pregnancy diagnosis at this early stage. However, raised plasma progesterone concentrations are not a good indication of pregnancy in the mare as spontaneous

prolongation of a functional corpus luteum commonly occurs
(Stabenfeldt et al, 1975).

Pregnancy was confirmed by a haemoglutination inhibition
test (M.I.P. Test) at approximately 42 days post-mating in four
of the six mares recorded as pregnant at 19 days.   The M.I.P.
Test depends on the presence of pregnant mare serum gonadotrophin
(PMSG) or equine chorionic gonadotrophin (eCG) as it is now called,
in the mares' serum.   The four pregnancies confirmed in this way,
at this stage, were in Sylvia, Misty, Dotty and Filly.

eCG is produced by the endometrial cups which develop in the
pregnant horn of the uterus and are of foetal origin (Allen and
Moor, 1972).   Therefore, a positive M.I.P. test confirms the presence
of a conceptus.   However, a positive diagnosis of pregnancy at this
stage will not always result in a foaling.   Embryonic loss in the mare
does occur after the establishment of the endometrial cups and in
these cases eCG production continues as in a normal pregnancy
(Allen and Moor, 1972).

In this study, a foetal mass was palpated in each mare said to
be pregnant, at 5/6 months of gestation.   Three of the original
six mares diagnosed pregnant at 19 days were confirmed in this way
(in Sylvia, Misty and Filly).   The two mares which were diagnosed
as pregnant at 19 days but gave a negative M.I.P. Test when blood
sampled at 42 days were N.T. and Hot Stuff.

In N.T., a distinct uterine swelling was palpable in the right
horn of the uterus at 42 days post-mating.   At 55 days post-mating
this swelling was still palpable but she was in standing oestrus.

Up to this time plasma progesterone concentrations had remained elevated.

From the results recorded, it is possible that N.T. was pregnant and embryonic death occurred. On the other hand, she may have been in prolonged dioestrus. However, the distention of the uterus suggests that the former situation was the most likely.

Early pregnancy failure is common in the mare (van Niekerk, 1965 b). One survey indicated that 75% of pregnancy losses in the mare occur before day 49 (Bain, 1969). Although the causes of many of these losses have not been established, it is thought that some may be the result of an abnormality in endocrine control (Mahaffey, 1968), as at this time the endometrial cups begin to form and secondary follicles and corpora lutea develop.

The mare is unusual in that the secretory activity of the primary corpus luteum of pregnancy can begin to decline from as early as day 14 - 16 of gestation, resulting in a slow and steady fall in plasma progesterone concentrations until day 35 - 45 where they increase again due to the formation of secondary corpora lutea (Allen, 1984).

Embryonic mortality is often attributed to a delay in the formation of these secondary corpora lutea with a resulting lack of circulating progesterone. This may have been the explanation for the loss of pregnancy in N.T. However, blood sampling for progesterone estimation was not carried out frequently enough at this stage to allow positive confirmation of this.

N.T. was covered on her return to positive oestrous behaviour in January, 1984 and subsequently diagnosed as pregnant. The pregnancy was confirmed at 5 months gestation by palpation of a

foetal mass.

Hot Stuff, the other mare who, although diagnosed as pregnant at 19 days, gave a negative M.I.P. test at 42 days, was covered in mid-December. At that time, twin follicles were present in her ovaries - one follicle of 3.5 cm in diameter on the right ovary and one of 4 cm on the left. Neither follicle was palpable on the last day of standing oestrus. Each had been replaced by a soft and spongy corpus haemorrhagicum.

At 19 days post-mating, Hot Stuff's uterus was very turgid and there were slight ventral swellings present in both uterine horns, to right and left of the uterine body. These findings would tend to suggest the presence of a twin pregnancy. However, no further increase in the size of the uterine swellings occurred and it would appear that endometrial cups did not develop. Plasma progesterone concentrations apparently remained elevated until early March.

The indications are that Hot Stuff was pregnant at 19 days but suffered embryonic death before the establishment of the endometrial cups. The mare subsequently demonstrated a period of prolonged dioestrus.

Twin ovulations and the establishment of twin pregnancies are relatively common in the T.B. mare but are less so in the pony mare (Arthur and Allen, 1972). Twin pregnancies are rarely carried to term in the equine and are considered to be one of the major causes of breeding losses in the T.B. industry. However, the majority of twin pregnancies are usually lost during the later

stages of gestation (Jeffcott and Whitwell, 1973) where two foetuses place a greater demand on uterine space and nutrition than one.

Other factors which could be considered, relating to embryonic mortality, in Hot Stuff's case are infection and poor nutrition. van Niekerk (1965 b) correlates malnutrition with early embryonic resorption in mares. However, this can be ruled out in this mare as she was in good condition and received adequate feeding. In addition, at no time did Hot Stuff give any clinical indication of a uterine infection. Therefore, if Hot Stuff did conceive, the reason for her early embryonic loss cannot be determined.

The seventh mare which demonstrated standing oestrus and was covered in December was Wellie. On rectal palpation at 19 days post-mating, she was thought not to be pregnant. At this time, the uterus was soft and flaccid and a swelling was palpable in the left horn of her uterus. However, this swelling was much larger than expected in a 19-day pregnancy, being approximately 10 - 15 cm in diameter. The swelling was fluid-filled and on manipulation of the uterus, the fluid traversed the length of the uterine horn. On vaginal examination, a trickle of greyish fluid was seen exuding through the cervix and pooling in the anterior vagina. When a catheter was inserted through the cervix, and the uterus massaged per rectum, approximately 500 ml of a greyish-white mucoid fluid was expelled. Unfortunately, bacteriological examination of this fluid was not carried out but, on examination of a stained smear of the fluid, only a few polymorphonuclear leucocytes were seen. This would tend to suggest that there was no acute bacterial infection

present.

Endometritis occurs in most mares after covering but is usually resolved within 72 hours (Peterson, McFeely and David, 1969). In those mares in which the natural defence mechanisms are inadequate, the infection continues. However, endometritis is usually associated with a uterine discharge in which numerous white blood cells are present (Wingfield-Digby, 1978).

Knudsen (1964 a) described a similar enlargement of the uterus, with the presence of copious amounts of greyish fluid and the absence of white blood cells, in fungal uterine infections in the mare. However, each mare in Knudsen's series had been treated previously with topical antibiotics. This was not the case in Wellie, therefore it was decided that she was unlikely to have a fungal infection of the uterus.

In Wellie's case, the unusual factor was that this dilatation and accumulation of fluid in the uterus occurred only in the dioestrous period following covering.

Again somewhat similar findings are described by Knudsen (1964 b) in a further series of mares. In the majority of these mares a dilatation of the uterus was always present and fluid accumulated in these dilated areas after covering. However, Knudsen (1964 b) describes one particular mare in which the dilatation was, as in Wellie, not persistent. In this mare, Knudsen suggested poor lymphatic drainage, found at post mortem, as the cause. In the other mares in the investigation, Knudsen suggested the cause as chronic atrophy of the endometrium. Perhaps in

Wellie's case poor lymphatic drainage could be considered and that this is an early stage of the condition which results in destruction of the endometrium. However, in all of the mares in Knudsen's series, numerous white blood cells were present in the fluid.

One other possible explanation for the condition found in Wellie is that of mucometra. Little details are available in the literature with regard to the condition of mucometra in mares. However, it is recognised in the cow, for example in white heifer syndrome, where the normal uterine secretions are unable to be voided to the exterior (Arthur et al, 1982). In the mare, mucometra may be due to an abnormal drainage from the uterus. Therefore, the explanation in Wellie's case could be that the normal accumulation of fluid after covering was not removed by lymphatic drainage and could not be expelled during dioestrus because of a closed cervix under the influence of progesterone.

Wellie was not covered subsequently since she did not demonstrate positive oestrous behaviour again during the period of investigation.

The remaining two mares in the investigation, Tula and Snowdrop, did not come into season of their own accord during the first half of December. Plasma progesterone concentrations indicated that, at this time, both mares were in prolonged dioestrus. Prolonged dioestrus is a common finding in mares, and is successfully treated with the administration of prostaglandin, during the breeding season. It was, therefore, decided to treat these two mares in this way.

Tula came into season three days later and was covered. She

was diagnosed pregnant at 19 days by rectal palpation. The
pregnancy was confirmed at 42 days by a positive M.I.P. test and
at 5 months of gestation by palpation of a foetal mass.

Snowdrop's results indicate that, after treatment with
prostaglandin, she had a silent oestrus. She was, therefore, not
covered. This mare subsequently progressed into anoestrus.

There have been reports that rectal palpation during oestrus
and early pregnancy may have deleterious effects on the establishment
of pregnancy in the mare (Squires, Wallace, Voss, Pickett and
Shideler, 1981). The examinations carried out in this investigation
would tend to indicate that this was not the case.

The results obtained during 1983/84 confirm the findings of
the previous two years in that mares, housed and fed, continued to
cycle throughout the winter months, demonstrating the same
variations in cyclical activity as are recognised during the breeding
season. Pregnancy was established in six of the eight mares covered
indicating that the majority of the "unseasonal" oestrous periods
demonstrated in these mares were indeed fertile.

Factors thought to influence reproductive function in the mare,
applicable to these particular mares, will now be considered.

CHAPTER III

PART II – PLASMA MELATONIN ESTIMATION
AND
PATTERNS OF MELATONIN SECRETION

3.5 - INTRODUCTION

Light is considered to be a major factor in the regulation of the onset of the breeding season in the mare (Burkhardt, 1947; Nishikawa, 1959; Loy, 1968; Arthur, 1969; Kooistra and Ginther, 1975; Palmer, 1978; Sharp, 1980) in that breeding commences in the spring as the balance moves towards greater daylight hours. Light is thought to exert its effect on reproductive function via the pineal gland (Reiter, 1980; Grubaugh, Sharp, Berglund, McDowell, Kilmer, Peck and Seamans, 1982). The pineal gland manufactures and secretes various substances, including melatonin, and at present pineal activity is usually investigated by monitoring circulating plasma melatonin concentrations. As stated previously, plasma melatonin estimations were carried out in specific blood samples from the mares investigated.

Melatonin has been assayed in the plasma of many species, including mares, using a variety of radioimmunoassay techniques and antisera (Kennaway, Frith, Phillipou, Matthews and Seamark, 1977; Arendt and Wilkinson, 1979; Geffard, Puizillout and Delaage, 1982).

The initial method used in this study was that of Rollag and Niswender (1976). As many problems were encountered in using this assay, when the method of Fraser, Cowen, Franklin, Franey and Arendt (1983) was published, it was decided to attempt it as well. Both methods are described and compared.

3.6 - BLOOD SAMPLING FOR MELATONIN ESTIMATION

Blood samples for melatonin estimation were collected hourly over 24-hour periods on five occasions.

An area, approximately 20 cm x 12 cm, directly over the jugular vein in mid-neck region, was shaved.   The shaved area was thoroughly cleaned with an iodine solution (Pevidine Scrub, Berk Pharmaceuticals) and swabbed with ethanol.

Then, 2 cc of local anaesthetic (Lignocaine; Lignol; Willington Medicals) was injected subcutaneously over the jugular vein using a $1\frac{1}{2}$" 21G needle (Terumo).

Cannulae (60 mm, 14G; Intraflon; Vigon) were inserted downwards into the jugular vein.

Blood samples were collected into heparinised Monovets (Sarstedt).   After collection, samples were centrifuged at 1500 g, 4° C for fifteen minutes and the plasma decanted into labelled glass bottles which were then stored at -20° C.

Immediately after insertion and after the removal of each sample, cannulae were primed with 5 ml of a heparinised saline solution (5000 i.u. heparin in 500 ml normal saline; Pularin, Duncan Flockhart & Co. Ltd.; Normal Saline, Travenol).

When sampling was completed, the cannulae were removed, the neck area cleansed and the insertion site sprayed with an antibiotic spray (Terramycine Aerosol Spray, Pfizer Ltd.).

TABLE 3.3

SUMMARY OF BLEEDS CARRIED OUT FOR MELATONIN ESTIMATION

Bleed No.	Date	No. of hours of darkness in Barn 3
1	4th/5th November, 1982	15
2	13th/14th January, 1983	16
3	10th/11th March, 1983	12
4	12th/13th May, 1983	8
5	8th/9th December, 1983	16

3.7 – ESTIMATION OF PLASMA MELATONIN – METHOD I

Determination of melatonin was carried out according to the method
of Rollag and Niswender (1976).

A. <u>MATERIALS AND METHODS</u>

Glass distilled water was used throughout.

All the substances used, unless stated otherwise, were obtained

from recognised firms and were of analar quality.

1. Phosphate Buffered Saline (P.B.S.) pH 7.0

   0.35 g Na_2HPO_4

   1.06 g NaH_2PO_4

   9.0 g NaCl

   0.10 g merthiolate

   Water to 1000 ml

   pH adjusted with either NaOH or HCl.

2. Phosphate Buffered Saline Gelatin (0.1% Gelatin) (P.B.S.G.)

3. Normal rabbit gamma globulin (NRGG) (Sigma, Poole, Dorset)

   50 µg/ml in P.B.S.

4. Antibody

   Supplied by Dr. G. D. Niswender.

   The antiserum was raised in rabbits to a bovine serum albumin

   conjugate of N-succinyl-5-methoxytryptamine and was supplied

   in the freeze-dried form.   It was reconstituted with distilled

   water and stored in an intermediate dilution of 1 : 400 at

   -20° C.   A working solution was prepared by adding 500 µl

   of the 1 : 400 solution to 20 ml NRGG to give a dilution

   of 1 : 16,000.   The working antibody concentration

was determined by incubating various dilutions of the antiserum with a fixed mass of ^{125}I-melatonin analogue. The dilution at which sufficient binding was achieved whilst still using an economically viable antibody concentration was found (see Fig. 3.8).

5. ^{125}I-N-3-(4-hydroxyphenyl)-propionyl-5-methoxytryptamine (^{125}I-melatonin analogue).

This was prepared according to the method of Rollag and Niswender (1976). 10 µl of a 1 mg/10 ml solution of N-3-(4 hydroxyphenyl)-propionyl-5-methoxytryptamine (melatonin analogue) in methanol were added to 50 µl of 0.1M phosphate buffer, pH 7.1, in the base of a 1 ml serum vial. To this was added 1 µg lactoperoxidase (Sigma, Poole, Dorset) in 1 µl deionised water, 2 mCi carrier free, high specific activity ^{125}I (Amersham International, Bucks.) and 40 ng hydrogen peroxide in 10 µl water. The quantity of hydrogen peroxide added was verified by spectrophotometric examination of a stock solution at 230 nm (molar absorptivity = 72.4).

After shaking the reaction mixture for 5 - 10 minutes, 100 µl of 16% sucrose in buffer containing 0.022 M boric acid, 0.0064M disodium EDTA and 0.014M Tris (pH 8.9) were added. The mixture was separated on a 7.5% polyacrylamide vertical slab gel at a constant current of 10 mA in a continuous buffer system of 0.011M boric
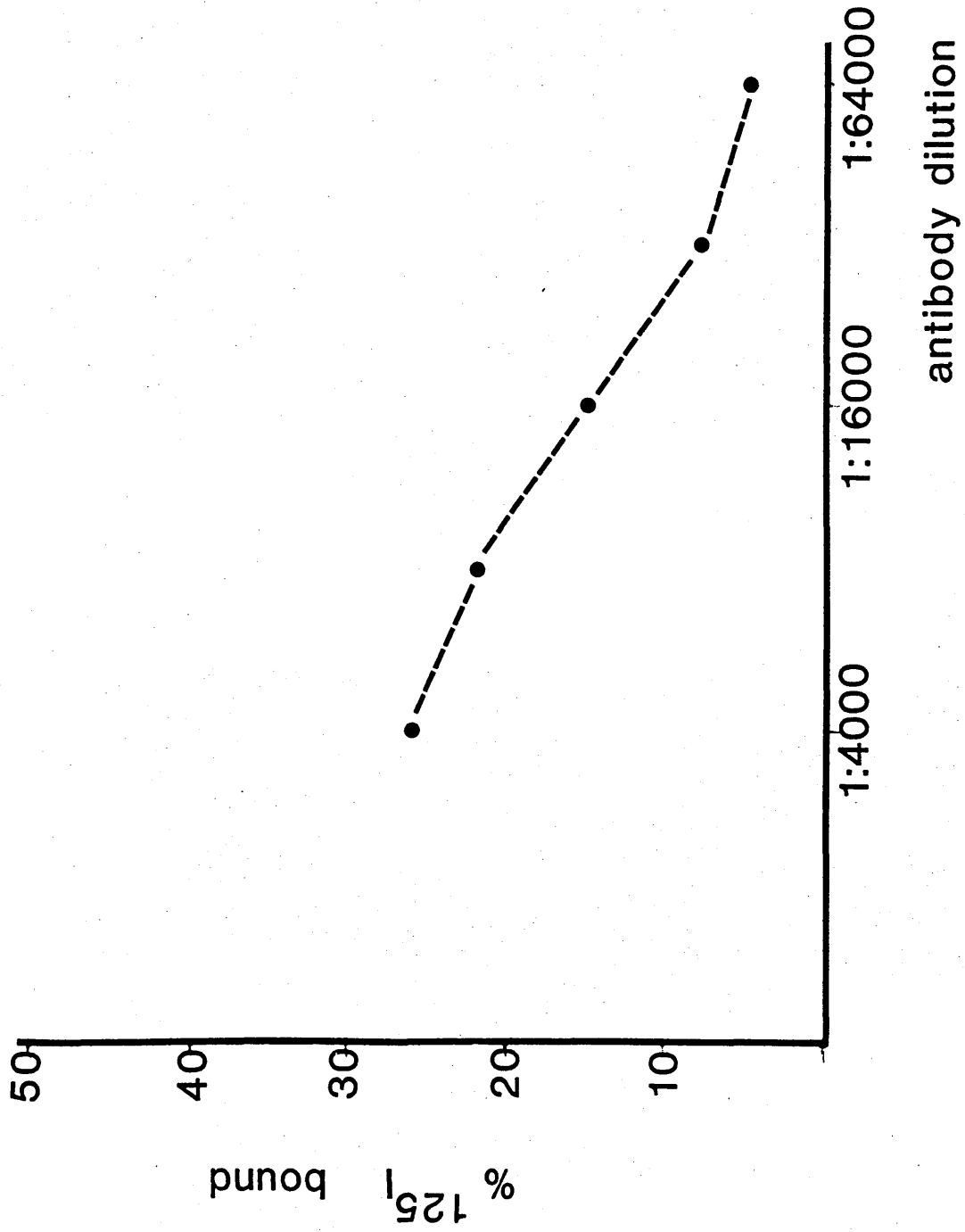
Fig. 3.8 — ANTIBODY DILUTION CURVE: METHOD 1

acid, 0.0032M disodium EDTA and 0.007M Tris (pH 8.9).

Electrophoresis continued until a BSA marker stained with

bromophenol blue had migrated 2/3 of the way through

the gel (approximately 90 - 120 minutes).   Following

electrophoresis, the gel was sectioned into 3 mm segments

and their radioactivity quantified.   Those segments

containing the ^{125}I-melatonin analogue were eluted

overnight in 1 ml of 0.05M phosphate buffer (pH 7.5)

containing 0.1% gelatin and 0.9% NaCl (PBSG).   A

working solution was made as required, by diluting this

stock solution with PBSG to give 20,000 - 30,000 cpm in

100 μl.

6. ^{3}H-melatonin (N.E.N., New Road, Southampton).

7.   Melatonin Standard

A stock solution of 1 mg/ml melatonin (Sigma, Poole,

Dorset) in methanol was prepared and stored at -20° C.

50 μl of this stock solution was diluted to 25 ml with PBSG

to give a 2000 pg/ml solution (Solution A).   This was made

weekly and stored at 4° C.   5 ml of Solution A were

diluted to 50 ml with PBSG to give a 200 pg/μl solution

(Solution B).   This was also made weekly and stored at

4° C.   The working solution of 1 pg/μl (Solution C) was

made by diluting 250 μl of Solution B to 50 ml with PBSG.

This was made daily.

8. 0.1M Carbonate Buffer, pH 10.25

   4.2 g $NaHCO_3$

   5.3 g Na_2CO_3

   Water to 1000 ml

   Check and adjust pH

9. $CHCl_3$

10. Petroleum Ether (40 - 60%).

11. Ethanol.

12. Assay tubes: Rimless, soda glass, 100 mm x 16 mm (Samco).

B. ASSAY PROTOCOL

To each assay were added control samples of high, intermediate and low values to allow the determination of inter- and intra-assay variation; tritiated melatonin in plasma to allow calculation of extraction values and assay buffer to allow identification of contaminants in the organic solvent. All samples and control samples were assayed in duplicate.

200 µl of each sample or control sample plus 500 µl of 0.1M carbonate buffer were aliquoted into labelled glass tubes. To each tube were added 2.0 ml $CHCl_3$. All tubes were vortexed for 20 minutes and the aqueous layer subsequently aspirated off. 500 µl of distilled water was then added to each tube, the tubes vortexed for 5 minutes and the aqueous layer again aspirated off. The $CHCl_3$ was then removed under a stream of N_2 gas. 500 µl of PBSG was added to each tube and these were incubated overnight at room temperature. The following morning all tubes received 2 ml petroleum ether, were vortexed briefly for 10 - 15 seconds and the aqueous layer frozen in a dry ice and ethanol ice bath. The petroleum ether was discarded and the aqueous layer allowed to thaw. At this point, a standard curve was prepared, in triplicate, by diluting standard Solution C in PBSG to give standards of 0, 1, 2.5, 5.0, 10.0, 25.0, 50.0, 100.0, 200.0, 500.0 pg/0.5 ml. Triplicate tubes of assay buffer only were also set up to allow determination of the total counts added (TC) and the non-specific binding (NSB). To all tubes

were added 200 μl antiserum (1 : 16,000).   The NSB tubes
received 200 μl NRGG.   The tubes were vortex mixed and
incubated overnight at 4° C.   100 μl of ^{125}I-melatonin
analogue (20,000 - 30,000 cpm) were added to each tube,
vortex mixed and incubated at 4° C for 48 hours.   To all
tubes, except TC's, were added 2.0 ml ethanol.   These were
vortexed for 30 seconds, allowed to stand at room temperature
for 2 - 10 minutes and then centrifuged at 1000 g for 30
minutes at 4° C.   The supernatant was discarded and the tubes
allowed to drain before counting in a gamma counter.
Results were determined by computer programme (Rodbard and
Lewald, 1970) and were presented in pg/ml uncorrected for
extraction.   Extraction values were calculated by determining
the percentage of the added ^{3}H-melatonin which was recovered
at the end of the extraction steps.

3.8 – ESTIMATION OF PLASMA MELATONIN – METHOD 2

Determination of melatonin was carried out according to the method of Fraser, Cowen, Franklin, Franey and Arendt (1983).

A. MATERIALS AND METHODS

Glass distilled water was used throughout.

1. Assay Buffer

   17.92 g Tricine (Sigma, Poole, Dorset)

   9.0 g Sodium Chloride

   1.0 g Gelatin

   Water to 1000 ml

2. Label

   ^{3}H-melatonin (N.E.N., New Road, Southampton).

   Specific activity 25 – 50 Ci/mmol.

   An intermediate solution was prepared by diluting 20 µl of stock label to 2.0 ml with ethanol and this was stored at -20° C. A working solution was prepared by further diluting the intermediate solution with assay buffer to provide 4,000 c.p.m. in 100 µl. The working solution was prepared freshly for each assay.

3. Antiserum

   Supplied by Guildhay Antisera (Dept. Biochemistry, University of Surrey). Batch numbers G/S/704/189-160779.

   Raised against N-acetyl-5-methoxytryptophan with thyroglobulin or bovine serum albumin as the protein carrier and conjugated with carbodiimide.

Supplied in freeze-dried form, the antiserum was
reconstituted with 2 ml water to provide an intermediate
dilution of 1 : 10. This was aliquoted to 100 µl
portions and stored at -20° C. Further dilution of this
intermediate solution provided a working solution at a
dilution of 1 : 1000. The concentration of the working
solution was determined by incubating various dilutions
of the antiserum with a fixed mass of tritiated melatonin
and with a fixed mass of tritiated melatonin plus a fixed
mass of melatonin (100 pg/ml). The dilution of the
working solution was that which bound 30 - 40% of the
tritiated melatonin and showed maximum displacement
with the addition of a known quantity of melatonin
(see Fig. 3.9).

4. Melatonin-free plasma

Blood samples were obtained on several occasions from a
number of mares during daylight hours. All samples
were individually assayed against a standard curve in
buffer and those samples reading less than 30 pg/ml were
pooled and used for preparing standards. This plasma
value read against a buffer standard curve effectively
represents assay "noise".

5. Standards

A stock solution (1 mg/ml) was prepared by dissolving
10 mg melatonin (Sigma, Poole, Dorset) in 0.5 ml ethanol
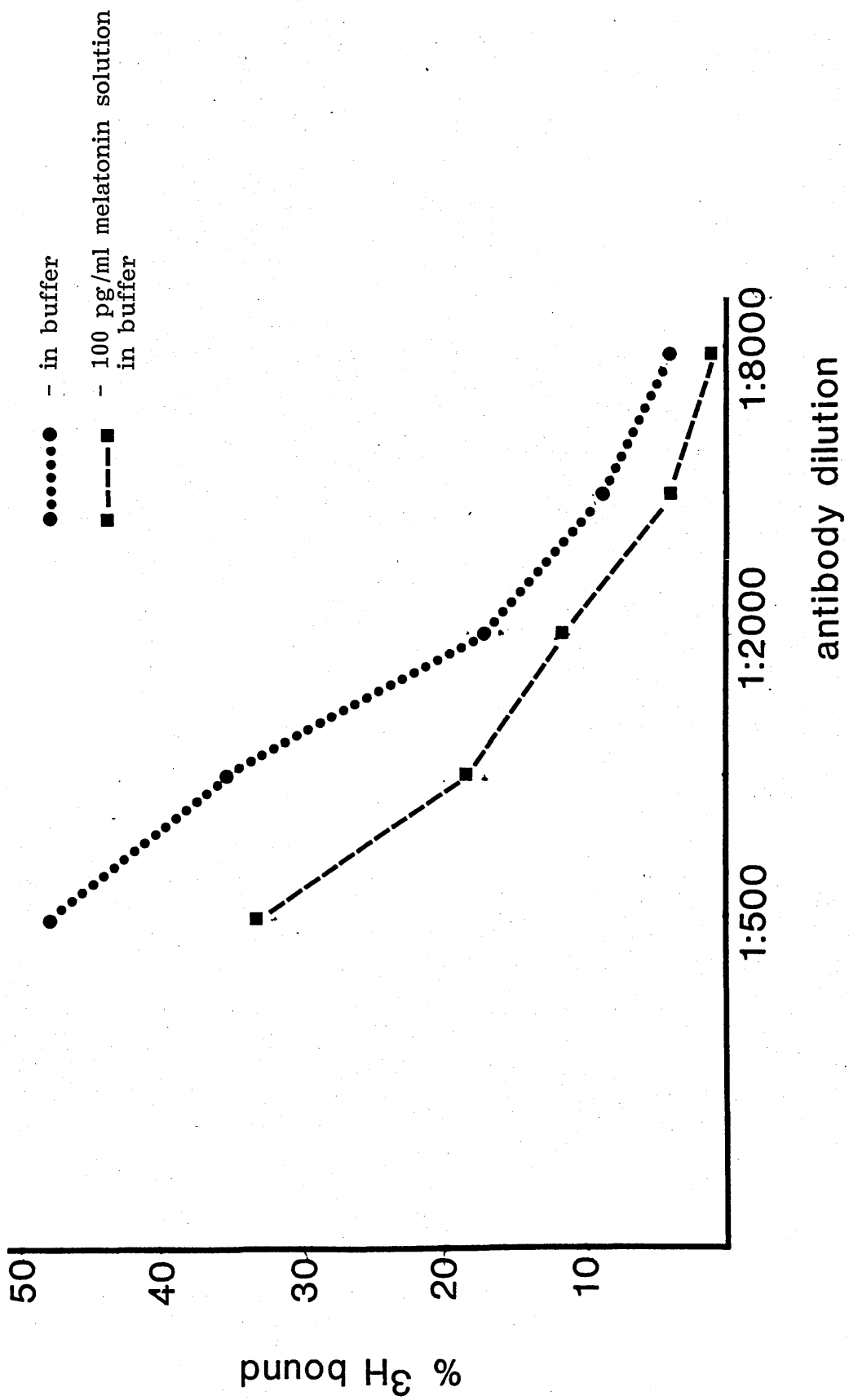and adjusting the volume to 10 ml with water. This was

Fig. 3.9 — ANTIBODY DILUTION CURVE: METHOD 2

stored at 4° C.   Standard solutions were freshly prepared
for each assay as follows.   50 µl of the stock solution
(1 mg/ml) was made up to 50 ml with water (Solution A –
1 µg/ml).   500 µl of Solution A was made up to 50 ml with
water (Solution B – 10 pg/µl).   125 µl of Solution B was
made up to 2.5 ml with melatonin-free plasma (Solution C –
0.5 pg/µl).

6. Activated Charcoal Solution

    10 g methanol washed activated charcoal (BDH Chemicals,
    Poole, Dorset) and 0.1 g Dextran 70 (Pharmacia Fine
    Chemicals, Uppsala, Sweden) were mixed with 500 ml assay
    buffer.   This was stored at 4° C and made fresh weekly.

7. Scintillator Fluid   –   Emulsifier Scintillator 229 (United
    Technologies, Packard).

8. Scintillator Vials, standard size (Packard).

9. Assay Tubes   –   Rimless soda glass 100 mm x 16 mm
    (Samco).

B. ASSAY PROTOCOL

All standards, samples and control samples were assayed in
duplicate and had a volume of 500 µl. Dilution of standard
Solution C with melatonin-free plasma provided standards of
0, 2.5, 5.0, 12.5, 25.0, 50.0, 100.0 and 250.0 pg/0.5 ml
respectively. Included in each assay were three control
samples of high, intermediate and low values. Duplicate
tubes containing 500 µl melatonin-free plasma were set up to
determine the total number of counts added (TC) and the non-
specific binding (NSB).

After aliquoting 500 µl of each sample or standard into assay
tubes, 200 µl of antiserum was added to each tube. The
NSB tubes received 200 µl of assay buffer. These were vortex
mixed and incubated for 30 minutes at room temperature. 100 µl
of ^{3}H-melatonin was then added to all tubes which were again
vortex mixed and incubated, this time for 18 hours at 4° C.
To separate the antibody-bound melatonin from the free melatonin,
500 µl dextran-coated charcoal was added to each assay tube.
The TC tubes received 500 µl of assay buffer. All tubes were
vortex mixed, incubated at 4° C for 15 minutes and centrifuged
at 1500 g for 15 minutes at 4° C. 700 µl of the supernatant
was aliquoted into vials containing 4.0 ml scintillant. These
were shaken and allowed to equilibrate in a cooled (4° C)
scintillation counter, for 1 hour, before counting.

Results were determined by computer using the programme
developed by Rodbard and Lewald (1970).

C. <u>ASSAY VALIDATION</u>

1. Specificity

From information supplied by Guildhay Antisera, the only measurable cross reactivity, expressed relative to the quantity of melatonin that displaced 50% of antibody bound (^{3}H) melatonin, was obtained using 6-hydroxymelatonin (0.4%) N-acetyltryptamine (0.35%) and N-acetylserotonin (0.03%). Cross reactivity of the 16 other indoles tested was < 0.01%.

2. Accuracy

For assessment of accuracy, known amounts (5 to 250 pg per tube) were assayed after addition to a horse plasma pool. The mean $\pm$ s.e.m. recovery figure was 97 $\pm$ 11.5%.

3. Parallelism

Standard curves made up in buffer and melatonin-free plasma were parallel to each other and to an inhibition curve of a plasma sample measured at different dilutions, and diluted in melatonin-free plasma (Fig. 3.10).

4. Repeatability of standard curve

The melatonin standard curve was highly reproducible (Fig. 3.11).

5. Precision

Intra-assay and inter-assay variations of three control samples of different values are presented in Table 3.4

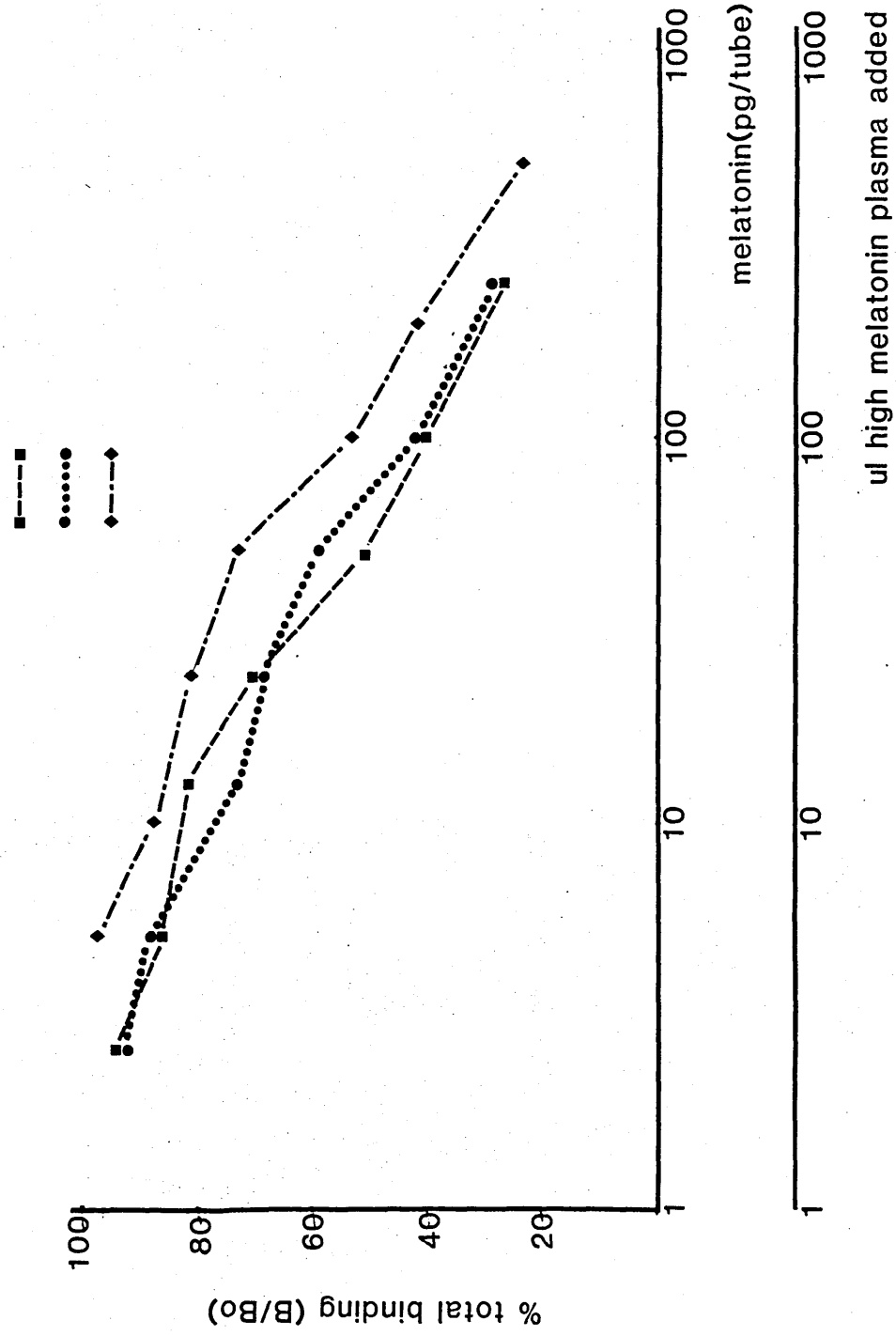FIG. 3.10   PARALLEL DILUTION CURVE   —   METHOD 2

FIG. 3.11 — COMPOSITE STANDARD CURVE from 10 melatonin assays — METHOD 2

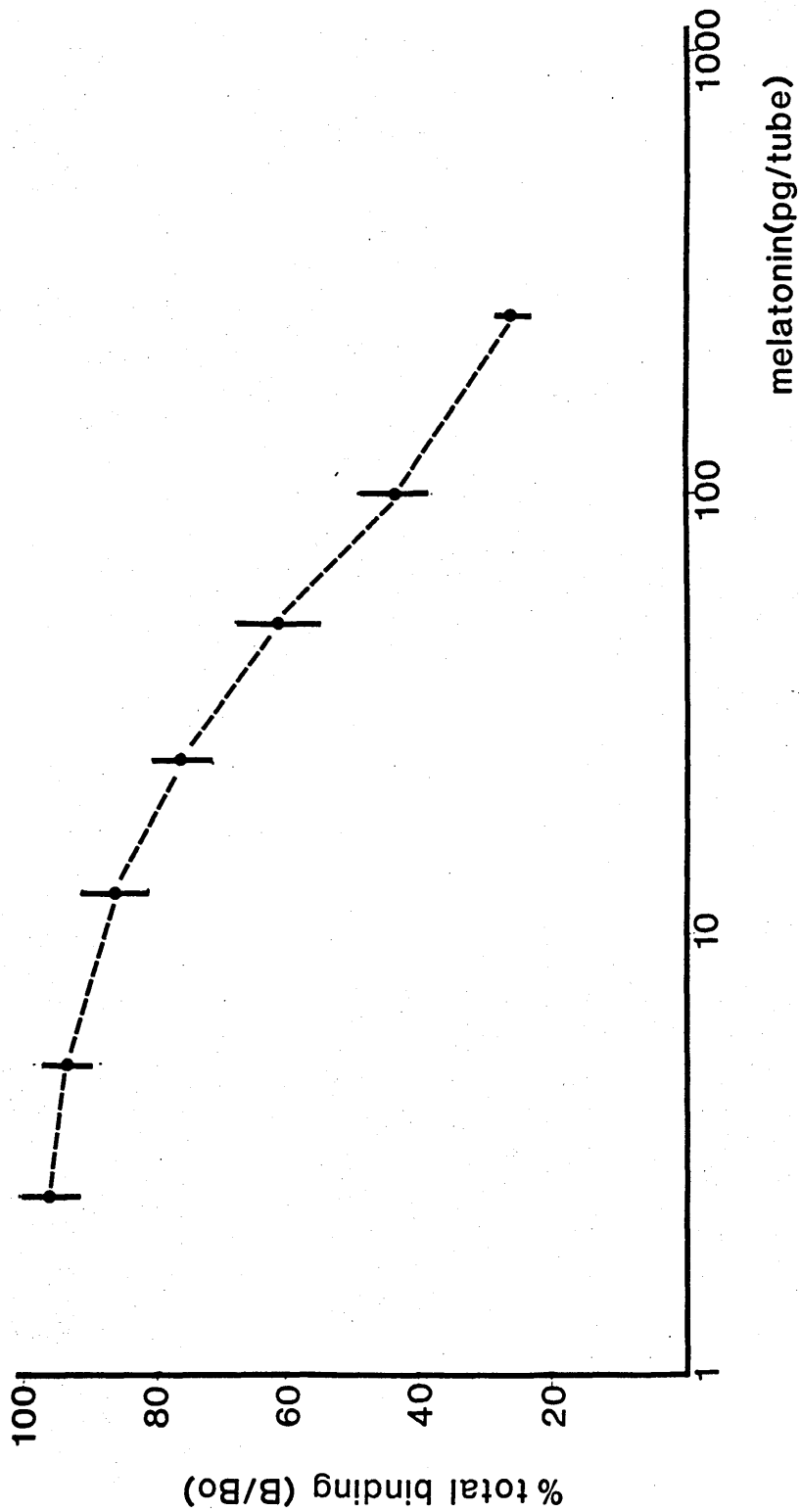Each point represents the mean +/- 1 S.D.

TABLE 3.4

PRECISION OF THE R.I.A. FOR MELATONIN

	Control Sample 1	Control Sample 2	Control Sample 3
Within Assay			
Mean, pg/ml	356.4	159.2	77.2
S.D., pg/ml	43.4	18.2	7.8
μ	5	5	5
C.V., %	12.2	11.5	10.1
Between Assay			
Mean, pg/ml	359.9	161.4	78.3
S.D., pg/ml	41.7	22.5	12.6
μ	27	27	27
C.V., %	11.6	13.9	16.1

6. Sensitivity

The limit of sensitivity of the assay, defined as the value at twice the standard deviation from the binding obtained with zero concentration of melatonin, was 7.0 pg per tube (14.0 pg/ml at a sample volume of 500 µl).

3.9 - COMPARISON OF R.I.A. TECHNIQUES

Fig.3.12 illustrates the melatonin levels of one mare over 24 hours assayed by both methods. From Fig.3.12 it can be seen that results obtained using Method 1 (Rollag and Niswender, 1976) were much higher than those using Method 2 (Fraser et al, 1983) although the pattern of melatonin secretion was similar in both cases. One of the problems found with Method 1 was that plasma melatonin values during daylight hours, although lower than night-time values, were still higher than expected when compared with those results reported in other species. Kennoway et al (1983) also report the finding of higher values using this antibody and suggest that it may cross-react with some other substance. Using Method 2 and the antibody supplied by Guildhay Antisera, daytime values were found to be much lower.

Method 1 had several other disadvantages. One of these was that the ^{125}I-labelled melatonin analogue required to be prepared every 5 - 6 weeks. The preparation of this product was not always reliable although various different methods were attempted to improve this. There was also the health hazard involved in utilising ^{125}I in large quantities. The major problem with the iodinated compound was in achieving sufficient binding to the antiserum to allow the development of a standard curve. Sufficient binding, although not as low as that recorded in the antibody dilution curve (Fig. 3.8) (This was incubated only over 6 hours at room temperature, not as in the assay, therefore was merely an indication), was only achieved using a final antibody dilution of 1 : 64,000 in comparison with the

recommended 1 : 256,000. Using Method 2, the ^{3}H-melatonin is available commercially and is very stable when stored at -20° C.

A further disadvantage of Method 1 is in the time taken to complete an assay – 5 days in comparison with only 24 hours in Method 2. Method 1 involved many steps at which an inexperienced operator could introduce errors. These errors, when taken together, can result in the assay being invalid. Method 2 had no complicated extraction steps, therefore the number of points at which errors could be made were reduced.

Overall, due to the simplicity of Method 2 and to the basal plasma melatonin values gained during daylight hours using this method, it was decided to use Method 2 rather than Method 1.

Fig. 3.12
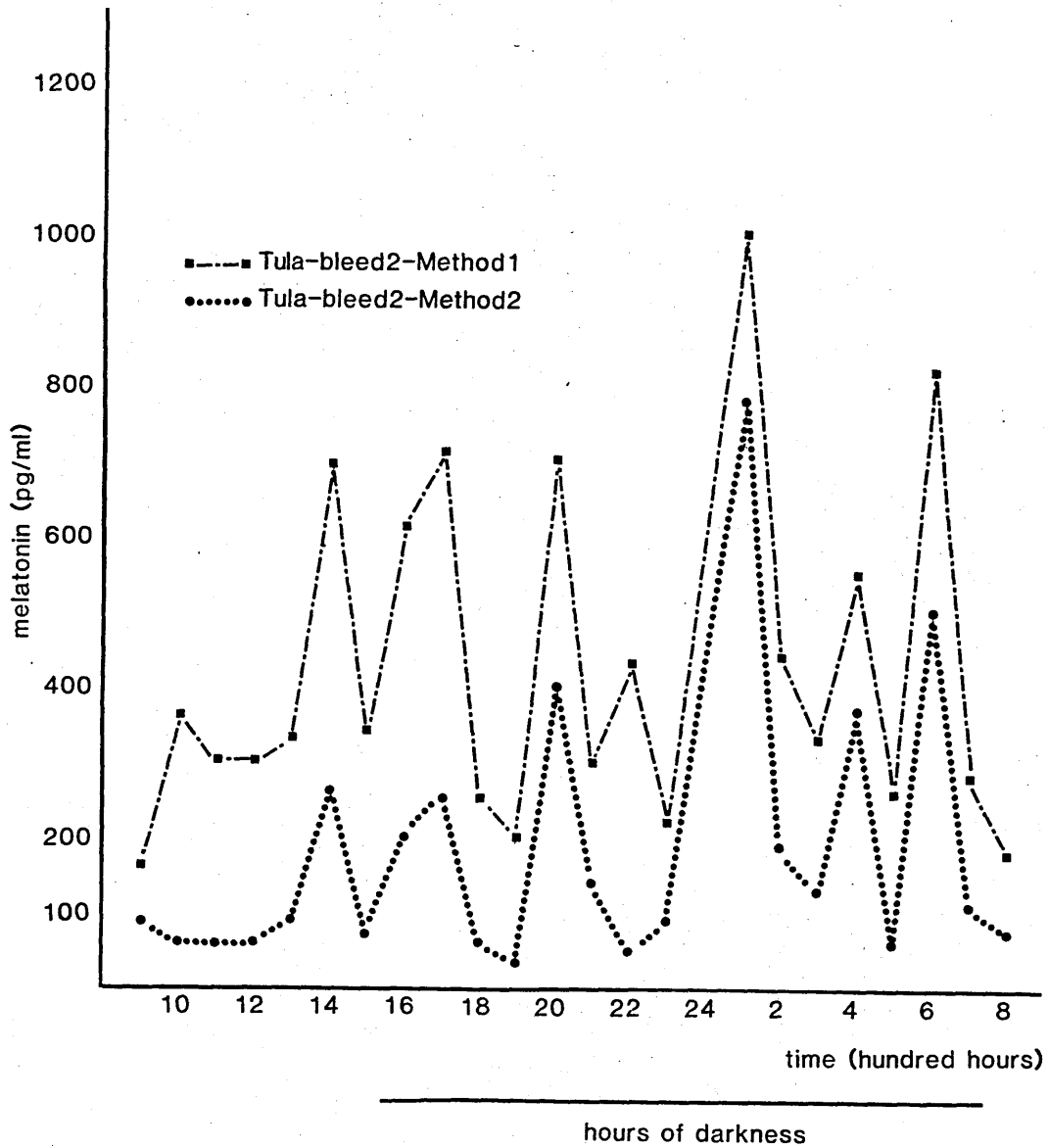
RESULTS OF PLASMA MELATONIN ESTIMATION OF SAMPLES
FROM ONE MARE, DURING ONE 24-HOUR BLEED, USING TWO
DIFFERENT METHODS.

3.10 - RESULTS

FIG. 3.13

GROUP 1, BLEED 1 (November, 19

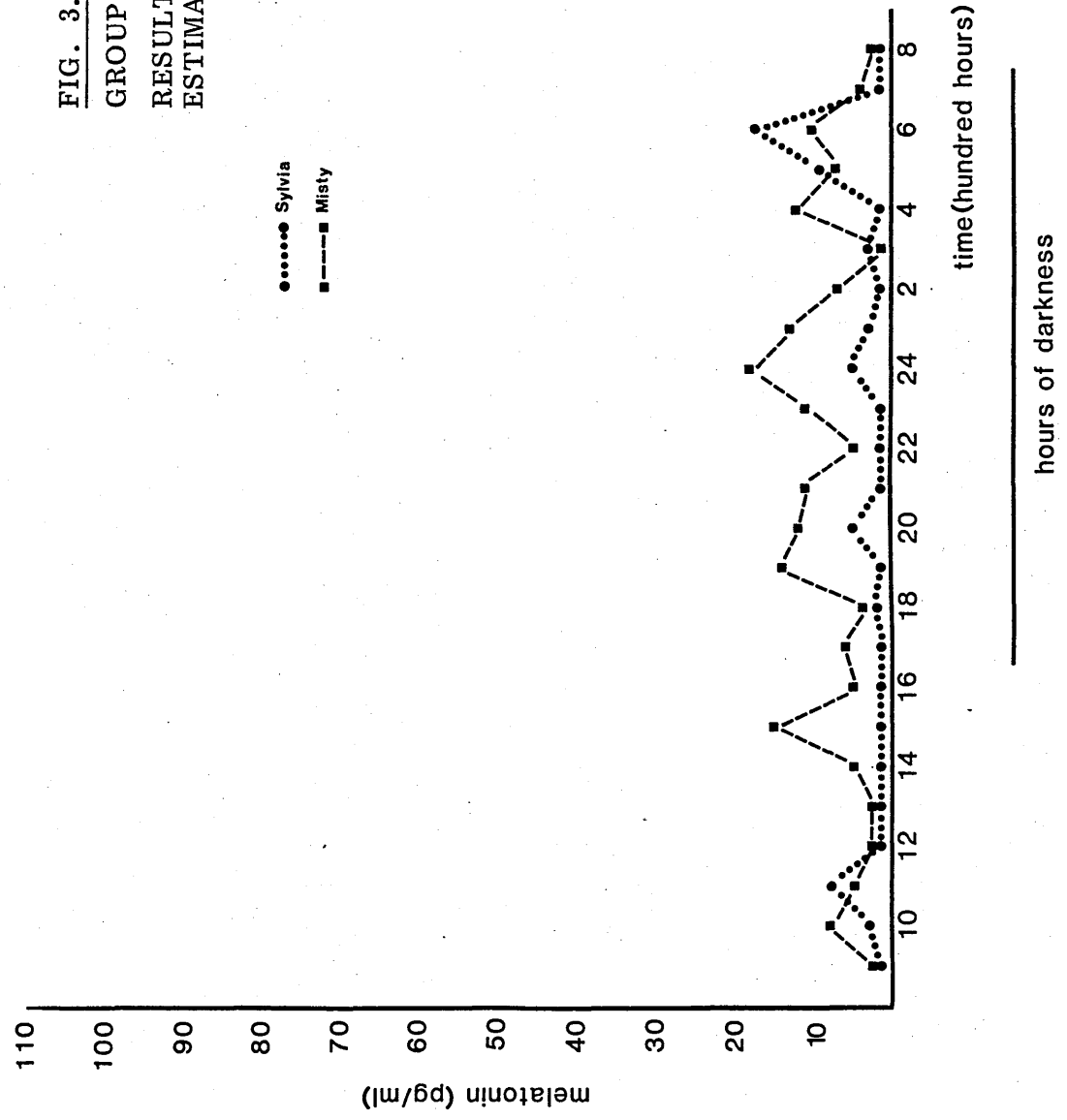RESULTS OF PLASMA MELATONIN
ESTIMATION

FIG. 3.14

GROUP 2, BLEED 1 (November, 1

RESULTS OF PLASMA MELATONII
ESTIMATION

■—---■ Filly
●●●●●● Polly
▲ ─ Value outwith range of
       Standard Curve

melatonin (pg/ml)

1200
1000
800
600
400
200
100

time(hundred hours)

10 12 14 16 18 20 22 24 2 4 6 8

hours of darkness

FIG. 3.15   -   GROUP 3, BLEED 1 (November, 1982)

RESULTS OF PLASMA MELATONIN ESTIMATION

FIG. 3.16

GROUP 1, BLEED 2 (January, 1983)

RESULTS OF PLASMA MELATONIN
ESTIMATION
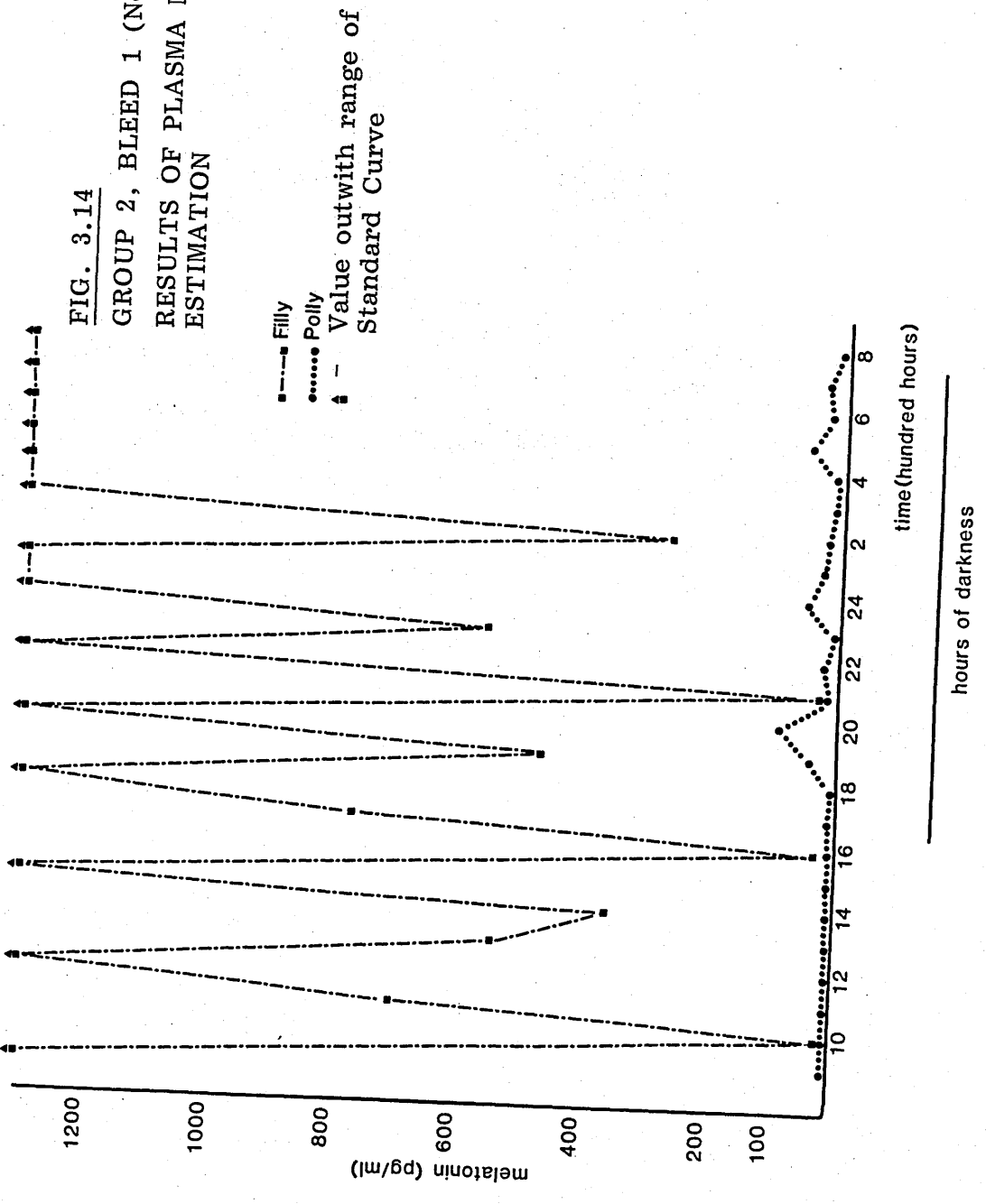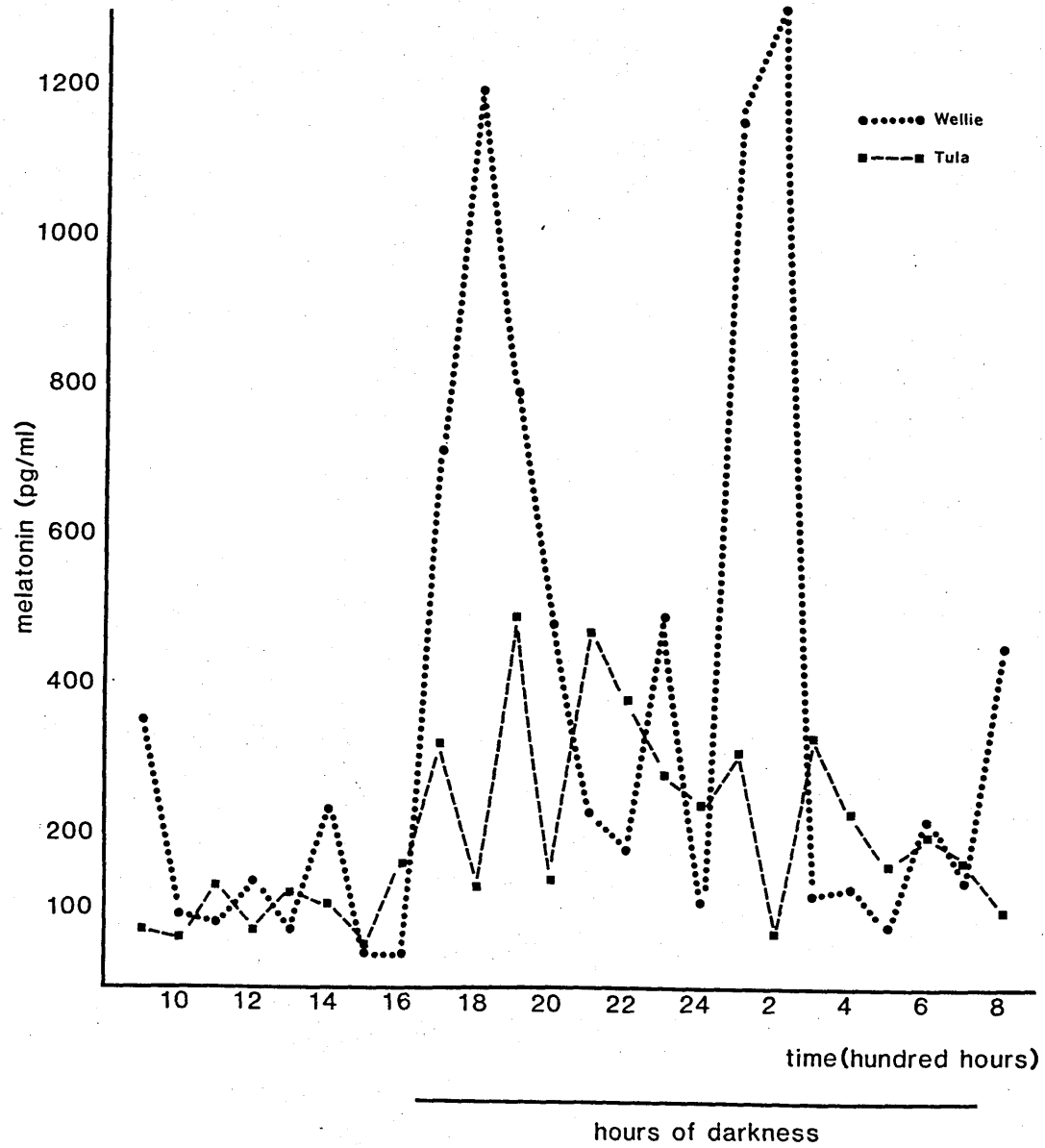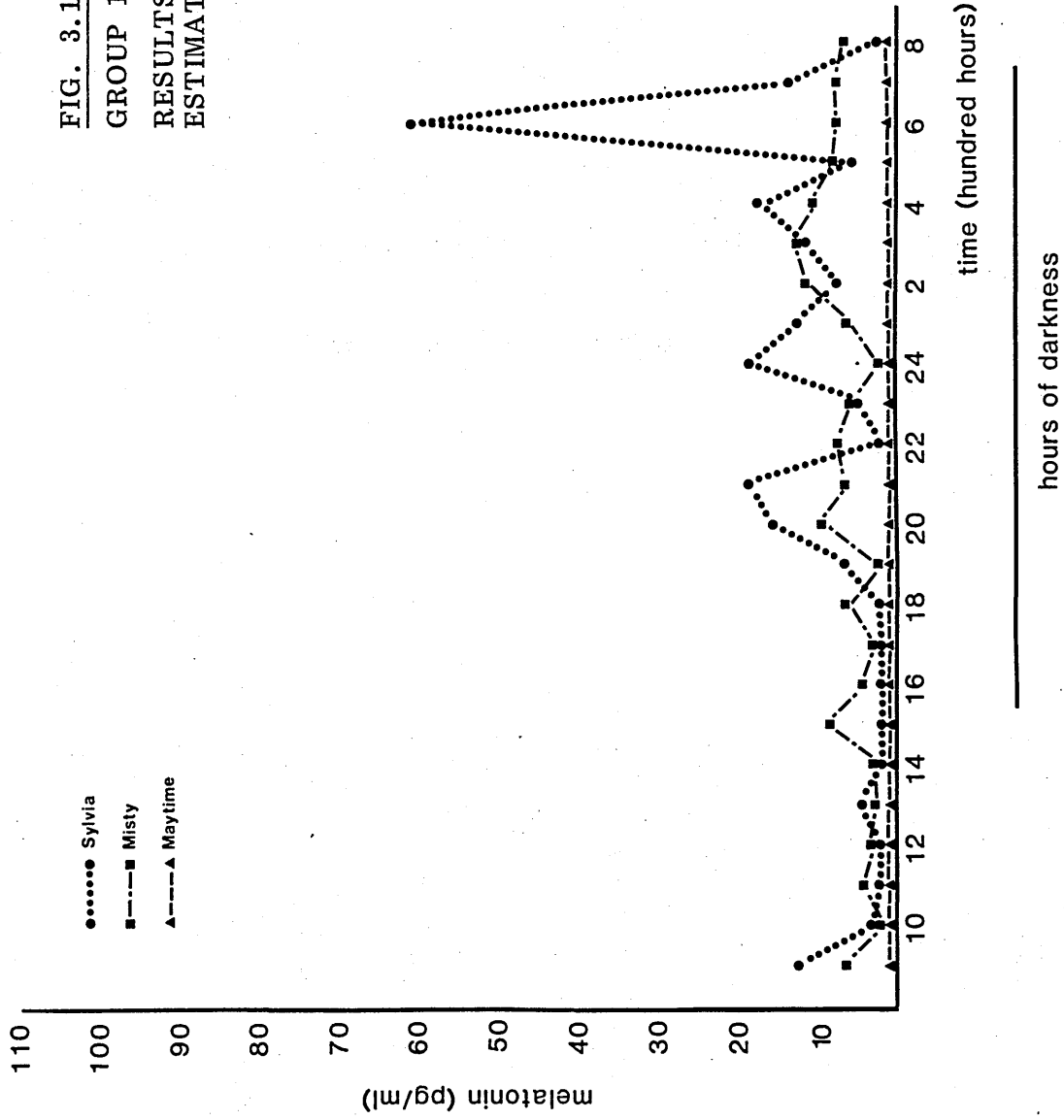
FIG. 3.17

GROUP 2, BLEED 2 (January, 1?

RESULTS OF PLASMA MELATON?
ESTIMATION

FIG. 3.18 - GROUP 3, BLEED 2 (January, 1983)
RESULTS OF PLASMA MELATONIN ESTIMATION

FIG. 3.19

GROUP 1, BLEED 3 (March, 1983)

RESULTS OF PLASMA MELATONIN ESTIMATION

FIG. 3.20   – GROUP 2, BLEED 3 (March, 1983)

RESULTS OF PLASMA MELATONIN ESTIMATION

300

250

200

melatonin (pg/ml)

150

100

50

●•••••● Wellie
■—••—■ Tula

10   12   14   16   18   20   22   24   2   4   6   8

time (hundred hours)

hours of darkness

FIG. 3.21   –   GROUP 3, BLEED 3 (March, 1983)
RESULTS OF PLASMA MELATONIN ESTIMATION

FIG. 3.22

GROUP 1, BLEED 4 (May, 1983)

RESULTS OF PLASMA MELATONIN
ESTIMATION

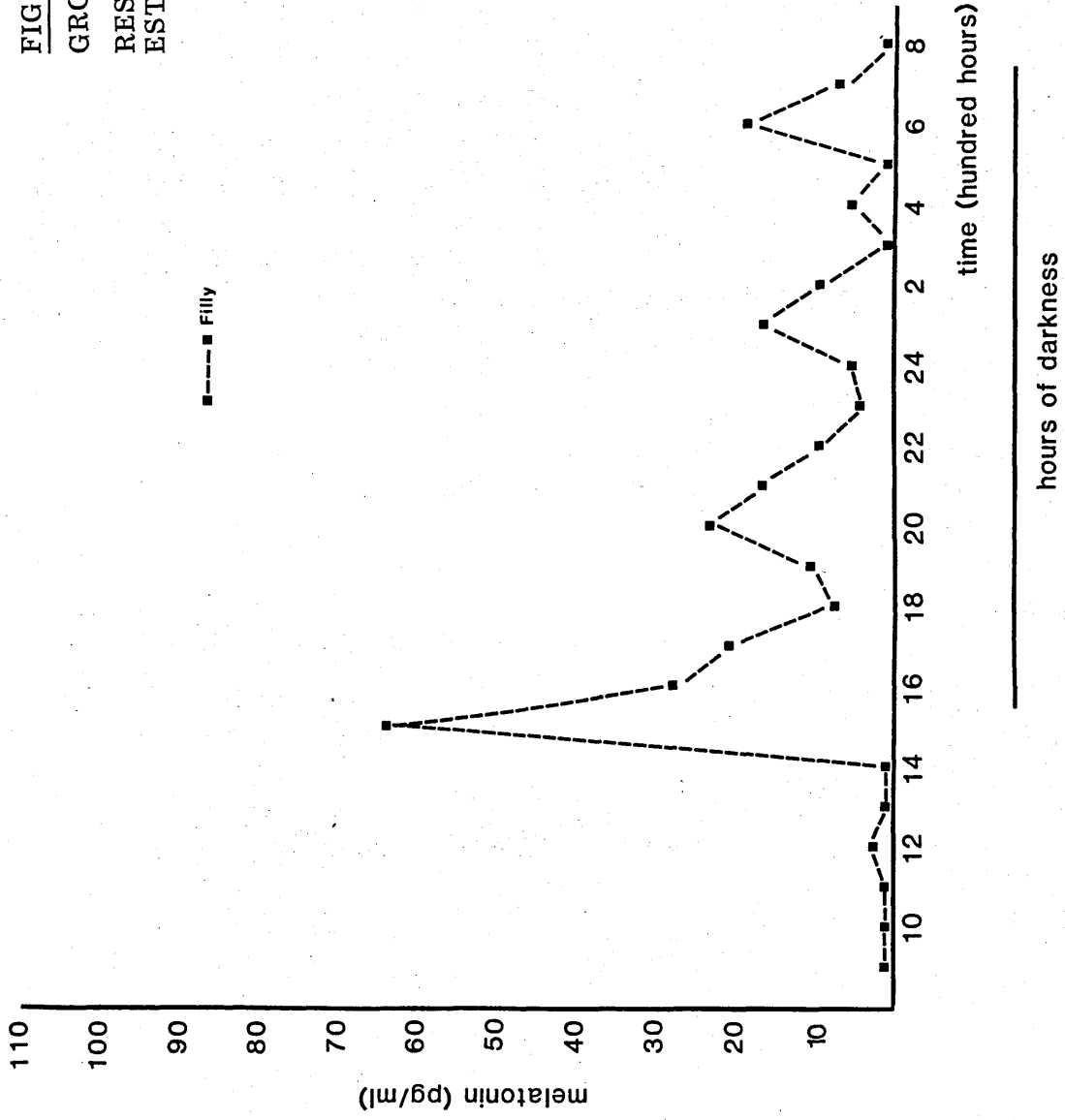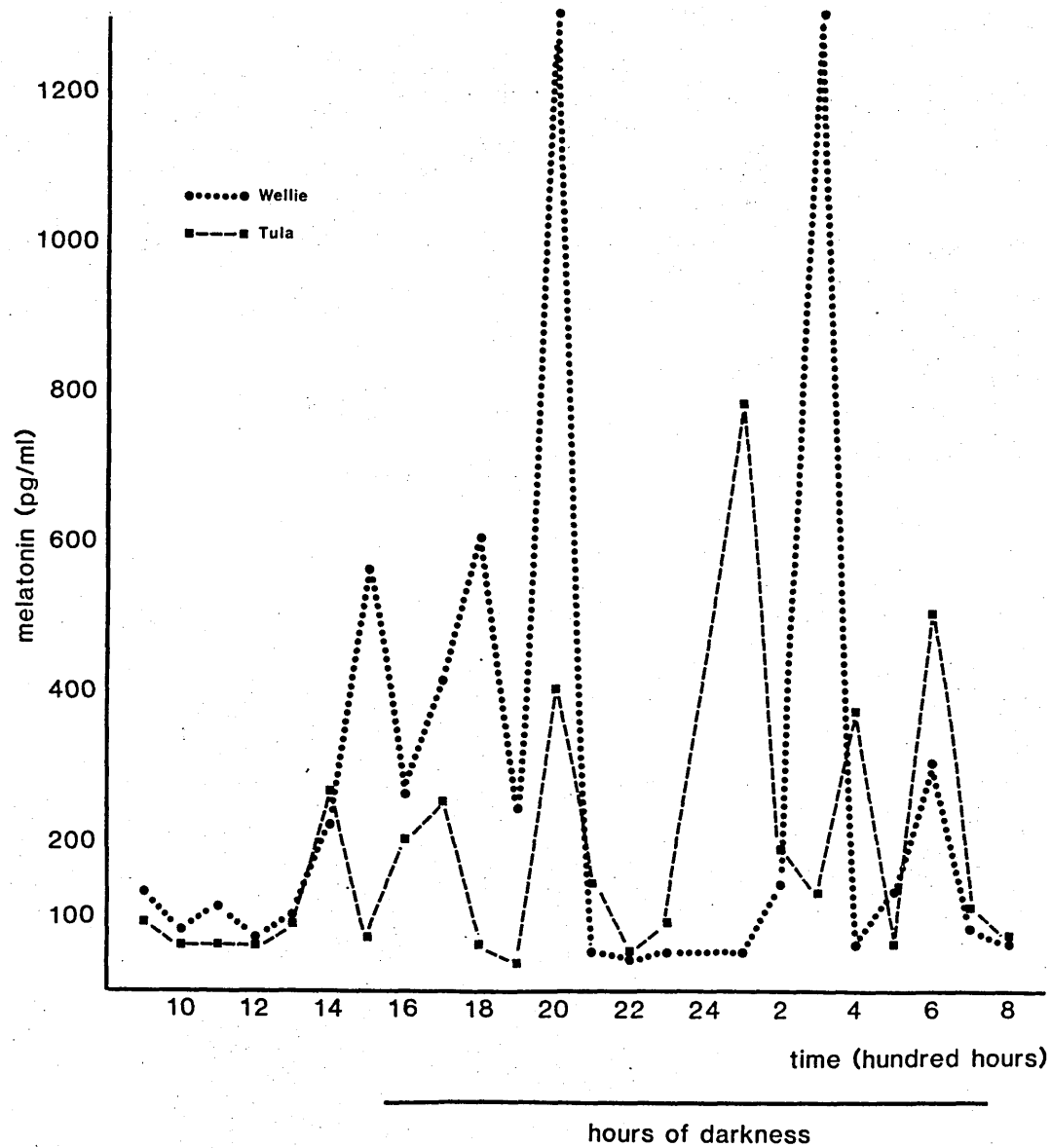FIG. 3.23

GROUP 2, BLEED 4 (May, 1983)

RESULTS OF PLASMA MELATONIN ESTIMATION

FIG. 3.24

GROUP 3, BLEED 4 (May, 1983)

RESULTS OF PLASMA MELATONIN ESTIMATION

melatonin (pg/ml)

hours of darkness

time (hundred hours)

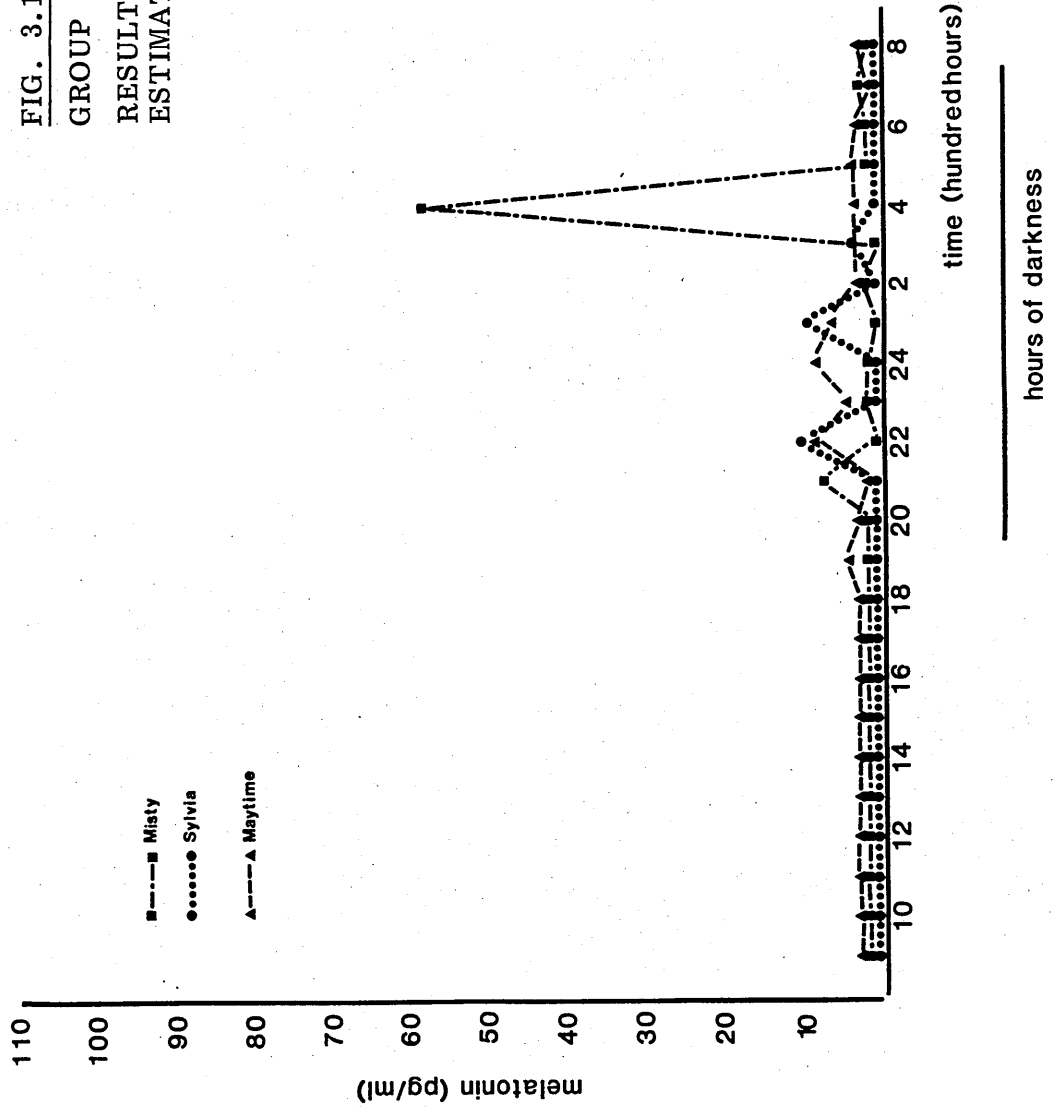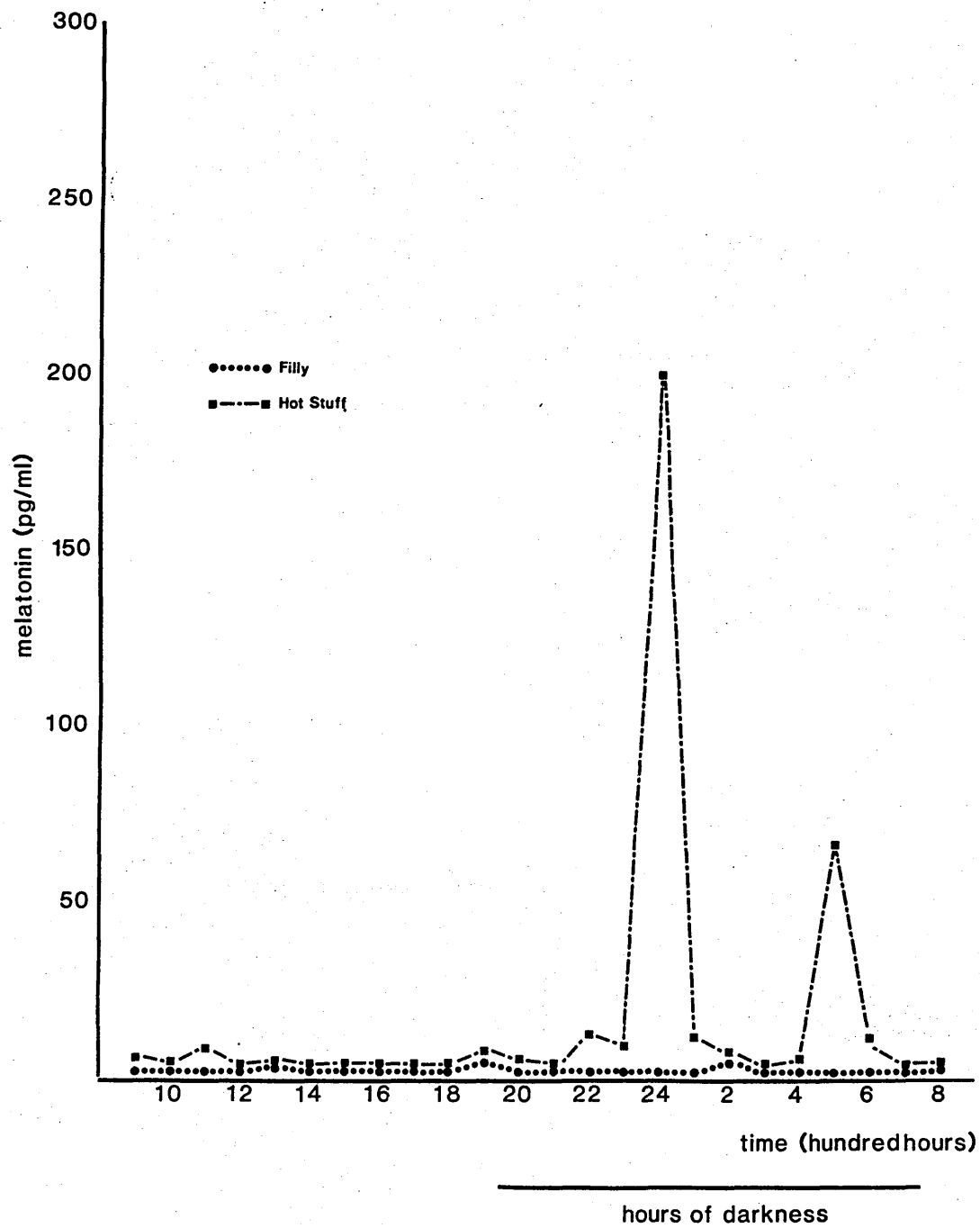Sylvia
Misty
Dotty
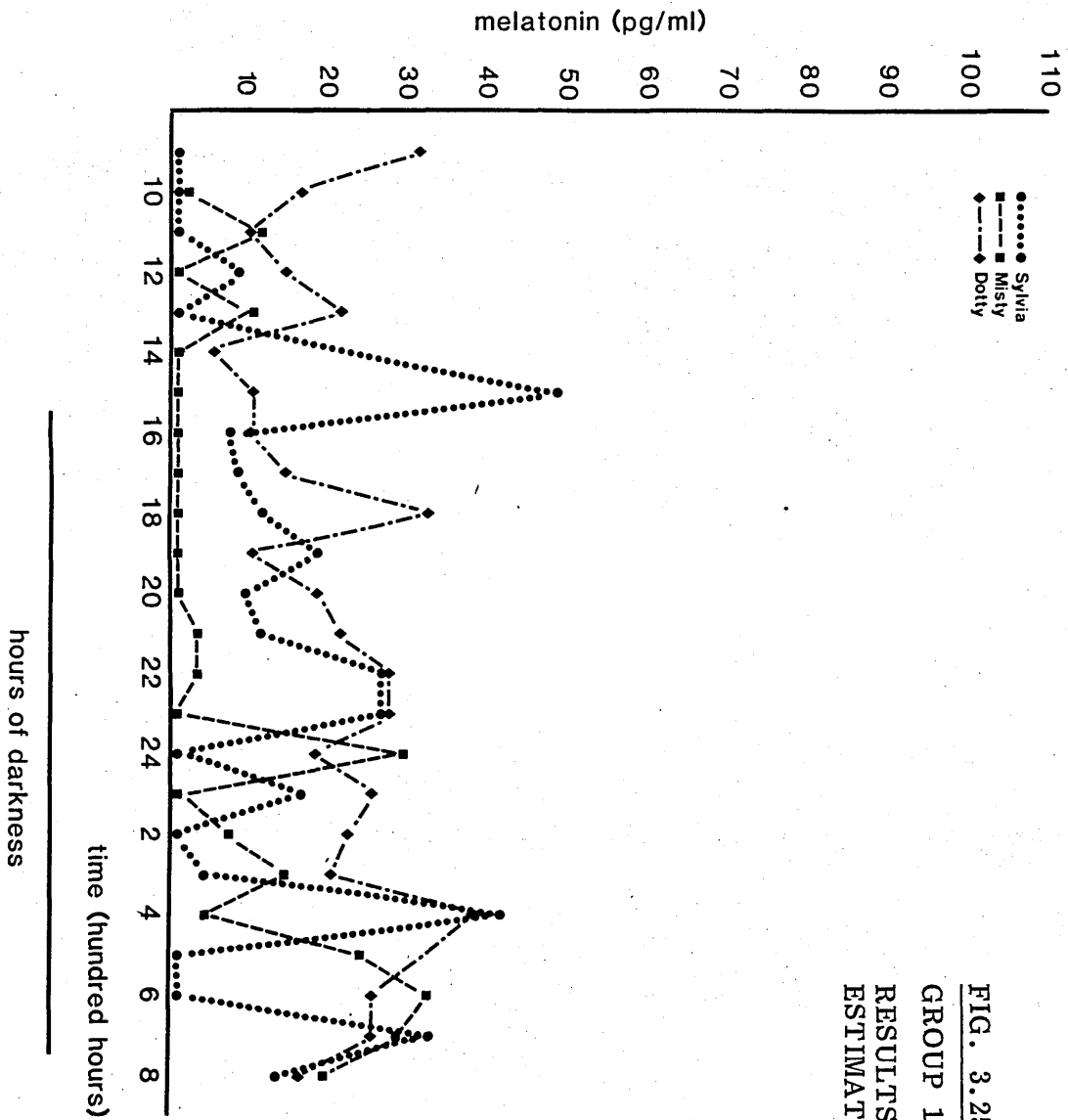
FIG. 3.25

GROUP 1, BLEED 5 (December, 1983)

RESULTS OF PLASMA MELATONIN
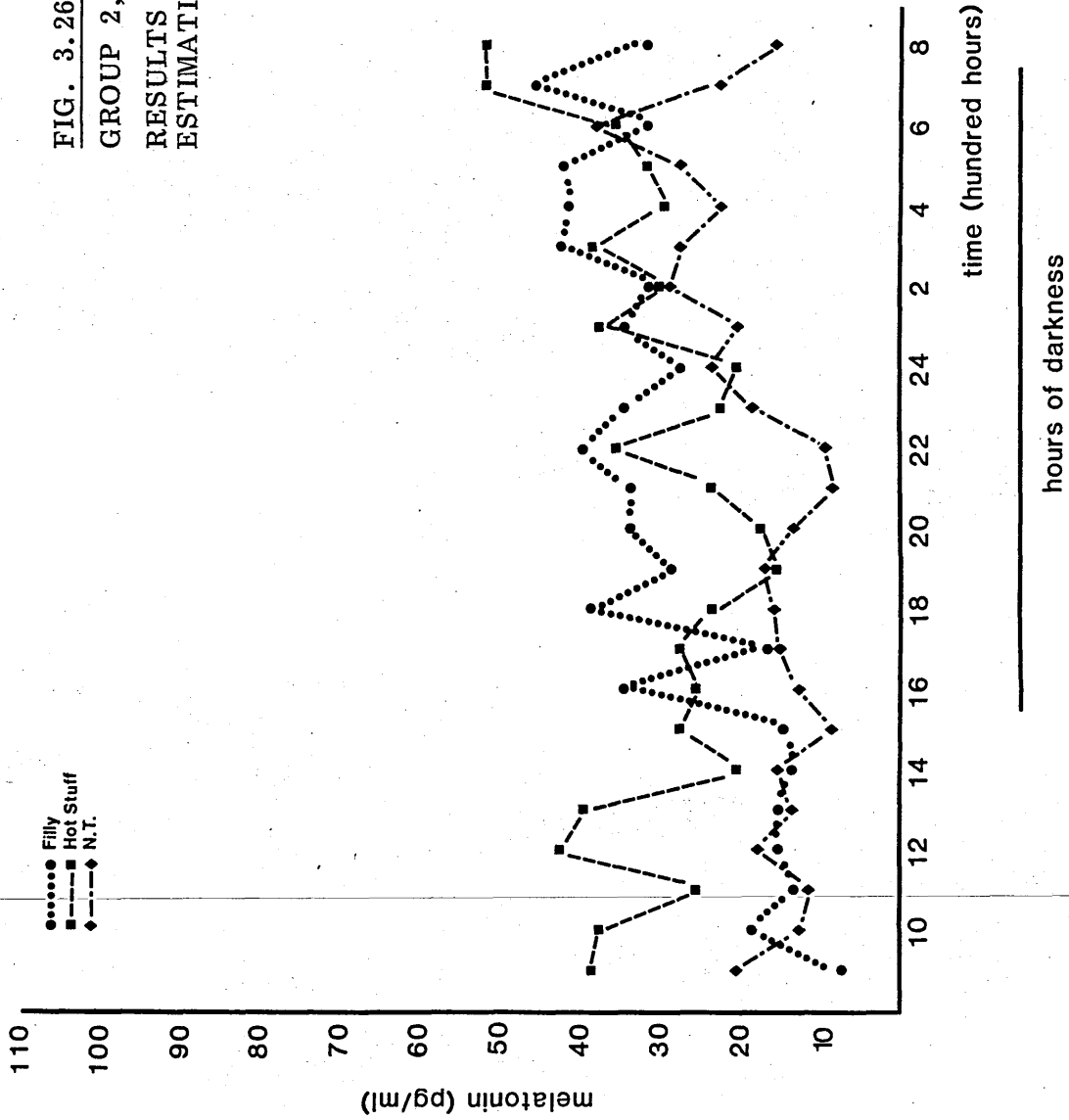
ESTIMATION

FIG. 3.26

GROUP 2, BLEED 5 (December, 198?

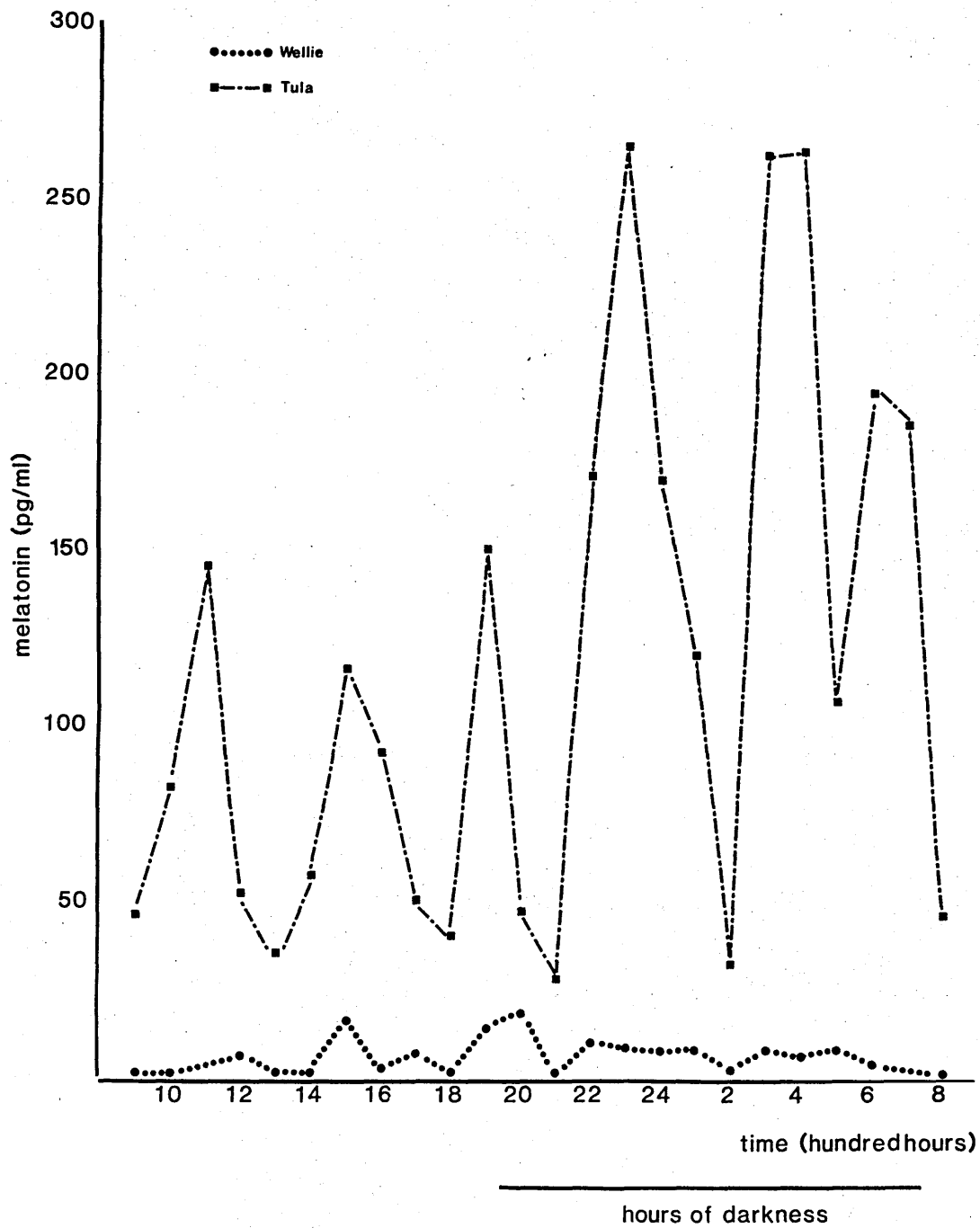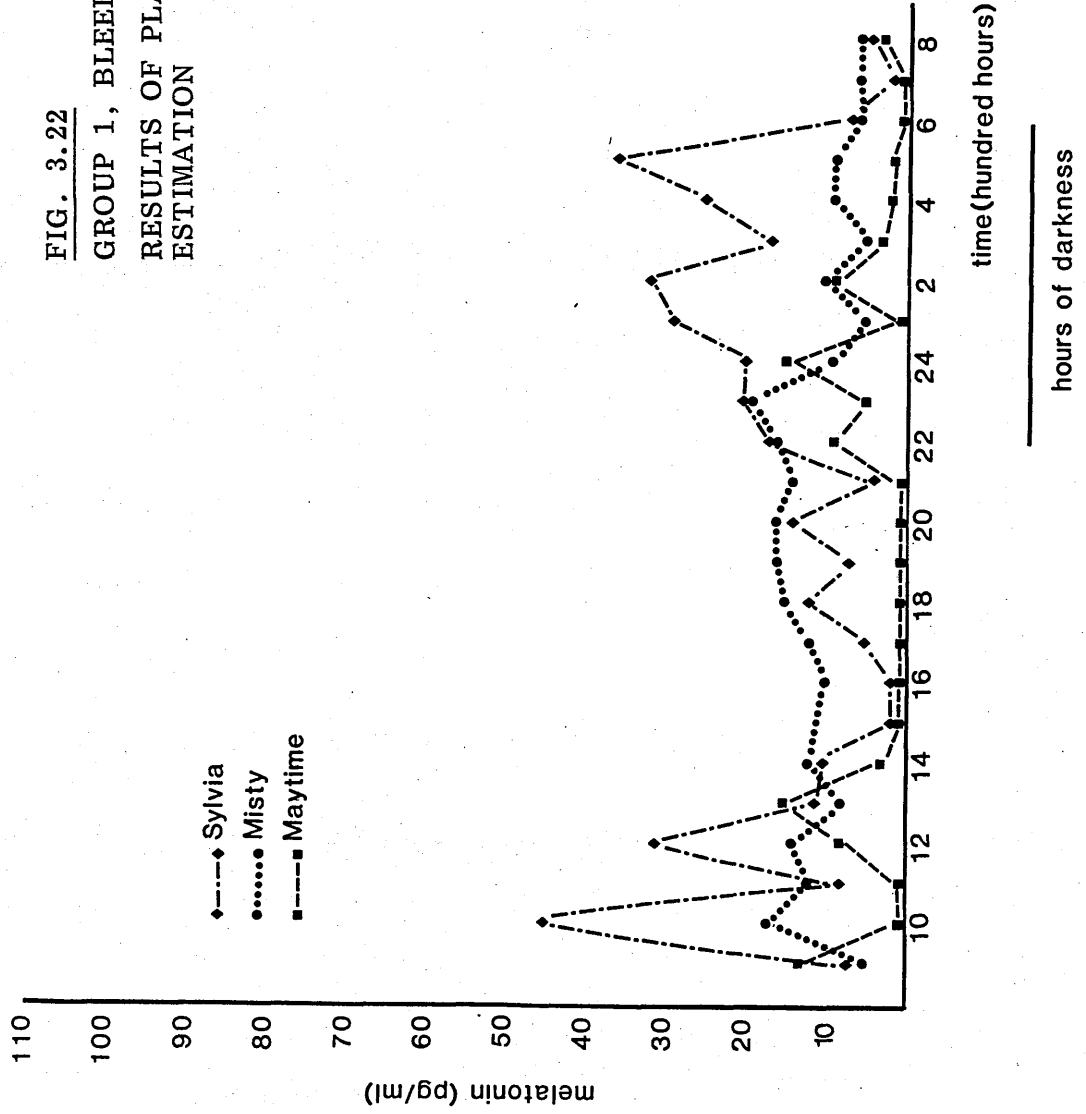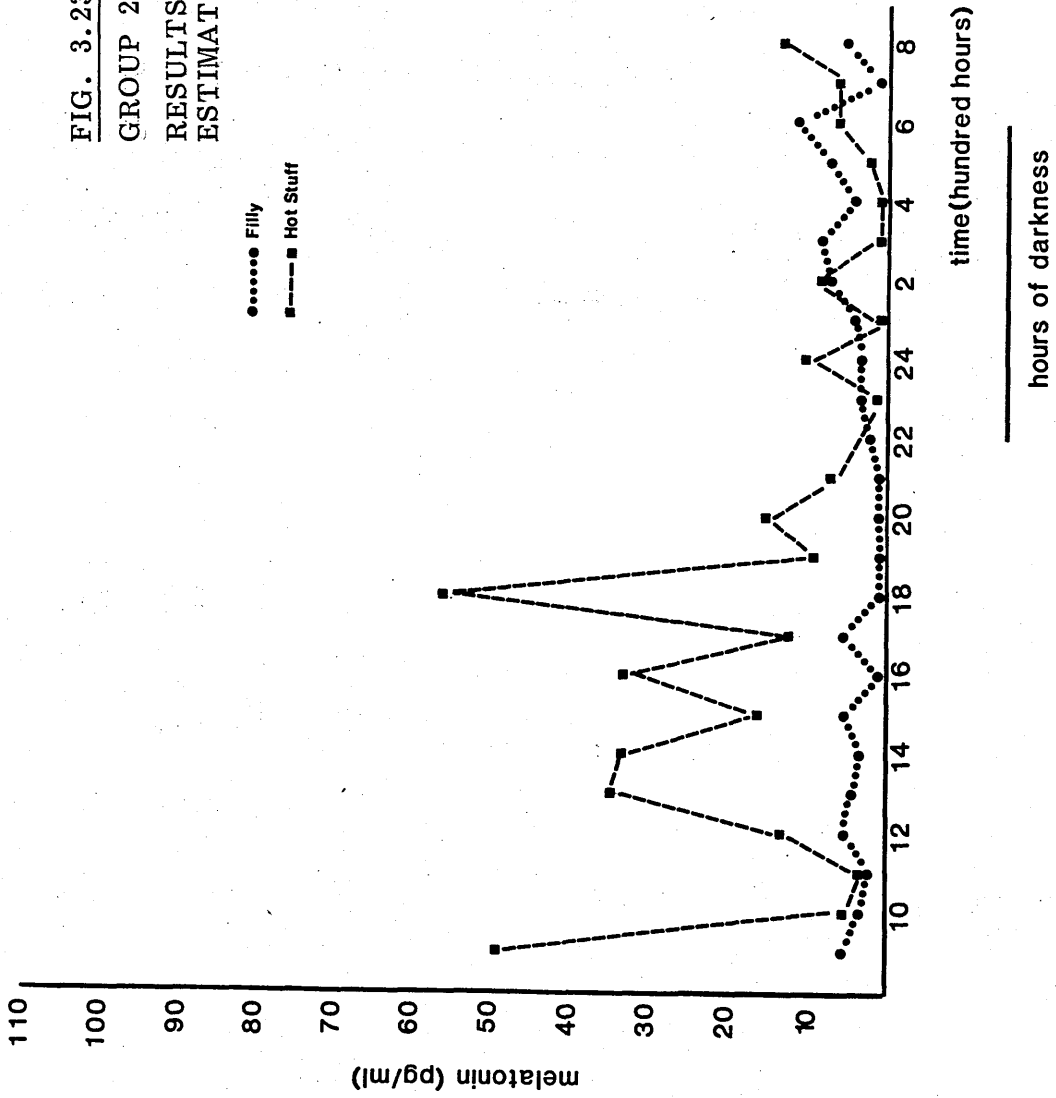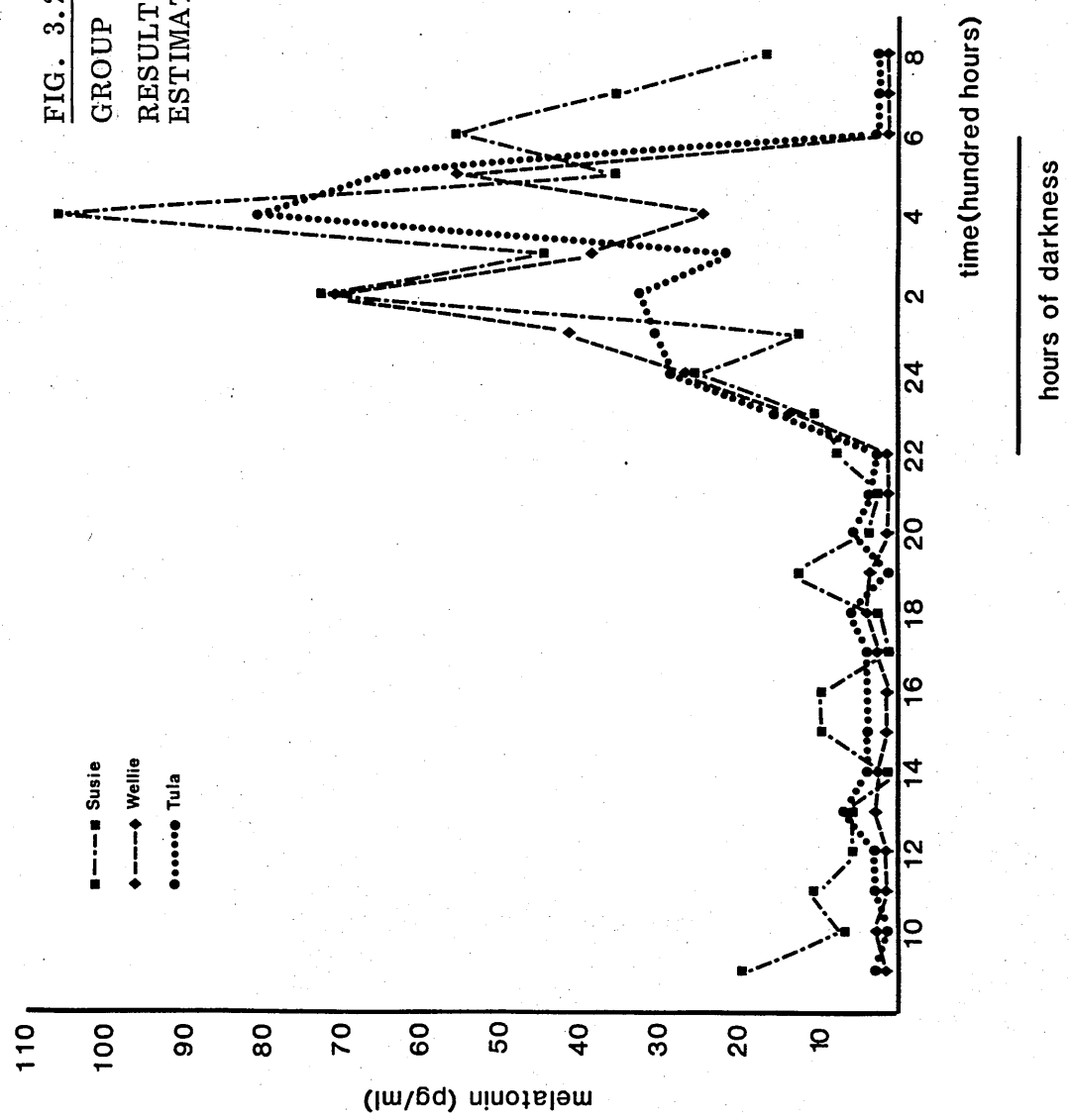RESULTS OF PLASMA MELATONIN
ESTIMATION
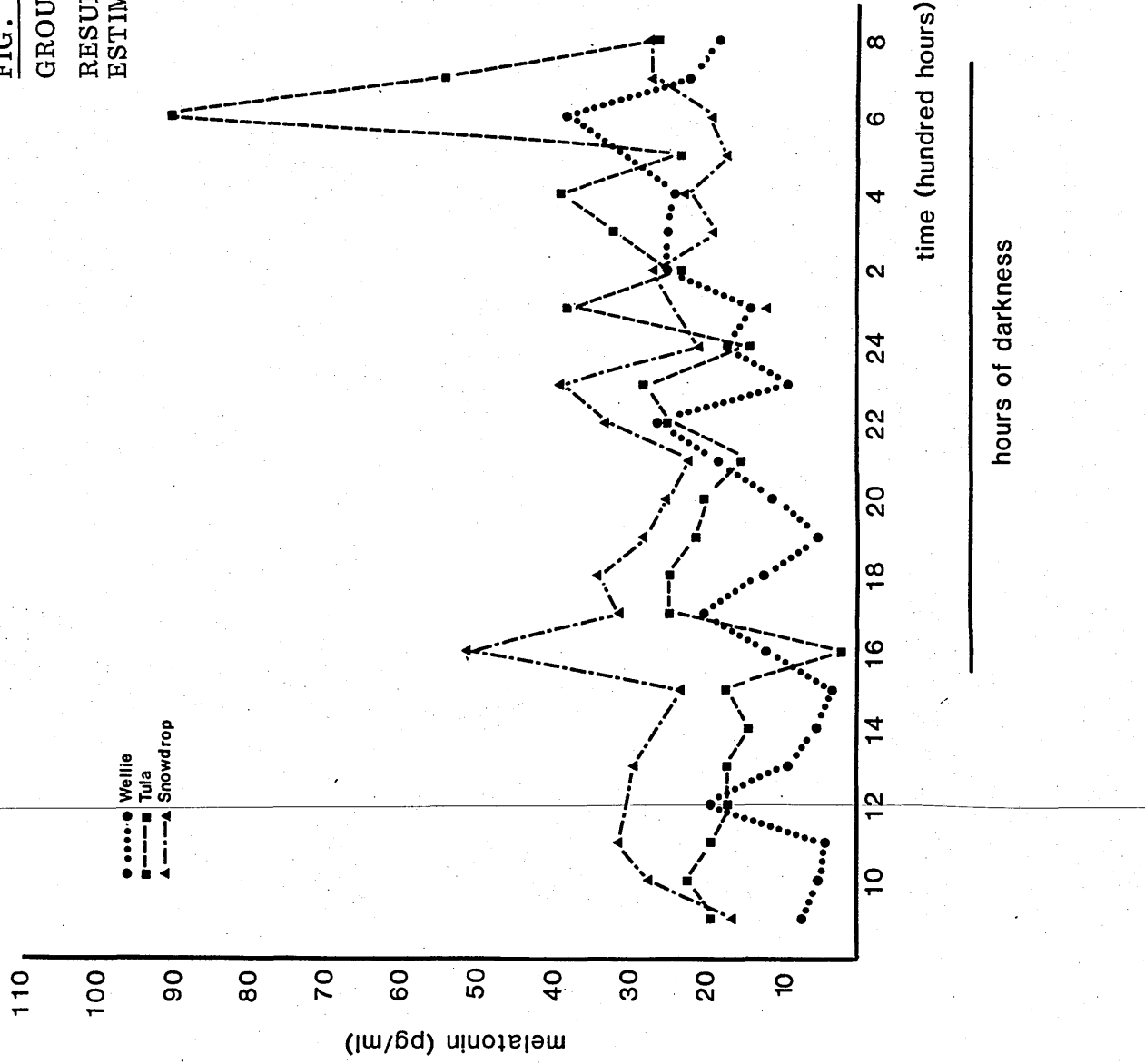
3.11 - DISCUSSION

Attempts to establish the pattern of melatonin secretion in the mares during these investigations were first undertaken during the winter of 1981/82, when no moves were made to control the amount of light received by the mares. In early December, 1981 it was recognised that the amount of light to which these mares were exposed during the night was greatly in excess of that present under natural conditions during the winter months. That this was indeed so was apparently borne out in the 1982/83 study by the pattern of melatonin secretion demonstrated by the group of pony mares exposed to similar conditions and which is referred to as the control group (Group 1). In this group there was little variation in circulating plasma melatonin throughout the twenty-four hour periods with melatonin concentrations remaining low. This would tend to suggest that, in this group, sufficient light was indeed present throughout the night to prevent synthesis and secretion of melatonin.

These patterns were in sharp contrast to those of the other two groups (Groups 2 and 3) in which darkness was experienced, where results showed definite increases in plasma melatonin concentration during the hours of darkness. However, there were differences in the patterns of melatonin secretion in Groups 2 and 3, both between groups and among individuals in each group.

In considering the general trend of melatonin secretion in the mares in Groups 2 and 3, it can be seen that melatonin was secreted in an episodic fashion during the hours of darkness, as a

wide variation in values between samples is present. These results agree with those of Sharp and Grubaugh (1983).

In the investigations reported here, there also appears to be a considerable variation in individual response by the mares to different lighting regimes. A similar occurrence has been recorded previously in mares (Sharp and Grubaugh, 1983) where although the majority of animals demonstrated the expected night-time rise in melatonin production, one failed to do so. That such individual responses are recognised should be taken into account when considering the patterns of melatonin secretion found in this study.

No results of melatonin patterns of secretion are available from Polly in Group 2 during bleeds 2, 3 and 4, or from Susie in Group 3 during bleeds 1, 2 and 3. Both of these mares were small Shetland Pony types and great difficulty was experienced in cannulating them. Hot Stuff, in Group 2, for whom no results during bleeds 1 and 2 are available, was newly acquired, young and virtually unhandled. It was realised that, at this time, attempts to tether or catch this mare in total darkness were not feasible and could have been dangerous. It was therefore decided not to sample this mare during the first two bleeds.

A striking feature of some of the melatonin profiles from certain of the mares is the extremely high circulating plasma melatonin concentration found in Filly (Group 2) during bleed 1 (Fig. 3.14) and in Tula and Wellie (Group 3) during bleeds 1 (Fig.3.15) and 2

(Fig. 3.18). These three mares were tethered initially for ease of bleeding. Because of the size of Barn 2, it was possible to release Filly after she became very restless and began to sweat profusely. However, Tula and Wellie in Barn 3, who showed the same symptoms, could not be untethered as it would have been impossible, due to the larger size of this barn, to locate them for sampling. It is significant that both the latter mares were new to the unit, were tethered for the first time and were subjected to handling and bleeding by a stranger, much of the time during total darkness.

All three mares were obviously under stress which could explain the very high concentrations of circulating melatonin. Stress results in a release of adrenaline and nor-adrenaline from the adrenals and in the generalised stimulation of the sympathetic nervous system. Lynch and Wurtman (1973) suggested that stress, in this way, results in an increase in pineal N-acetyltransferase (NAT) activity and, therefore, in an increase in melatonin production. However, Parfitt and Klein (1976) suggested that only in cases of severe stress is the sympathetic nervous system supplying the pineal unable to cope with the re-uptake of adrenaline and nor-adrenaline, and that most forms of stress result in only a minor increase in NAT activity and plasma melatonin concentration.

There was no immediate response to untethering in Filly during bleed 1 with plasma melatonin concentrations remaining high. However, such high values were never recorded again in this mare after bleed 1, nor in Tula and Wellie after bleed 2. This would tend

to support the theory that stress may well have played a part in the very high melatonin concentrations reached and that as the mares became accustomed to the routine, the procedures became less stressful.

On the assumption that stress does influence melatonin production, increased circulating melatonin concentrations could occur at any time of the day or night which could explain several of the other unexpected findings in the mares investigated.

Stress could account for the transient rises occurring in two mares in the control group who exhibited a definite but transient rise in plasma melatonin concentration during one particular bleed - Misty during bleed 3, Fig. 3.19, and Sylvia during bleed 2, Fig. 3.16.   These rises were unexpected in that, at all other times, the plasma melatonin concentrations in this group remained low.   Sylvia, in particular, is a very nervous mare and can be awkward to catch, therefore stress may well have played a part in this result.

Another mare, Hot Stuff (Group 2) during bleed 4 (Fig. 3.23) recorded higher plasma melatonin concentrations during daylight hours than during the hours of darkness.   This mare always appeared calmer during sampling at night, so that stress again may have contributed to this odd result.

On the other hand, (some of) the increases in plasma melatonin concentrations in the control group (Fig. 3.16 & 3.19)   occurred only during the hours of "darkness", which could indicate that stress was not the explanation.   It was noted that mares, like

other animals, sleep and doze on occasions throughout the night. Therefore, it could be suggested that when their eyes were shut, they were subjected to an increased level of darkness and responded with increased production and secretion of melatonin. On awakening, the time taken to withdraw the sample was not sufficient to allow the circulating melatonin concentration to return to basal values in the presence of light. This, rather than stress, may explain some of the sporadic increases in melatonin production in the control group who were subjected to a constant degree of lighting throughout the night.

If this was so, then the mares in this group, on any night other than the night when samples were taken, could have been exposed to variable durations of increased melatonin concentrations, depending on the length of time the eyes remained closed.

Another explanation for these unexpected peaks of melatonin secretion throughout the day or night could be that, in the absence of a definite light/dark pattern, where either light is present throughout the 24-hour period or where there is only a very short period of darkness, melatonin in the mare is produced and secreted at random. This could apply to several of the unexpected increases in plasma melatonin concentration already discussed.

In Group 2, in March, 1983 when the light pattern was 12D : 12L, (Fig. 3.20) one mare, Hot Stuff, demonstrated the expected night-time rise in plasma melatonin concentrations whereas the other, Filly, did not. At this time, Hot Stuff was partitioned in the darkest part of the barn, furthest from the

half-door leading into the passageway. Filly was loose and had access to this passageway which connected with the lighted yard. This access to light may explain Filly's low plasma melatonin concentrations during this bleed. In support of this explanation, during the next bleed in May with an increased natural daylight length, perhaps greater than the artificial 16L : 8D, light would have appeared to penetrate the depth of the barn and plasma melatonin concentrations in both mares remained low throughout.

Finally, a rather odd set of results was recorded in the three mares in Barn 3 during bleed 5 in January, 1984 (Fig. 3.27), when 16 hours of darkness and 8 hours of light were present, in that although higher plasma melatonin concentrations were present during the hours of darkness, they were not as high as had been recorded previously. It might be suggested that some light was present at this time. However, this was definitely not so. Checks have also been made to rule out the possibility of these lower results being due simply to assay variation. Therefore, an explanation for these lower than expected results in this particular group of mares is not obvious.

Sharp et al (1980) report a seasonal variation in melatonin production in the mare. Wellie, in Group 3, did appear to show such a variation, as peak values for night-time plasma melatonin decreased with increasing daylight hours and then increased on return to autumnal and therefore decreasing daylight hours. However, Tula, another mare in the same group, although demonstrating an apparent decrease in peak melatonin concentrations

when progressing from winter to summer, did not show a return to higher values the following autumn. Too few results are available from the other mares in the almost identical environment (Group 2) to deduce whether or not a seasonal pattern for melatonin secretion was present. Overall, it is therefore impossible to draw any conclusions from these studies with regard to the effect of season on melatonin production in the mare.

One interesting finding reported relates to mares which have been pinealectomised. In these mares it has been found that whereas the onset of the first breeding season after pinealectomy is not altered, subsequent breeding seasons are (Grubaugh et al, 1982). This tends to suggest that the effect of the pineal on reproductive activity in a particular year is programmed by the lighting changes experienced during the previous year.

When the clinical results, of those of our mares investigated during 1983/84, are examined, it can be seen that the same variations in sexual status are present as were in evidence during 1982/83. Therefore, it can be concluded that the strict lighting regimes in Barn 2 and Barn 3 in 1982/83 had no long-term effect on reproduction in the mares housed therein.

Since it has been suggested that decreasing daylight length, resulting in an increased melatonin production, causes cessation of the breeding season in the mare (Sharp, Grubaugh, Zavy and Vernon, 1980), melatonin secretion in those mares which entered anoestrus must be considered.

In Tula, (Group 3) plasma melatonin concentrations showed a

diurnal variation and were high during the hours of darkness,
prior to the onset of anoestrus.   In the control group, where a
degree of light was always present and melatonin concentrations
remained low throughout, anoestrus was experienced by two out of
three mares.   These results tend to indicate that high levels of
melatonin were not always present and therefore not necessary prior
to the onset of anoestrus.

That melatonin may not play the same role in the mare as in
the other long-day breeder, the hamster, has been demonstrated
by Thompson, Godke and Nett (1983) where the constant
administration of melatonin, via intra-vaginal sponges, to anoestrous
mares failed to stimulate gonadal function.

The results gained in these investigations, over two years,
seem to indicate that the majority of pony mares will continue to
cycle throughout the winter months independent of daylight length
and/or circulating plasma melatonin concentrations.   In addition, it
would appear that factors which could affect melatonin production in
the mare make it very difficult to assess the importance of the
results reported here and elsewhere.

Therefore, according to our results, it would appear that
factors other than light and melatonin secretion are involved in the
continuation of reproductive activity, fertile cycles, and the delay
in entering, and the curtailment of, anoestrus.

The major difference in the handling of the mares in this
investigation and the majority of pony mares during the winter

months was that the mares studied here were housed and well fed.

At the beginning of each study period the mares investigated were all in good condition and were fed to maintain this. Indeed, one of the main worries during 1983/84 was that the mares were too fat as there have been reports that overweight mares can have problems conceiving (Bellinge, personal communication). However, this would not appear to have been so in this study as few fertility problems occurred in these mares.

The continuation of reproductive function during the winter months could therefore be an energy balance situation. By housing the mares, energy, which would have gone into maintenance of body temperature and body condition outside, could be channelled into the maintenance of reproductive cyclicity.

Reproduction is not essential for life and only after the basic body energy requirements are met, and extra energy is available, does reproductive function take place. Evidence for this is that, normally, reproductive function is absent prior to puberty which does not occur until a certain degree of maturity and development is reached. Energy in the prepubertal stage is required for growth. As the growth rate slows down so does its claim on the nutritional input and reproductive function commences. Larger species and larger breeds which take longer to mature are later reaching sexual competence than their smaller contemporaries.

As mares tend not to be bred until well after puberty has occurred, little information with regard to the effects of nutrition on its onset are available although Arthur and Allen (1972) do

state that puberty occurs earlier in well-fed fillies. That nutrition plays an important part in the onset of puberty has been well established in the bovine (Bond, Wiltbank and Cook, 1958).

After puberty, reproductive function can cease when energy supplies are limited either due to malnutrition or chronic illness.

Little work has been carried out to investigate the role of nutrition in reproductive activity in the mare as breeding tends to occur when food supplies are greatest. However, Oxender et al (1977), although stating that nutrition and ambient temperature have not been shown to be of major significance in the onset of oestrus in mares, comment that oestrous activity can be delayed when nutrition is restricted and body weight loss significant. More recent work (Henneke, 1982) states that in barren and maiden mares who are in poor condition at the beginning of the breeding season, there is a significant delay in the onset of oestrus when compared with mares in good condition.

In contrast to the mare, a great deal of work has been carried out in this area in the bovine where the relationship of feeding to pregnancy and lactation is of greater economic importance than in the equine. In this species, it has been shown that in the cycling heifer cessation of ovarian activity and ovulation can result from underfeeding (Bond et al, 1958). In mature cows (Wiltbank, Rowden, Ingalls, Gregory and Koch, 1962) and heifers (Dunn, Ingalls, Zimmerman and Wiltbank, 1969) low energy diets pre- and post-calving result in low pregnancy rates mainly due to the failure

of these animals to demonstrate positive oestrous behaviour.

Therefore, it would appear that, from the literature and from the results of the studies discussed in this thesis, available energy, nutrition and body condition could allow the continuation of reproductive function in the mare.

The effect of nutrition on the reproductive cycle of the mare is likely to be a complicated process, with energy being involved in more than one link in the chain of events. However, it is most likely that nutrition has its effect on reproductive function at the level of hypothalamic sensitivity, hypothalamic production of GnRH and/or pituitary production of FSH and LH.

From the studies undertaken in this work it would appear that excess available energy was the factor of most significance in the continued reproductive function in these mares. In addition, the persistent teasing with a stallion may also have played a part. Workers with sheep and goats (Schinkel, 1954; Knight, Peterson and Payne, 1978; Ott, Nelson and Hixon, 1980) have found that ewes and does can be stimulated to ovulate, at the onset of the breeding season, by the introduction of rams or billy goats. However, Schinkel (1954) suggested that this stimulation to reproductive activity is operative only during the transitional period and has no effect if rams are run with ewes all year round. One reference to the onset of oestrus in the mare in the presence of a stallion is that of Ginther (1974) who found that anoestrous mares penned with a vasectomised stallion did not enter the breeding season any earlier than control mares. However, stallion presence

in this study cannot be discounted as a contributory factor.

It can be hypothesised that the mare, with increasing domestication, and the assurance of a year-round food supply, is making the transition from seasonal breeder to non-seasonal breeder and, in doing so, the nutritional and energy balance situation would appear to over-ride the photoperiodic stimulus for anoestrus.

It is interesting to note that, although the majority of writers consider the mare to be a seasonal breeder and that increasing daylight length is necessary for the breeding season to commence, many report that with housing, feeding and careful handling, some mares will continue to cycle throughout the year (Day, 1939; Burkhardt, 1947; Quinlan, van Rensburg and Steyn, 1951; Nishikawa, 1959; Arthur and Allen, 1972; Ginther, 1979; Rossdale and Ricketts, 1982).

CHAPTER IV

GENERAL DISCUSSION

A great deal of research has recently been undertaken into the effect of daylight length on reproductive function in certain species. The hypothesis is that light achieves its effect via the pineal gland (Reiter, 1980; Reppert and Klein, 1980; Sharp et al, 1981). One of the products of the pineal gland, secreted in diurnal rhythm, with highest levels present in the bloodstream during the hours of darkness, is melatonin (Reiter, 1980; Reppert and Klein, 1980). Much of the research associated with the role played by light in reproduction has included investigation of circulating plasma melatonin concentrations.

In the mare it has been suggested that melatonin suppresses the output of gonadotrophins (Sharp et al, 1981) and therefore by decreasing the hours of darkness using artificial lighting, the duration of elevated plasma melatonin concentration is reduced, production of gonadotrophins begins and reproductive activity ensues. However, in direct contrast to this hypothesis there is the ewe in which the exact opposite appears to occur. In the ewe, the breeding season begins when daylight length is decreasing, i.e. as the duration of elevated plasma melatonin concentrations increases (Rollag, O'Callaghan and Niswender, 1978; Arendt et al, 1979; Bittman and Karsch, 1984).

The general rule of metabolism in nature is to be conservative in that the same chemical reactions are found in many different metabolic sequences. It would therefore seem to be against the general trend that a compound like melatonin should have directly opposing effects in two different species.

In the groups of mares investigated, the whole spectrum of light was included. Some of the mares were maintained in total darkness for periods equivalent to those experienced during the shortest day, some were kept in an environment which closely resembled the natural situation where moon and stars contribute some light during the hours of darkness and others received a level of light during the night which was little different to that experienced during the day.

Almost all the mares in all three groups continued to cycle, and many conceived, during the winter months irrespective of the light patterns received. Indeed, in the few mares in which anoestrus occurred, it tended to be later and of a shorter duration than expected.

In conjunction with these clinical happenings, the duration of elevated melatonin concentrations agreed in the main with the amount of darkness but even the very high levels of melatonin secreted in some mares did not apparently cause cessation of gonadotrophin release and reproductive activity. This would suggest that melatonin per se, in these mares, did not assume the role suggested for it in the literature.

An important finding with regard to the melatonin profiles demonstrated in this thesis is the unexpected day-time rises recorded. Such increases have been reported by other workers (Arendt et al, 1977; Almeida and Lincoln, 1984), but no real explanation for such rises has yet been established. Such findings make translation of the actual role of melatonin in studies associated

with reproductive performance difficult.

Melatonin is only one of the many substances produced by the pineal gland (Benson, 1977; Reiter, 1980). It may be that one of these other substances is more important in the regulation of breeding patterns in the mare or that the effect of melatonin may require associated variations in other CNS products. Prolactin, produced in the anterior pituitary, is reported to show a marked response to changes in daylight length in the sheep (Lincoln et al, 1982; Kennaway et al, 1983). Therefore it will be of interest to consider the patterns of prolactin secretion, obtained from samples collected at the same time as those in which plasma melatonin estimation were carried out, which are under investigation at this time. However, irrespective of these findings, one must accept that light would not appear to have played any major part in the reproductive performance of the groups of mares investigated.

Nutrition and ambient temperature may have been the reason for the continuation of reproductive function in these mares by making energy available for the continuation of the integrated processes required for cycling and conception.

The mares due to foal in November and December, 1984 will do so in the three different environments in which they were maintained when covered. It will be of interest to discover if they demonstrate, at this time, a foal-heat and a subsequent return to cyclical reproductive activity within the period acceptable during the recognised breeding season.

APPENDIX I

SUNNY - GROUP 2

Results of plasma melatonin estimation, from samples collected during a 24-hou bleed in June, 1982, using Method 1
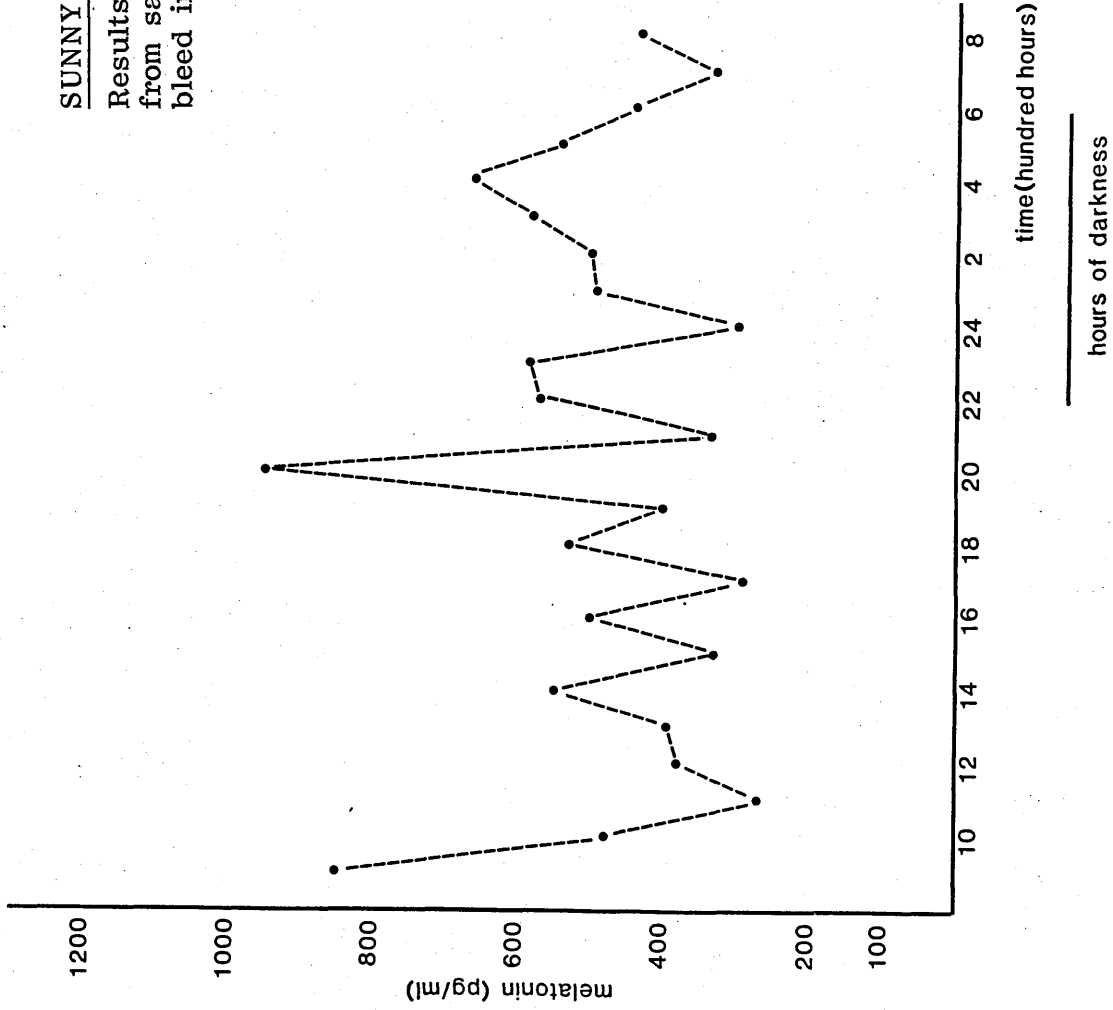
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