STUDIES OF NEUROENDOCRINE MECHANISMS INFLUENCING

SEASONAL VARIATIONS IN SEMEN PRODUCTION AND PLASMA

HORMONE LEVELS IN RAMS

A thesis presented in partial fulfilment

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GRAHAM KEITH BARRELL

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AND PLASMA HORMONE LEVELS IN RAMS

by Graham Keith Barrell

Many workers have shown that sheep are seasonal breeders with peak reproductive activity occurring during the autumn months. The initial experiment in this thesis was designed to define the seasonality of reproduction in rams of the N.Z. Romney breed as determined by repeated measurements of semen characteristics and of plasma hormone levels. These parameters were studied for 16 months in six N.Z. Romney rams on pasture, with five Merino and four Polled Dorset rams included for comparison.

Semen from all three breeds showed relatively regular seasonal changes in ejaculate volumes and seminal fructose levels with peak values being recorded during March. Likewise, monthly hormone levels varied in a regular manner with plasma LH, testosterone and prolactin concentration being elevated during the summer months. Many of the other semen parameters measured showed little tendency for seasonal variations, however a change in semen collection technique, from predominantly artificial vagina to entirely electro-ejaculation, may have masked some seasonal changes. All three breeds showed similar seasonal changes in the parameters studied although semen from the Polled Dorsets did not exhibit regular seasonal variations in fructose levels.

Further experiments were carried out to define the neuroendoc-
rine mechanisms which regulate the seasonal reproductive changes in
N.Z. Romney rams.   Three olfactory bulbectomized rams, three
cranial cervical ganglionectomized rams and four rams which had
undergone both of these surgical modifications, were grazed to-
gether with the rams mentioned above.   These surgical treatments
disrupted the regular seasonal changes in plasma levels of LH and
prolactin, but not, of testosterone.   Spermatozoal concentrations
in ejaculates from operated rams were higher than those from un-
operated controls, whereas mean fructose concentrations were lower;
however the pattern of seasonal changes in seminal fructose levels
was similar in all groups of rams.   Cranial cervical ganglionectomy
reduced hydroxyindole-O-methyl transferase activity and cell vol-
umes in the pineal glands, so it was concluded that disrupted
seasonal patterns of changes in plasma LH and prolactin levels,
plus the altered semen production in the surgically treated rams,
resulted from modified pineal gland and/or olfactory system activity.

A preliminary investigation into the role of changes in daily
photoperiod as the stimulus for seasonality of reproduction, was
carried out by placing rams in light-controlled rooms at the time
of the March equinox.   Over the following nine months rams exposed
to a phase-reversed annual lighting cycle showed earlier elevations
of seminal fructose and plasma testosterone levels than rams on
either the normal annual or a constant equinoctial lighting regime.
In all three groups plasma prolactin levels were directly related
to the length of daily photoperiod.

The findings of the above experiments were extended by a
final study in which both pinealectomized and sham-operated rams
were exposed to normal or reversed annual lighting cycles.

Effects of lighting on plasma testosterone and prolactin levels, and on seminal fructose levels, were diminished by pinealectomy. Autopsy data related to gonadal and accessory sex gland function showed significant operations x lighting regimes interactions, which supported the conclusion that in rams pineal gland function mediates endocrine and gonadal responses to changes in daily photoperiod.

Three short-term investigations of hormonal secretion profiles conducted during the latter experiment, showed that major fluctuations in the release of LH, testosterone, prolactin and cortisol occurred irregularly during the day. A nocturnal elevation of plasma prolactin levels was abolished by pinealectomy. These acute studies tended to confirm the findings of the latter experiment, but in particular they highlighted the pulsatile nature of hormonal secretion.

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Finally, I thank my wife Rosalind and daughters, Sarah and Rebecca, for their encouragement, support and perserverance whilst I was engaged in these studies.

In these studies, the candidate made the major contribution to the conception and execution of the experiments. The chief supervisor assisted with the experimental design and in the development of surgical techniques and hormone assays. Otherwise, apart from the execution of cortisol assays and histological processing performed by others, and the development of an ovine-LH radioimmunoassay where the candidate's contribution was minimal, all remaining work described in this thesis involved the full participation of the candidate.

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CHAPTER I

INTRODUCTION

Most animals are exposed to rhythmic environmental influences which probably are responsible for the induction of cyclic reproductive activity. Examples of cyclic activity include the oestrous cycles of many species, menstrual cycles in humans and higher primates, and the seasonal patterns of reproduction seen in many species. Although cyclic changes may result from intrinsic rhythmic mechanisms, it is likely that environmental factors regulate or modify these mechanisms. Such factors are the seasonal changes in temperature and daily photo-period, and the daily light-dark sequence. The latter produces the so-called circadian rhythms (Latin circa diem : about one day) which have been the subject of many investigations into the control of cyclic phenomena in living organisms (Bünning, 1967). Other rhythmic environmental influences may arise from the twenty-eight day lunar cycle, and for marine animals, from tidal rhythms.

In this thesis the rhythms studied were related to annual changes in reproduction.

1. SEASONALITY OF REPRODUCTION

(1) General

In any evolutionary scheme the ability to reproduce is of paramount importance. The most vulnerable stage in reproduction is the survival of the new-born, which is dependent on environmental influences such as the supply of nutrition. As such factors are governed largely by seasonal conditions, it has been stated that "the survival of a species therefore ultimately depends on the ability of the individual

to bring forth its young in due season" (Short, 1973a). In turn the ability to determine the date of parturition requires control over the time of conception and its associated reproductive activity. Such control manifests itself as a seasonality of reproduction in many species of wild and domesticated animals. A comprehensive review on the topic of seasonality of reproduction in birds and mammals has been provided by Amoroso and Marshall (1960). Additional information about mammalian reproductive patterns has been recorded by Asdell (1964).

(2) Manifestations of Reproductive Seasonality in Female Mammals

(a) General. Among the domestic animals sheep, goats and horses exhibit marked seasonal variations in parturition dates, whilst swine and cattle exhibit a lesser tendency to such variations (Dutt, 1960).

Information regarding the seasonality of oestrous activity and mating behaviour of domestic animals has been reviewed by a number of authors (Ortavant, Mauleon and Thibault, 1964; Thibault et al., 1966; Fraser, 1968).

(b) Sheep. Hafez reported on the breeding seasons of wild goats, wild sheep and domesticated sheep (1952), while more recently Van Tonder (1972) reviewed this topic with respect to domesticated sheep. These authors have concluded that most sheep breeds have a breeding season corresponding to the period of decreasing daylight hours. Although sheep generally show a seasonal pattern in the timing of parturition, this can vary according to the locality (Hulet et al., 1974).

A number of authors have reported on seasonal changes in fertility. Ortavant, Mauleon and Thibault (1964) reviewed literature showing that fertility of sheep, which is often estimated by the lowest proportion of barren ewes, or highest proportion of ewes leaving more

than one lamb, showed pronounced seasonal variation. Such studies indicated that ewe fertility was maximal midway through the breeding season.

Generally, the same conclusions are reached in classification of female animals as seasonal or non-seasonal breeders, regardless of whether such classification is based on observations of parturition dates, oestrous activity, fertility measurements or other parameters. In this manner the autumn breeding season of the ewes of most sheep breeds has been clearly defined.

(3) <u>Manifestations of Reproductive Seasonality in Male Mammals</u>

(a) <u>General</u>. Seasonal changes in reproductive activity of male mammals have not been as widely reported as for females. However, much information has been listed by Asdell (1964) and in the reviews by Ortavant, Mauleon and Thibault (1964), Thibault <u>et al</u>. (1966), and Lodge and Salisbury (1970).

Male mammals do not display any event as dramatic and characteristic as oestrus in the female, nor of course do they undergo the events of gestation and parturition. Nevertheless seasonality of reproduction can be recognised by observation of a wide number of parameters. Apart from rodents, the animals which have received most attention as seasonal breeders are deer, goats, cattle, horses and sheep, all of which are ungulates.

(b) <u>Antlers of Deer</u>. Stags of most species of deer display characteristic seasonal changes in the status of their antlers which have been shown to reflect testicular activity (Meschaks and Nordkvist, 1962; Markwald, Davis and Kainer, 1971; Mitchell, 1973). According to these authors, antlers become hard (shed velvet) during the period of maximal testicular activity. Short and Mann (1966) demonstrated that testosterone injections could induce shedding of the velvet in roe-bucks, and concluded that seasonal changes in circulating

androgen levels were involved in the natural antler cycle.

(c) <u>Gonads and Accessory Sex Glands.</u>   Other male mammals lack
such obvious indicators as the antlers of deer, thus information
regarding the seasonality of reproduction has been obtained by
examination of the gonads, the accessory sex organs, and the semen.
This research usually has involved weighing the various organs,
estimating levels of activity by histological or enzymatic analyses,
as well as semen studies.

There have been few studies on rams involving examination of
sex glands, although three separate reports have demonstrated seasonal
changes in testicular and accessory sex gland activity (Pelletier,
1971; Gupta, 1972; Lemay and Corrivault, 1973).   Pelletier described
a marked increase in testicular and seminal vesicular weights, and
seminal vesicular fructose content during June in Ile-de-France rams.
Gupta found that histological data indicated a similar pattern of
increased activity during the breeding season.   These results were
confirmed by the seasonal changes in testicular weights and seminiferous
tubule diameters reported by Lemay and Corrivault (1973).

Likewise there have been very few studies on male goats,
although Leidl, Hoffmann and Karg (1970) reported a seasonal pattern of
accessory sex gland activity and semen quality.   Wild goats in New
Zealand breed throughout the whole year according to Rudge (1969), but
show a slight increase in sexual activity in the early summer.

The reports of Skinner and his colleagues from South Africa have
usually contained data for numerous parameters including : weights of
testes, epididymides, seminal vesicles, ampullae, and bulbo-urethral
glands;   epididymal spermatozoal reserves;   seminiferous tubule
diameters;   seminal vesicular fructose concentrations;   testicular
histochemical changes (lipid content and $\Delta 5$-3β -hydroxysteroid
dehydrogenase activity);   as well as observations of the rut and mating

activity. These authors have produced a considerable amount of information on several of the African ungulates which breed in the autumn (Skinner and van Zyl, 1970; Skinner, 1971; Skinner, van Zyl and van Heerden, 1973; Skinner, van Zyl and Oates, 1974).

Other authors have measured some of the same parameters in deer, many species of which have been shown to be autumn breeders on the basis of studies with male animals (Wislocki, 1949; Meschaks and Nordkvist, 1962; Short and Mann, 1966; Aughey, 1969; Bramley, 1970; Chapman, 1970; Chapman and Chapman, 1970; Lincoln, 1971; Chaplin and White, 1972).

(d) Male Libido. The very distinctive behaviour patterns of many male mammals during the breeding season is well exemplified by the rutting and mating behaviour of ungulates described by Fraser (1968).

It has been a popular belief, that male mammals exhibit little seasonal variation in libido. However this opinion has not always been borne out by the results of controlled investigations of this aspect of male reproduction. Although Mies Filho and Ramos (1956) were unable to detect significant seasonal differences for rams of the Romney Marsh, Australian Merino, Corriedale and South Down breeds, McKenzie and Berliner (1937), Pepelko and Clegg (1965), Symington (1961), and Holmberg and Karlsson (1965) have reported distinct seasonal variations in libido in rams of several breeds, usually with peak activity occurring in the autumn. Lees (1965) on the other hand reported that Hampshire and Suffolk rams displayed a distinct breeding season in the autumn, whereas Kerry rams showed mounting activity all year round.

Fraser (1968) summarised the situation, rather generally, with the statements : "in seasonally breeding species the breeding drive is intensive in the breeding season and subdued or reduced, or absent during the remainder of the seasons" and, "when the breeding season is a very limited one the intensification of mating drive is more evident in the male animals. This provides the phenomenon of rutting."

(e) <u>Semen Production.</u> Much information on the seasonality of reproduction in male animals has been contained in reports on the seasonality of semen production. Semen can be obtained with relative ease from most species, and unlike the discontinuous events of oestrus and pregnancy in females, provides a number of continuously occurring variables from which quantitative and qualitative estimates of the degree of seasonal changes can be made.

(i) <u>Bull and Stallion.</u> In spite of the large quantity of data available, evidence that bull semen shows a seasonal improvement in quality during the spring is still inconclusive (Ortavant, Mauleon and Thibault, 1964; Lodge and Salisbury, 1970). On the other hand the same reviewers and, more recently Pickett, Faulkner and Sutherland (1970) reported a seasonal improvement in the quality of stallion semen in the spring months.

(ii) <u>Goat.</u> Studies with goats have yielded consistent evidence for seasonal changes in semen production with the highest quality samples being obtained in autumn. Eaton and Simmonds (1952) reported an autumnal peak for semen volume, spermatozoal numbers, and spermatozoal motility, whilst other caprine studies have shown that peak levels of seminal fructose and citrate occur during this period of the year (Leidl and Bronsch, 1959; Masaki and Masuda, 1968).

(iii) <u>Ram.</u> A large volume of research on the seasonality of production of semen by rams has been reviewed on several occasions (Ortavant, Mauleon and Thibault, 1964; Smyth and Gordon, 1967; Lodge and Salisbury, 1970) with the general finding that semen quality was highest in autumn and winter. Rams of breeds in which the ewes displayed long or continuous breeding seasons tended to have less pronounced seasonal fluctuations in semen quality than did rams of the more seasonal breeds.

A number of reports have appeared since the most recent of the

above reviews.   Land (1970) found no marked seasonal variations in semen of Finnish Landrace and Scottish Blackface rams, except for changes in spermatozoal motility, spermatozoal numbers and per cent live spermatozoa, which showed peak values during the autumn. Further evidence for autumnal peaks of semen quality, but with considerable breed variations, have been reported by Hogue, Hashem and Rahim (1970), William et al. (1970) and Sahni and Roy (1972a,b).   On the other hand, ejaculates collected from Ile-de-France rams during the spring and autumn did not differ significantly in fertility as evaluated by the percentage of ewe lambs pregnant after artificial insemination with fresh semen (Colas et al., 1972).

(4)   Hormonal Changes

An increasing volume of information has been obtained over recent years on changes in peripheral plasma levels of gonadotrophins and sex steroids, as well as pituitary gonadotrophin and hypothalamic GnRH levels, in both male and female animals.

(a)   Pituitary and Hypothalamic Hormone Levels.   Although a number of investigations on pituitary gonadotrophin content have been carried out in sheep, most of these have been confined to ewes.   Also, many of these studies only have considered differences between the oestrous and anoestrous periods, rather than between the breeding and non-breeding seasons.   Results from such experiments have been conflicting. Kammlade et al. (1952) reported a high pituitary gonadotrophin content during the non-breeding season in ewes, but Lamond, Radford and Wallace (1959) were unable to duplicate this result.   Nevertheless, work with rams has demonstrated that pituitary LH levels in Ile-de-France rams showed clear evidence for seasonal changes, being highest in summer and lowest in winter (Thibault et al., 1964).   FSH levels in the same study showed a similar pattern but with an extra peak in the winter months.

Relevant results from another species were provided by Brüggemann, Adam and Karg (1965) who reported that the highest pituitary levels of LH in stags occurred ten weeks before the rutting season.

The only work on hypothalamic GnRH levels in grazing sheep was based on an oestrous cycle study in ewes (Jackson et al., 1971) which indicated that hypothalamic GnRH levels were as high or higher during anoestrum than at any time during the oestrous cycle. This finding indicated that anoestrum in the ewe was not related to an absence of GnRH in the hypothalamus, but to the absence of a signal for its release.

(b) Plasma Hormone Levels. It is difficult to interpret changes in pituitary levels of gonadotrophins, as it is impossible to distinguish between the changes in rate of synthesis or release of a hormone from glandular content alone. On the other hand measurement of blood levels of a hormone may be considered to provide information regarding the rate of release from an endocrine gland, providing that clearance rates do not alter greatly. Nevertheless, in spite of the widespread use of radioimmunoassays, the literature on seasonal changes in blood levels of hormones is still deficient.

(i) LH and FSH. Short (1973b) suggested that the increased output of LH in rams during the autumn took the form of an increased frequency of discharge rather than any change in the amplitude of peaks. This statement was supported by twenty-four hour secretion pattern data obtained from Suffolk rams during the summer (one ram) and autumn (two rams) (Katongole, Naftolin and Short, 1974). The maximum LH levels of serum taken at bi-monthly intervals from Suffolk and Hampshire rams in Oklahoma were obtained during October (Johnson, Desjardins and Ewing, 1973). In Canada Sanford, Palmer and Howland (1974a) were unable to detect seasonal changes in serum LH levels in six cross-bred rams sampled

at two to three week intervals, yet the same group of workers
(Sanford et al., 1974b) reported seasonal differences in two Finnish
Landrace rams by measuring twenty-four hour secretion patterns.
Their results showed that in January LH peaks were of greater
frequency but smaller magnitude than in May or August. Weekly LH
levels in plasma collected from one ram lamb, starting during June at
three months of age and continuing for fourteen months, showed marked
peaks during June, July and August (Pelletier, 1971). Also, more
recently, Hochereau-de Reviers, Loir and Pelletier (1976) reported
peak plasma LH levels in adult rams during June.

There have been no reports on seasonal variations in blood
levels of FSH in the higher mammals.

(ii) Androgens. Reports of seasonal changes in androgen
levels in males have been more numerous than for gonadotrophins.

Elevated plasma androgen levels have been found during the
breeding season of a number of different species (Saumande and Rouger,
1972; Whitehead and McEwan, 1973; Berndtson, Pickett and Nett, 1974;
McMillin et al., 1974; Plant et al., 1974; Giménez et al., 1975;
Mirarchi et al., 1975).

In rams seasonal changes in plasma testosterone levels have been
reported for the Suffolk breed (Katongole et al., 1974), Suffolk and
Hampshire breeds (Johnson, Desjardins and Ewing, 1973), Finnish Landrace
and cross-bred rams (Sanford, Palmer and Howland, 1974a; Sanford et al.,
1974b) and Southdown, Shropshire and Targhee rams (Gomes and Joyce,
1976). In each case the plasma testosterone levels were higher during
or shortly before the breeding season, than in the rest of the year.
In Britain plasma testosterone levels obtained over long sampling periods
from four Hampshire rams, were much lower in late March than in January
(Purvis, Illius and Haynes, 1974).

(5) <u>Male Sexual Cycles</u>

The various changes described above relate to annual seasonal rhythms in males. Kihlström (1966) has postulated that in rats, rabbits, bulls and men there is a male sexual cycle similar to that of the female of the species. He based this postulate on data obtained from daily ejaculates from rabbits, and from a number of reports in the literature. However, the existence of male sexual cyclicity of this nature has not been widely reported.

(6) <u>Male-female Reproductive Seasonality Interrelationships</u>

As male mammals require two or three months to pass from a state of testicular inactivity to full fertility, whereas in females an inactive ovary can be stimulated to the point of ovulation within two or three days (Short, 1973<u>a</u>), it would seem reasonable to postulate that there may be sex differences in seasonality of reproduction. This idea was supported by Grubb and Jewell (1973) who noted that rutting activity amongst the Soay rams on the island of Hirta started well before the appearance of oestrus amongst the ewes. Short (1973<u>a</u>) reviewed evidence from deer which indicated that spermatogenesis in males recommences about four months before the females start to ovulate.

Nevertheless it is difficult to attribute seasonal changes in fertility to the male or to the female in any particular species. In cattle attempts to overcome this difficulty by the use of liquid and frozen semen and artificial insemination have not been successful, according to Lodge and Salisbury (1970). In their review they showed that some reports attributed seasonal changes in fertility to variations in semen quality, whereas other reports stated that the changes were primarily attributable to the female.

For sheep it has been stated that "the breeding season is controlled more by the female than the male since females normally have an anoestrus period, whereas the male produces semen throughout the year"

(Lodge and Salisbury, 1970). This statement may seem rather sweeping in light of the evidence for seasonality of reproduction in rams, but it does indicate a possible sex difference in the magnitude of seasonality within one species.

(7) Summary

The seasonality of reproduction in animals has been summarised by Ortavant, Mauleon and Thibault (1964), who divided domestic species into three categories :

(a) those that reproduce during the seasons of long daylight : horses, donkeys;

(b) those that reproduce during the seasons of short daylight : sheep, goats;

(c) those, finally, whose long domestication has caused their sensitivity to photoperiodic stimulation to degenerate : cattle, buffaloes, pigs, rabbits.

2. FACTORS INVOLVED IN THE SEASONALITY OF REPRODUCTION

The various environmental factors which probably are involved in inducing seasonality of reproduction include : light, temperature, nutrition and olfactory stimuli. These will be discussed in the present section along with other factors which may be involved in reproductive seasonality.

(1) Light

(a) General. Daylight produces two major cyclical influences : the regular sequence of light and dark every twenty-four hours, and the annual seasonal change in daily photoperiod. This latter has been described by Menaker (1971) as being absolutely regular and thus a much more reliable indicator of season than other quasi-annual environmental cycles.

The major influence of daylight on reproduction is produced by

the annual seasonal change in daylight. Variation in daily photo-period is minimal at the equator (two minutes throughout the year), and maximal at the poles where periods of constant daylight and dark-ness are experienced. Between these extremes seasonal variations in photoperiod are determined by the distance from the equator (latitude). Accordingly the finding that seasonal breeding is less apparent near the equator is not surprising (Fairall, 1970; Spinage, 1973). However the sun does move across the equator twice each year and causes two peaks of solar radiation. This has been suggested as an explanation for the two peaks of breeding activity noted in both sexes of the dikdik by Kellas (1955), who carried out observations in equatorial Africa.

Change in length of daylight is not the only factor to be considered because it follows that there must be concurrent, but inversely related changes in the length of darkness, and changes in the light : dark ratio throughout the year.

Daylight consists of solar radiation, the biologically significant regions of which are the higher wavelength ultraviolet and visible light. Ultraviolet light has been proposed as a controlling factor in animal reproduction by Amoroso and Marshall (1960), however their evidence was based only on a few experiments using ferrets and marmosets.

There have been a number of attempts to define the portions of the visible spectrum which are most effective in producing reproductive responses, particularly in birds. In reviews on this topic (Amoroso and Marshall, 1960; Farner, 1961; Bünning, 1967; Wurtman, 1967) it has generally been agreed that the optimal wavelengths for responses in birds were in the red region (580 nm to 750 nm).

Little work has been done in this area with mammals although Amoroso and Marshall (1960) cited reports in which red and yellow

radiation had stimulated sexual activity in field mice and in Romney Marsh ewes. In his review on the endocrine significance of light spectra, Wurtman (1967) concluded that "there has been little supporting evidence presented to show that the endocrine responses of mammals to visible light are related to particular wave lengths within its spectrum."

Usually it has been considered that the effects of light on animals are mediated via the retinal photoreceptors, and that full vision requires the integrity of the retinae, optic nerves and tracts, superior colliculi, lateral geniculate bodies, and the occipital cortex (Wurtman, 1967). Research on the neural connections between the retinae and the regions of the brain involved in endocrine regulation has been summarized by Critchlow (1963) and Wurtman (1967). From the optic chiasm impulses may travel in the optic tracts or the accessory optic system. The latter includes the anterior and posterior accessory tracts as well as direct retino-hypothalamic connections (Printz and Hall, 1974). Evidence for direct retino-hypothalamic connections has yet to be substantiated.

An attempt to determine the role of the eyes in the photo-periodicity of breeding in ewes was reported by Clegg, Cole and Ganong (1964). Using Suffolk-Hampshire cross-bred ewes, they performed optic nerve sections, or destroyed the posterior portion of the optic chiasm together with the suprachiasmatic region. The former operation had no effect on seasonal oestrous activity whereas the latter tended to reduce the incidence of oestrus after a year or more. The lack of response to optic nerve sectioning indicated that the sheep either reverted to an inherent biological rhythm, used environmental cues other than lighting, or had light receptors other than those of the eyes.

There is a considerable body of evidence for light perception

involving receptors other than those of the retinae. For example, amphibians and some reptiles have photoreceptor cells in their subcutaneously located pineal glands (Collin, 1971; Oksche, 1971). The role of the pineal in reproductive regulation is elaborated later. Extraretinal receptors in the house sparrow have been described by Menaker (1974) : light entrainment of locomotor activity in these birds persisted when they were blinded and the feathers plucked from the top of the head, but was lost when India ink was injected under the head skin. Also Reiter (1973a) has suggested that in rats responses to constant light involved non-retinal photoreceptors, or possibly even a humoral agent.

Some attention has focussed on the possibility that light may reach neural structures in the brain directly. Benoit and Assenmacher (1959) produced gonadal stimulation in blinded ducks by directing light into the pituitary, hypothalamus, or rhinencephalon. Using glass rods, Lisk and Kannwischer (1964) directed light on to the suprachiasmatic region of blinded rats and produced a constant oestrus-like vaginal response. This result may have been due to the local application of heat, especially in view of the lack of photopigments or morphological photoreceptor units in this region, and the fact that exposure to intense light had no effect on blinded rats (Wurtman, 1967). Penetration of light into the brain has been established by Ganong et al. (1963), who recorded responses from a light-sensitive cell placed into the temporal lobe and hypothalamus of sheep, dogs, rabbits and rats. Responses to external light were abolished when aluminium foil was placed over the skull. Visible light, particularly red, can penetrate the skin readily, and even ultraviolet light can reach superficial capillaries where unknown effects of solar radiation may occur (Daniels, 1974).

For changes in daily photoperiod to regulate the seasonality of

reproduction, mere perception of light may not provide sufficient information. Menaker (1971) summarized this topic : "In most of the photoperiodic systems which have been investigated in any detail the organism appears to measure daylength not by measuring the length of the light period, nor by measuring the length of the dark period nor again by measuring the ratio of the light period to the dark period. Rather, organisms distinguish between inductive and non-inductive day-lengths by assessing whether or not light is present at a particular phase point on an endogenous circadian rhythm of sensitivity to its inductive effects." This mechanism was first postulated by Erwin Bünning and recently has received support (Farner and Lewis, 1973; Follett, 1973; Gwinner, 1973; Lofts and Lam, 1973) as an explanation for the control of annual rhythms of reproductive activity in birds. This mechanism depends on the existence of a circadian rhythm on which the annual seasonal change acts as an entraining agent - often termed a Zeitgeber.

Possible mechanisms for photic control of annual endocrine rhythms in mammals were listed by Wurtman (1967) and included : firstly, a response to increased light (or dark) period; secondly, a response to any light period exceeding a critical length; and finally a response following a set number of consecutive light-dark sequences. Although Amoroso and Marshall (1960), Farner (1961) and Wurtman (1967) reviewed literature showing that the reproductive system of the ferret responded when it had received sufficient light exposure, they pointed out that the intervening periods of darkness were equally important. This suggested that a mechanism similar to the final proposal of Wurtman (1967, above) may be present in ferrets.

A number of other questions relating to the mechanism of action of light remain unanswered. These have been outlined by Bowman (1973) and include the question of whether photoperiodic changes are

stimulatory, inhibitory, or both. Also, there has been no explanation for the fact that after prolonged exposure to continuous illumination or continuous darkness, some mammals become refractory to treatment and re-establish their normal cyclical activity.

A major criticism of the view that light has direct influences on endocrine functions has been that light also influences the muscular activity, wakefulness, and feeding habits of most animals, so that any effect on the reproductive system may be the result of such activity, and only arise indirectly from light per se. Evidence against this criticism has been reviewed by Amoroso and Marshall (1960) and Wurtman (1967) who cited experiments in which these other factors could not account for the effects of light. Nevertheless, indirect influences must be borne in mind when experiments involving lighting effects are being evaluated.

(b) Photoperiodicity of Reproduction in Mammals. Literature on the effects of light on reproduction is now extensive and has been reviewed for animals generally (Hammond, 1954; Farner, 1961; Critchlow, 1963; Wurtman, 1967; Luce, 1971) and for domestic animals (Yeates, 1954; Dutt, 1960; Ortavant, Mauleon and Thibault, 1964; Thibault et al., 1966). Research on this topic has involved studies of the reproductive effects of both natural and artificial lighting.

(i) Effects of Natural Lighting. The fact that seasonally breeding animals which originated in the Northern Hemisphere breed during the same seasons in the Southern Hemisphere, is good evidence that such animals respond to photoperiodic influences (Marshall, 1937).

As the amplitude of seasonal photoperiodic changes is greater at higher latitudes, a greater degree of seasonality of breeding may be expected from animals living in such regions. This deduction was made by Hafez (1952) in an extensive review of the topic; he concluded that wild sheep, goats and thar have a restricted breeding season, the length

of which is dependent on the latitude of the place of origin of the species or breed. In domestic sheep the breeding season gets gradually shorter nearer the poles, even for a particular breed. The occurrence of polyoestrous ewes increases with domestication and in equatorial regions. Similar relationships between the timing and length of breeding seasons, and latitude, have been described for both sexes of fox (Lloyd and Englund, 1973), and the rock hyrax (Millar and Glover, 1973). On the other hand, Fletcher (1974) reported that red deer have the same breeding season in the Northern Hemisphere regardless of the latitude and that there was no increase in the length of the breeding season nearer the equator.

Williams (1975) recently published data from British breeds of sheep maintained at Pasto, Columbia, South America (lat. 1° N), which has a temperate climate because of its elevation, but nevertheless an equatorial photoperiod. Over a two year period the dates of onset of mating activity were gradually but significantly advanced in ewes of Welsh Mountain, Scottish Blackface, and North Country Cheviot breeds, but not in Romney Marsh, Dorset Down or Border Leicester ewes. It must be noted that the latter three breeds were not kept at quite the same location as the former. Williams suggested that the results were due to the lack of fluctuation in the photoperiod.

Onset of oestrus in Rambouillet ewes was approximately two months earlier in Texas than in Idaho (Hulet et al., 1974), however the two locations did have different altitudes and annual temperature changes. Wodzicka-Tomaszewska, Hutchinson and Bennett (1967) indicated that reversal of thermal seasons had no effect on breeding in ewes maintained on an equatorial lighting regime, thus temperature differences probably did not account for the results of Hulet et al.

Because such experiments do not allow evaluation of the reproductive effects of photoperiodic changes in isolation from changes

in other factors, precise information can be derived only from experiments using controlled conditions and artificial lighting.

(ii) <u>Effects of Artificial Lighting.</u> Research on the reproductive responses of birds to artificial lighting regimes has been reviewed by Follet (1973), while earlier, Critchlow (1963) summarized similar work on rodents. In general, light stimulated the gonads of these animals as long as it was periodic.

Work in which domestic animals were subjected to artificial lighting has been reviewed by Ortavant, Mauleon and Thibault (1964). They found that provision of additional periods of lighting stimulated the reproductive systems of horses, pigs and cattle. Kordts and Gravert (1972) have since found that eighteen hours of light per day caused a reduction in conception rate in cows, which suggested that there is a limit to the amount of supplementary illumination which will stimulate the reproductive system in this species.

In the case of autumn breeding animals, decreasing photoperiods brought forward the oestrous period in goats (Ortavant, Mauleon and Thibault, 1964). Reversed lighting regimes reversed the antler cycle in deer, whereas a constant twelve hours per day lighting regime abolished the antler cycle (Goss, 1969<u>a</u>, <u>b</u>). Considerably more data has been obtained from experiments on sheep.

<u>Ewes.</u> Ovine research up to 1954 was reviewed by Yeates (1954) and Hammond (1954). More recent reviews have been provided by Ortavant, Mauleon and Thibault (1964) and Clegg and Ganong (1969). General findings of these reviewers have been that decreasing daily lighting regimes, or daily photoperiods less than twelve hours, stimulated the onset of oestrous behaviour. These conclusions have been confirmed by Newton and Betts (1972), Ducker, Bowman and Temple (1973), Ducker and Boyd (1974), and Horak (1974).

By compressing the normal annual photoperiodic rhythm into six months, Mauleon and Rougeot (1962) induced two breeding seasons per year in ewes of various French breeds. Peaks of breeding activity corresponded to the longest daily photoperiods, which contrasted with the normal autumn breeding season of these ewes. However, Williams and Jackson (1971) found that supplementary lighting during the summer also advanced the date of first oestrus in ewes. On the other hand, Williams (1974) and Williams and Thwaites (1974) have since reported that daily photoperiods above twelve hours per day tended to suppress oestrous activity.

Thwaites (1965) reversed the breeding pattern of Southdown ewes by using a reversed lighting regime, while the pattern was abolished in an even (equatorial) regime. A marked decline in ovulation rate occurred when Rambouillet, Targhee and Columbia ewes were exposed continuously to a lighting regime of two hours light and two hours dark (Hulet, Price and Foote, 1968); the effect of continuous darkness being intermediate between this treatment and normal lighting.

To date, the only endocrinological study of ewes under artificial lighting was reported by Palmer, Howland and Ibrahim (1972). They did not find any significant differences in serum LH levels between Western Whiteface ewes on rapidly reducing daily photoperiods or those on gradually decreasing photoperiods; neither were there any differences in the dates of onset of breeding activity.

Knowledge of the photoperiodic responsiveness of ewes has been utilised as a means of producing lambings every seven or eight months after exposing ewes to regimes of decreasing artificial light (Williams, 1970; Ducker and Bowman, 1972; Ducker, 1974). Likewise Newton and Betts (1972) have reduced the interval from parturition to the next conception.

Semen Production in Rams. Results of investigations into

artificial photoperiodic influences on semen production in rams have been reviewed by Yeates (1949), Ortavant, Mauleon and Thibault (1964), and Lodge and Salisbury (1970). In most cases, decreasing the photoperiod had favourable influences on both androgen-production related, and spermatogenic, characteristics of semen, whereas increased illumination had deleterious effects on semen production.

Increased libido also has been reported in rams subjected to decreasing lighting regimes (Moule, 1950, Brückner, 1972; S.J. Miller, pers. comm.).

By using lighting regimes in which the annual photoperiodic cycle was compressed into six months, Jackson and Williams (1973) obtained cyclic changes in semen production from Suffolk rams which corresponded to the lighting cycles. The normal annual fluctuations persisted, superimposed on the six-monthly cycles, although they diminished during the experiment.

French workers have reported the effects of different lighting regimes on spermatogenesis. The Feulgen-DNA content of germ cells from rams submitted to long daily photoperiods was lower than that from rams submitted to short photoperiods (Esnault, Fautrez and Ortavant, 1964). The decrease in Feulgen-DNA corresponded with an increased number of degenerating spermatozoa.

Ortavant and his colleagues (Ortavant, 1956, 1959, 1961; Ortavant and Thibault, 1956; Ortavant, Mauleon and Thibault, 1964) have used lighting regimes which compress the annual cycle into six months. Such "shortened" photoperiodic cycles were chosen so that artificial lighting experiments could incorporate more than one cycle per year of study. These authors have shown that there is a delay of about forty-eight days between the initiation of spermatogenesis and the liberation of new spermatozoa from the seminiferous tubules. This result must be taken into account when conclusions about

spermatogenic activity are to be made on the basis of semen examinations or estimates of spermatozoal stores, as the spermatogenic status of rams may reflect events which occurred some time previously. By quantitative studies of the spermatogenic epithelium these authors have been able to determine that the optimum daily photoperiod for spermatogenesis in Ile-de-France rams was between ten and twelve hours, provided that the rams had been exposed to longer daily photoperiods beforehand. An important finding of these workers has been that the maximum rate of spermatogenesis can not be sustained for any length of time. The reason for this has eluded them.

Sex Hormone Levels in Rams. Soay rams displayed marked elevations in plasma LH levels followed by similar elevations in plasma testosterone levels when their daily lighting schedule was reduced from eighteen hours to eight hours (Lincoln, 1976a). Pelletier and colleagues have described marked effects of different lighting regimes on pituitary levels of FSH and LH, as well as on plasma levels of prolactin and LH in rams of French breeds. The pituitary content of FSH and LH was greater in rams exposed to eight hours of light per day rather than to sixteen hours per day; also forty-eight hours of continuous light caused a marked reduction in pituitary levels of the gonadotrophins (Pelletier and Ortavant, 1964). The authors suggested that light stimulated the release of hormones from the pituitary but inhibited synthesis, which was favoured by darkness.

When the normal annual photoperiodic cycle was compressed to six months, plasma LH levels decreased with increasing photoperiod, but then showed a short-lived, marked elevation when the photoperiod began to decrease; thereafter plasma LH levels declined until the photoperiod reached minimum levels. The same pattern of response was seen, but with higher hormone levels, in castrated rams (Pelletier,

1971, 1972; Pelletier and Ortavant, 1975a). A change in daily photoperiod from eighteen hours to eight hours induced a rise in plasma FSH and LH levels in Soay rams (Lincoln, 1976b), this being the first report of lighting effects on FSH secretion in rams. In contrast to FSH and LH, plasma prolactin levels exhibited a positive relationship with the length of daylight (Pelletier, 1973).

Pelletier and Ortavant (1975b) found that intramuscular injections of testosterone propionate were more effective at inhibiting LH release in rams on sixteen hours light per day than in rams on eight hours light per day. They postulated that, in rams a decreasing light photoperiod acts in two ways :

(1) by stimulating the activity of the hypothalamo-hypophysial system,

(2) by decreasing the intensity of the negative feedback effect of steroids.

(2) Temperature

The effects of temperature on mammalian reproduction have been reviewed by Dutt (1960) and VanDemark and Free (1970).

Most interest in the direct effects of temperature on reproduction has been related to its deleterious effects rather than to any regulatory or controlling capacity it may have on animal reproduction. Plant growth, however, is dependent on the annual cycle of temperature changes, especially in the temperate regions. Temperature effects on plant growth may influence animal reproduction through effects on nutrition (see next section).

When ambient temperature rises above about $31^{o}C$, body temperatures of animals will also rise, and to prevent hyperthermia, metabolic activity becomes depressed. Under such conditions adverse effects on the reproductive tract may be observed in both males and females.

In males effects of hyperthermia on testicular function can be
limited by the heat-regulating ability of the scrotum, the mechanism
of which has been studied in the ram (Waites and Moule, 1961). The
scrotum provides a mechanism for isolating the testes from the higher
body temperature by relaxation of the dartos and cremaster muscles which
lower the testes away from the body and thin the scrotal skin. A
countercurrent heat-exchange system operates between the internal
spermatic artery and pampiniform plexus in the spermatic cord. Heat
is also lost from the scrotal skin by the evaporation of sweat. These
mechanisms usually ensure that the testes are held at a temperature
about $4^{o}C$ lower than the rectal temperature. This mechanism can not
operate in cryptorchidism, nor if the scrotum is insulated such as by
the presence of excess wool.

In rams the detrimentral effect of high ambient temperatures on
semen production has been termed "summer sterility". The general effect
on testicular function is impaired spermatogenesis and androgen
production (Gomes and Johnson, 1967; Waites and Ortavant, 1969).

These effects of elevated temperatures can also be produced by
heating the scrotum directly. In early studies on the direct effects
of heat on the scrota of various mammalian species, temperatures
between $44^{o}C$ and $50^{o}C$ were used (VanDemark and Free, 1970). Such
temperatures would have impaired testicular function as a consequence
of inhibiting enzyme systems. It has since been shown that
temperatures at or near body temperature are sufficient to impair
testicular function in rams (Glover, 1955; Fowler and Dun, 1966).

Dutt (1960) suggested that some effects of elevated temperatures
might have arisen indirectly from hypothyroidism such temperatures
would have produced. Treatment of such cases with thyroxine, however,
has produced equivocable results. Other indirect effects could have
arisen from depressed food intake. For example, Newsome (1973)
postulated that the deleterious effects of hot weather on

spermatogenesis in red kangaroos resulted from the combination of raised temperatures and reduced food intake, due to the fact that these animals remained prostrate on hot days instead of feeding. Also the availability of green fodder was reduced because of the dry conditions prevailing.

Although the classic effect of elevated non-lethal ambient temperatures is impaired reproductive function in the male, Dutt (1960) has reviewed evidence of effects on female farm animals, particularly the ewe. He reported that elevated temperatures caused delayed onset of oestrus, lowered fertilization rates, raised percentages of abnormal ova, and increased embryonic deaths.

Effects of cold temperatures on testicular function are much less severe than those of high temperatures and are only manifest near freezing point (VanDemark and Free, 1970). On the other hand Dutt (1960) claimed that the effects of cooler temperatures on ewes may provide a mechanism for controlling the onset of the breeding season.

During the summer months in Kentucky twenty Hampshire-cross Western ewes held in rooms at 45°F to 48°F came into oestrus about eight weeks earlier than control ewes which experienced an average maximum daily temperature of 88.7°F (Dutt and Bush, 1955). In the same experiment Southdown rams housed at the lower temperature produced semen with a significantly higher per cent motile spermatozoa and a lower per cent abnormal spermatozoa than rams at ambient temperatures. The authors concluded that the reduced temperature prevented summer sterility in the rams, but did not consider that a similar explanation applied to the earlier onset of oestrus in the ewes. They also overlooked the possibility that the presence of oestrous ewes in the cooled rooms may have influenced semen quality.

As there have been few experiments which confirm or refute the findings of Dutt and Bush, the role of annual temperature changes in

controlling the seasonality of reproduction in animals remains unclear.
However Clegg and Ganong (1969) stated that although temperature was an
environmental factor modifying reproductive activity, its role was of
secondary importance in birds and most mammals..

(3) Nutrition

Nutritional effects on reproduction in domestic animals have been
reviewed by Reid (1960) and Moustgaard (1969), in male animals by
Leathem (1970), and in sheep by Cloete (1972). The development and
function of the female reproductive system are impaired by underfeeding.
Conversely, increased ovulation rates can be obtained by providing
increased food supplies for a certain period prior to breeding.

In male animals, reduction in both testicular function and
sexual activity results from underfeeding. Parker and Thwaites (1972)
reported the effects of feeding diets at seventy-five per cent and fifty
per cent of maintenance to rams : libido, as assessed by reaction time,
number of mounts per ejaculate, frequency of failure to ejaculate, and
vigour of the ejaculatory thrust, was progressively and markedly depressed;
semen quality was also reduced.

According to the reviews by Moustgaard (1969) and Leathem (1970),
undernutrition causes reduced production and/or release of gonadotrophins
and/or hypothalamic hormones. As a consequence of the lack of
gonadotrophic stimulation, reduction in gonadal activity follows. This
theory was contested by Gombe and Hansel (1973) who reported that while
restricted energy intake in heifers produced ovarian hypofunction,
plasma LH levels were elevated. They suggested that undernutrition
prevented ovarian tissue from responding to gonadotrophic stimulation.

Although major changes in the supply of food can alter
reproductive function in grazing animals, there is no evidence that
nutrition is involved in the seasonality of reproduction in such animals.
On the other hand, some desert dwelling species are opportunistic rather

than seasonal breeders, possibly because their nutrition supply is
very dependent on rainfall in the desert regions.

(4) Olfaction

Although external factors such as light and nutrition provide
primary information about seasons, finer signals may be required to
synchronize reproductive behaviour in groups of animals, and between
the two sexes.   In ungulates such signals involve tactile, auditory,
visual and olfactory stimuli (Fraser, 1968).   Considerable interest
has centred on olfaction which has been presumed to provide a
significant environmental stimulus to behavioural and endocrine
responses concerned with reproduction (Whitten and Champlin, 1973).
The relationship between olfaction and reproduction has been studied
in depth in mice and three distinct reactions reported.   These are :

(a)   the Lee-Boot effect - inhibition of oestrus in females kept
      apart from males

(b)   the Whitten effect - stimulation of oestrus by the odour from a
      male;   and

(c)   the Bruce effect - blocking of pregnancy by the odour of a strange
      male (Mykytowycz, 1973).

The substances involved in such olfactory effects have been termed
pheromones (Whitten, 1966;   Bronson, 1971) and are present in urine
and secretions of the cutaneous glands of most animals.   The precise
chemical nature of the pheromones influencing reproduction in higher
animals has not been elucidated.   There is some evidence that steroids
or their metabolites may be involved,   however Jones and Nowell
(1974) claimed that the five day delay before administration of
androgens produced urine with aversive properties in mice indicated
that production of a new compound, rather than an androgen metabolite,
was responsible.

Much evidence relating to the role of olfactory stimuli in

reproduction has been obtained from studies with animals which have had their olfactory bulbs extirpated, or which have been rendered anosmic by chemical means. Removal of the olfactory bulbs produced ovarian atrophy and infertility in female mice (Whitten, 1956; Lamond, 1958) and terminated sexual behaviour in male golden hamsters (Lisk, Zeiss, and Ciaccio, 1972). French workers have shown that olfactory bulbectomy of Large White sows inhibited oestrous activity by interfering with pituitary discharge of FSH (Signoret, 1964; Mauleon and Signoret, 1964).

Attention has focussed on sheep since Schinckel (1954) reported that the presence of rams early in the breeding season advanced and synchronised the first oestrous cycle. The effects of olfactory bulbectomizing Ile-de-France ewes were investigated by Signoret (1964) and Mauleon and Signoret (1964), who reported that normal oestrous cycles continued. In contrast Morgan, Arnold, and Lindsay (1972) reported a significant drop in the number of Border Leicester or Merino ewes which mated after olfactory bulbectomy.

Olfactory stimuli may play an important role in the mating behaviour of rams, but deprivation of the olfactory sense does not necessarily preclude successful mating. Lindsay (1965) reported that olfactory bulbectomized Merino rams could only distinguish between non-oestrous and oestrous ewes by attempting to mount them, successfully mating those ewes which did not move away. Whilst their mating ability was similar to that of normal rams, mating behaviour was modified in that there was an increased display of nudging. Earlier Banks (1963) had reported a similar result from rams rendered temporarily anosmic with local anaesthetic spray, but Rouger (1973) found that this type of treatment reduced sexual activity of rams.

Perception of olfactory stimuli involves the olfactory organ system which has a number of distinct and separate components - namely,

the olfactory nerve system (olfactory system), the vomeronasal system, the trigeminal nerve system and the nervus terminalis system (Alberts, 1974). This author pointed out that removal of the olfactory bulbs interferes with all the above systems, as well as causing widespread damage to other regions of the brain. Thus in the experiments involving olfactory bulbectomy, it is not possible to determine which component(s) of the olfactory organ system were primarily responsible for the results obtained.

There has been a recent upsurge of interest in the possible role of the vomeronasal organ (Jacobson's organ) in the perception of pheromones. Estes (1972) reviewed this topic and suggested that the "flehmen" response of most ungulates when exposed to the odour of conspecific urine, represented a mechanism by which air could be drawn in through the incisive ducts over the vomeronasal organ. The characteristic lip curl associated with flehmen blocks the external nares to assist this air movement.

Recently Powers and Winans (1975) were able to deafferent the vomeronasal system without disturbing the olfactory system in the male hamster. Cutting the vomeronasal nerves produced severe sexual behaviour deficits in one-third of the treated animals. All sexual activity was eliminated when this treatment was combined with destruction of the olfactory epithelium by zinc sulphate, a result comparable to the effects of olfactory bulbectomy. There have been no reports of similar experiments in domestic animals.

On the basis of the literature available, it is possible to suggest that olfaction may be involved in the seasonality of reproduction in ewes, but there is little evidence to indicate a similar function of olfaction in rams.

(5) Other factors

It has been difficult to evaluate the relative importance of

olfactory stimuli as compared to auditory or visual stimuli, or
social contact, in the induction of reproductive activity during the
breeding season of any species. The role of these stimuli, plus
genital manipulation, in reproductive processes has been discussed
by Clegg and Doyle (1967). These authors concluded that although
the evidence for a role seemed irrefutable, these senses involved
were interrelated, but no one of them was absolutely essential.
The extent to which these stimuli are involved in the induction of
seasonal reproductive activity, as distinct from reproductive
behaviour within the breeding season, was not made clear by these
authors.

Fletcher and Lindsay (1968) showed that the sexual activity
of rams was affected slightly by loss of hearing, more by inability to
smell, and most by loss of vision, but partner seeking was affected
specifically only in those rams unable to smell.

On the evidence available it is assumed that the factors
mentioned in this section are less important than light, temperature,
nutrition and olfaction, in the control of seasonal breeding patterns.

3. NEUROENDOCRINOLOGY OF REPRODUCTION IN MALE MAMMALS

(1) General

The reproductive system of mammals is controlled by neural and
hormonal interactions between the hypothalamus, anterior pituitary,
and gonads. These interactions can be influenced by stimuli from
other neural structures, usually acting at the level of the hypothalamus,
as well as by other endocrine organs.

(2) Hypothalamus

The hypothalamus releases one or more releasing hormones from
nerve endings in the median eminence. It/they are transported by the

hypothalamo-hypophysial system to the anterior pituitary to cause the synthesis and release of gonadotrophins. Secretion of releasing hormone(s) from the hypothalamus, as well as of gonadotrophins from the anterior pituitary, can be inhibited by the negative feedback of the sex steroids, although there are circumstances in which a positive feedback can occur.

(3) Hypothalamic Regions Involved in Gonadotrophin Release

Experiments involving precisely placed electrochemical stimuli, and in vitro assays of frozen sections of hypothalamic tissue, have shown that gonadotrophin releasing activity was localized in a band of tissue extending from the preoptic area (POA), through the anterior hypothalamus to the medial basal hypothalamus (MBH), in the region of the arcuate nucleus and median eminence (McCann et al., 1973).

Conversely, inhibition of gonadotrophin release has resulted from the production of electrolytic lesions, or placement of implants of inhibitors of protein synthesis, into these hypothalamic areas (Motta, Piva, and Martini, 1973). The majority of these studies have been performed on laboratory rats but similar observations have been made on rabbits, guinea pigs, ferrets, dogs and monkeys (Barraclough, 1973).

(4) Extrahypothalamic Areas Involved in Gonadotrophin Release

The involvement of extrahypothalamic areas in pituitary gonadotrophin regulation usually has been studied by the technique of surgical deafferentation of regions of the hypothalamus using the knife developed by Halasz and colleagues (Halasz and Gorski, 1967; Davidson and Bloch, 1969; Gorski, 1971; Barraclough, 1973). In the female rat the MBH, which includes the ventromedial and arcuate nuclei, stimulates a tonic basal level of gonadotrophin release, even when completely deafferentated. However, massive release of gonadotrophins,

such as precedes ovulation, requires the integrity of afferent neurons from the POA. The POA thus provides the stimuli for the ovulatory release of LH, and is also the site at which other neural stimuli which modify gonadotrophin release, are integrated in both sexes.

Recent work has shown that the rhesus monkey differs from the rat in that the control systems which govern both tonic and surge secretion of gonadotrophins are resident in the MBH, and do not require any signal from the POA to initiate ovulation (Knobil, 1974).

(5) Mechanisms of GnRH Release

The release of gonadotrophin releasing hormone (GnRH) from the hypothalamus into the hypothalamo-hypophysial portal system has been claimed to involve catecholaminergic, cholinergic, or indoleaminergic neural mechanisms (Kamberi, 1972). Using intraventricular infusions or systemic injections, as well as in vitro studies, this author was able to demonstrate that the catecholamines (particularly dopamine) stimulated the release of gonadotrophins in the rat. Further, blockage of the proestrous surge of LH and FSH by atropine indicated a stimulatory role for the cholinergic system, whereas the indoleamines, serotonin and melatonin, were inhibitory.

Serotonin levels in the median eminence of the ewe were shown to be low when LH was being released from the pituitary, supporting the hypothesis that serotonin is inhibitory to GnRH release (Wheaton et al., 1972). It has been suggested that compounds, such as the biogenic amines, may be secreted into the cerebrospinal fluid by neural structures distant from the hypothalamus, to reach the median eminence by way of the third ventricle (Anand Kumar, 1973). Consequently interest has concentrated on the ependymal cells lining the ventricle in the region of the median eminence (Gorski, 1971). These cells may be specialized for absorption of substances such as

the biogenic amines from the cerebrospinal fluid, and thereby provide a means for stimulating (or inhibiting) the GnRH release mechanism.

(6) <u>Negative Feedback of Steroids</u>

Research by several workers, summarized by Barraclough (1973), has shown that large doses of steroids usually are inhibitory to gonadotrophin release, whereas small doses may be stimulatory. Also, progesterone never shows a positive feedback action on gonadotrophin secretion in the absence of oestrogen.

Whilst it has generally been accepted that androgens produce a negative feedback on gonadotrophin secretion (Davidson and Bloch, 1969), there have been some reports indicating a positive feedback action of low doses of androgen. In pubertal rats doses of 6 μg of testosterone propionate had stimulatory effects on LH release (Bloch, Kragt and Masken, 1971), and higher doses increased FSH levels in female rats (Johnson and Naqvi, 1969). More recently Osland (1972) reported increased LH levels in wethers following testosterone treatment with low doses, as well as the characteristic decrease in LH levels following high doses of testosterone.

(7) <u>Sites of Steroid Feedback</u>

Recent literature describing the effects of sex steroids on pituitary responsiveness to GnRH has led to conflicting conclusions about the relative importance of the hypothalamus and anterior pituitary as sites of steroid feedback. For example Cumming <u>et al</u>., (1972) concluded that because progesterone had no effect on the LH response to GnRH in the ewe, this steroid must act at the hypothalamus rather than the pituitary. This finding was supported by Reeves, Tarnavsky, and Chakraborty (1974), Rippel <u>et al</u>. (1974), and Symons, Cunningham, and Saba (1974). However, testosterone propionate pretreatment of rams (Hopkinson, Pant, and Fitzpatrick, 1974) and

wethers (Pelletier, 1974) caused a reduced LH response to GnRH, which the authors considered indicated an effect on the pituitary. Later Galloway and Pelletier (1975) provided evidence that testosterone pretreatment acted on the hypothalamus to depress basal LH secretion rates in the ram, but also acted at the pituitary to delay and reduce the response to GnRH.

The existence of a testicular factor called "inhibin" (McCullagh, 1932), which inhibits the release of FSH, has been postulated by several authors (see review by Setchell and Main, 1974), but has not been isolated or characterised.

(8) Hypothalamic Sites of Steroid Feedback

Davidson and Bloch (1969) proposed that the negative feedback responses to testosterone are mediated by the hypothalamus. Likewise, in the female, oestrogens and progesterone must interact with sites in the hypothalamus and anterior pituitary to cause both stimulation and inhibition of gonadotrophin release (McCann et al., 1971). Information on the sites in the hypothalamus with which steroids interact has been obtained by autoradiographic mapping studies using tritiated steroids (Pfaff, 1971; Stumpf and Sar, 1973), by recording electrical activity during steroid infusions (Faure and Vincent, 1971; Kawakami et al., 1971), and by implantation of crystalline steroids (Gorski, 1971; Stumpf and Sar, (1973). The sites at which steroids feedback on the hypothalamus are located in the region from the POA to the median eminence. According to Stumpf and Sar (1973), the median eminence may be involved in the negative feedback effect of steroids, whereas in other regions of the hypothalamus, sites for both positive and negative feedback effects of steroids exist.

(9) Role of Steroids in Hypothalamic Differentiation

Sex steroids not only have an important feedback influence on

hypothalamic releasing hormone secretion, but also affect hypothalamic differentiation.

Development of a male-type gonadotrophin secretion pattern in the adult hypothalamus of the rat depends on the presence of gonadal androgen during the first 8 - 10 days of neonatal life (Barraclough, 1971). However, in primates (Goy and Phoenix, 1971; Resko, 1975) and in sheep (Short, 1974) the critical period during which androgen-induced male hypothalamic differentiation occurs is during the first half of prenatal life. In the absence of androgen, female-type development takes place so that the POA exerts a cyclic influence on GnRH release. The production of sex steroids in these critical periods is thus of paramount importance to normal adult reproductive function.

(10) Synthetic GnRH

Schally and his coworkers have led the intensive research effort on the isolation, characterization, synthesis, and physiological effects of GnRH (Schally, Kastin and Arimura, 1972a).

Although porcine GnRH was isolated by Schally et al. (1971), ovine GnRH had been isolated previously in a highly purified form by Guillemin, Jutisz and Sakiz (1963), Dhariwal, Antunes-Rodriguez and McCann (1965), and Fawcett, Charlton and Harris (1968). Matsuo et al. (1971b) determined the primary structure of porcine GnRH and soon after, synthesized the molecule and showed that it had the same chemical and biological properties as natural porcine GnRH (Matsuo et al., 1971a). The same polypeptide was found to be the principal LH releasing hormone of ovine hypothalami (Burgus et al., 1972).

Synthetic GnRH has been shown to elevate plasma LH levels and to cause ovulation in ewes (Arimura et al., 1972; Cumming et al., 1972; Reeves, Tarnavsky and Chakraborty, 1974; Rippel et al., 1974), and to raise plasma LH levels in rams (Pelletier, 1974: Falvo et al., 1975;

Galloway and Pelletier, 1975; Lee et al., 1976; Lincoln, 1976a).
Other workers have recorded an elevation of plasma FSH levels
following administration of synthetic GnRH to ewes (Jonas et al.,
1973; Hopkinson, Pant and Fitzpatrick, 1974; Symons, Cunningham
and Saba, 1974).

(11) Actions of GnRH

The best known actions of GnRH are the release of FSH and LH
from the anterior pituitary (Schally et al., 1973). Tissue culture
studies have indicated that both synthetic and natural porcine GnRH
also stimulate the synthesis of FSH and LH by the anterior pituitary
(Schally et al., 1972b).

Galloway and Pelletier (1975) have shown that since wethers
have a ten to twelve times greater response to GnRH injections than
entire rams, LH release from the anterior pituitary in rams is
influenced by the levels of circulating androgens.

It is still not absolutely certain that the decapeptide isolated
by Schally and his colleagues is the only physiological gonadotrophin-
releasing hormone. Pant (1973) suggested that as a single injection
of oestradiol-17β produced a marked increase in plasma LH in the ewe
without any change in plasma FSH levels, two releasing hormones could
exist. Similar implications have been made by Franchimont et al.
(1974) and Schams et al. (1974a) as a consequence of experiments on
human and bovine subjects, respectively. However, since the release
of gonadotrophins is modified by steroid levels (see discussion above),
the findings of Pant, Franchimont et al. and Schams et al. do not
exclude the possibility that the differential release of FSH and LH
could depend on differential sensitivity to steroid feedback and
involve only one releasing hormone. The existence of an antigenically
distinct FSH-releasing hormone in the rat has been reported by Shin
and Kraicer (1974) who found that only LH release was inhibited by

LH-releasing hormone antisera. However, this result could have arisen from the production of antisera specific only for the LH-releasing moiety of GnRH, which would not eliminate FSH-releasing activity from the same molecule.

(12) Gonadotrophins

(a) General. The nature and role of the gonadotrophins has been reviewed by Greep (1973) who reported that both LH and FSH were glycoproteins consisting of two non-identical subunits. Both LH and FSH have the same α subunit, which also is identical to that of thyroid-stimulating hormone and human chorionic gonadotrophin. Each subunit has very little biological activity alone, but when the two subunits are recombined, biological activity is largely or completely restored (Papkoff and Li, 1970; Pierce et al., 1971).

There is a paucity of data on the dynamics of secretion and the plasma clearance rates of gonadotrophins in domestic animals. This is particularly true of FSH for which the development of specific radioimmunoassays has presented major problems. Geschwind (1972) reported that bovine LH had a plasma disappearance rate equivalent to a half-life of approximately thirty minutes. Metabolic clearance rates for LH, FSH and prolactin in ewes were reported by Akbar, Nett and Niswender (1974), being about 46, 25, and 100 ml/minute, respectively. These results were equivalent to half-lives of 43, 102, and 23 minutes, respectively.

The actions of gonadotrophins on the testes in mammals have been reviewed (with extensive bibliography) by Setchell and Hinton (1973).

(b) FSH and LH. FSH influences spermatogenesis only in prepubertal males, LH being the sole spermatogenic gonadotrophin of the adult (Ortavant, Courot and de Reviers, 1969; Courot, Ortavant and de Reviers, 1971; Davies, Courot and Gresham, 1974). Davies,

Courot and Gresham postulated that previous reports of major spermatogenic effects of FSH in adults arose from LH-contamination of many FSH preparations. They concluded that in the adult, FSH only has a secondary role in the control of spermatogenesis. In prepubertal males it has been shown that FSH alone stimulates Sertoli cells and the early stages of germ cell development (spermatogonia and primary spermatocytes); meiosis and the full development of spermatids require testicular androgens as well as FSH (Lostroh, 1969).

Testicular androgen formation is largely under control of LH, and several authors have demonstrated a positive relationship between LH levels and testosterone release in rams (Katongole, Naftolin and Short, 1974; Sanford et al., 1974b; Falvo et al., 1975). A possible synergistic role of FSH in steroidogenesis was suggested by the effect of FSH plus LH on seminal vesicular weights in hypophysectomized adult rats (Ortavant, Courot and de Reviers, 1969).

Although the precise mechanisms of action of the gonadotrophins are not known, it seems certain that FSH stimulates protein synthesis in testicular tissue (Means and Hall, 1967), and LH stimulates the production of testicular androgens by increasing the conversion of cholesterol to 20β-hydroxy cholesterol (Hall and Young, 1968).

(c) Prolactin. Prolactin is luteotrophic in rats (Evans et al., 1941) but there is little evidence that it has a similar role in other mammals. However, Denamur and coauthors (Denamur, Martinet and Short, 1973; Kann and Denamur, 1974) considered that it is a luteotrophic factor in ewes, and claimed that prolactin and LH formed the "luteotrophic complex". Also, Davis and Borger (1974) reported that prolactin and LH levels were depressed by progesterone injection in ewes. On the other hand prolactin displayed little or or no luteotrophic activity in the sow relative to that of LH

(Hoffmann et al., 1974).

Bartke (1973a, b) suggested that prolactin could also be gonadotrophic in males, after recording that this hormone stimulated mouse-testis testosterone production in the presence of LH, which confirmed an earlier finding in hypophysectomized rats (Hafiez, Lloyd and Bartke, 1972). Also, prolactin displayed a synergistic action with testosterone by increasing the weight and zinc uptake of rat prostate glands (Moger and Geschwind, 1972), and by increasing their nucleic acid content (Thomas and Manandhar, 1975).

Conversely, a steroidogenic role for prolactin in bulls was ruled out by Smith et al. (1973), as prolactin injections had no effect on serum testosterone levels. However, Schams, Reinhardt and Karg (1974b) showed that both progesterone and oestradiol-17β stimulated prolactin release in bulls, which indicated that there was at least a steroid feedback on prolactin secretion.

(13) Androgens

The actions of testicular androgens on accessory sex glands and secondary sexual characteristics are particularly evident from castration studies, and have been described by Parkes (1966). These include the development of masculine musculature and external genitalia, and male behavioural patterns.

Although the endocrine regulation of spermatogenesis is not fully understood, it is clear that androgens are required for the normal function of the germinal epithelium. Hall (1970) pointed out that the spermatogenic action of LH was the result of stimulated Leydig cell production of androgens. Also, the regression in spermatogenesis which follows hypophysectomy in rats can be prevented by the administration of androgens (Clermont and Morgentaler, 1955).

An increasing body of evidence has accumulated for the existence of a testicular androgen-concentrating mechanism which

maintains high tissue levels of androgens within testes and epididymides of the rat (Free, Jaffe and Jain, 1973; Einer-Jensen, 1974a, b). These authors postulated the existence of a counter-current exchange mechanism between the internal spermatic artery and pampiniform plexus, allowing local recirculation of androgens. A similar exchange between venous and arterial blood in the spermatic cord was described in rams and wallabies by Jacks and Setchell (1973), and in men by Bayard et al. (1975).

The mechanism of action of the androgens has been recently reviewed by Minguell and Sierralta (1975). Androgen-sensitive tissues appear to be those which have a high ability to bind testosterone then metabolize it to 5α-dihydrotestosterone, which probably is the active principle and which enhances RNA synthesis.

4. THE ROLE OF THE PINEAL GLAND IN MAMMALIAN REPRODUCTION

(1) General

The pineal gland has been regarded by some workers as a possible modifier of the hypothalamo-hypophysial-gonadal axis as a consequence of its secretion of antigonadotrophic compounds. Inhibitory effects of the pineal gland on FSH and LH secretion have been reviewed on a number of occasions (Fraschini, Collu and Martini, 1971; Moszkowska, Kordon and Ebels, 1971; Reiter, 1973b), and the pineal has particularly been implicated in reproductive responses to photoperiodic stimuli (Reiter, 1973b, 1974a).

(2) Effects of Pinealectomy

Unlike the clear-cut responses which follow removal of other endocrine glands, pinealectomy sometimes has produced no detectable effects. However the characteristic response of rodents has been a slightly accelerated rate of gonadal growth (Reiter, 1973b). This

author commented that if it is true that light inhibits pineal antigonadotrophic activity, lack of response to surgical removal of the gland in laboratory animals, may be accounted for by the fact that the commonly used twelve to sixteen hour lighting schedules effectively "pinealectomized" these animals.

(3) Pineal Indoleamines

Much research into the role of the pineal gland has centred on the actions of pineal principles, particularly melatonin. Melatonin is one of a group of indoleamines formed in the pineal gland from tryptophan (Wurtman, Axelrod, and Kelly, 1968). The last step in the melatonin biosynthetic pathway is the transmethylation of N-acetyl serotonin by the enzyme hydroxyindole-O-methyl transferase (HIOMT). This enzyme was thought to be unique to the pineal gland, but recently Cardinali and Wurtman (1972) reported its presence in the retinae and harderian glands of the rat, as well as in the pineal gland. Melatonin has been named for its lightening action on the skin of amphibians, but its principle function in mammals is considered to be antigonadotrophic, acting at the level of the hypothalamus, or the anterior pituitary, or both. Serotonin (5-hydroxytryptamine), a melatonin precursor, also is biologically active and found in high concentrations in the pineal gland (Owman, 1968). The effects of light on pineal content of melatonin and serotonin, and on HIOMT activity, have been summarized by Wurtman, Axelrod and Kelly (1968) and Quay (1970). In the rat, pineal gland enzyme activity and amine levels appear to be at their greatest during the hours of darkness and reach a minimum during daylight hours. The production of serotonin by the rat pineal gland is an exception in that it is greatest during daylight, and an increase in serotonin levels occurs within ten to fourteen minutes of switching on the

lights (Illnerová, 1971).

In ewes, melatonin infusion prevented the post castration rise in LH levels which occurred in saline-infused control animals (Roche et al., 1970b). Infusions of melatonin or serotonin into the third cerebral ventricle of ewes prolonged oestrous cycles and delayed ovulation by several days, serotonin being more effective than melatonin (Domański et al., 1975). Indoleamine metabolism, estimated in the pineals of mixed-breed ewes by measuring levels of serotonin and HIOMT activity, was maximal at oestrus and day four of the oestrous cycle, and minimal during the mid-luteal phase of the cycle (Cardinali, Nagle and Rosner, 1974a).

There have been even fewer reports on the effects of the indoleamines in male domestic animals. Subcutaneous implants of melatonin in beeswax, or injections of melatonin in sesame oil, had no effect on antler growth in five sika deer (Goss and Rosen, 1973). However, infusions of serotonin into the third cerebral ventricle of castrate male sheep caused a significant depression of spontaneous LH release, as did norepinephrine, while dopamine was without effect (Riggs and Malven, 1974).

(4) Pineal Peptides

Lately, the antigonadotrophic role of the pineal indoleamines has tended to be over-shadowed by increasing support for a similar, but more potent, property of some pineal peptides. Pavel and his co-workers (Pavel and Petrescu, 1966; Pavel et al., 1973a; Pavel, Petrescu, and Vicoleanu, 1973b) have isolated a peptide from bovine and fetal human pineals with potent antigonadotrophic properties, and which they claimed was chemically identical to arginine vasotocin. Cheesman and Fariss (1970) recorded a similar conclusion. On the other hand, Benson and his colleagues have isolated an antigonadotrophic

peptide from bovine pineals which they stated was not arginine vasotocin (Benson, Matthews, and Rodin, 1972). French and Dutch investigators have isolated antigonadotrophic fractions from sheep pineal glands, which contained low molecular weight compounds (M.W. <500) which were not melatonin (Moszkowska et al., 1974; Ebels, 1975).

A recent development has been the report by White et al. (1974) of the isolation of high levels of immunoassayable GnRH in ovine, bovine, and porcine pineal glands. This could mean that the pineal gland is a source of both progonadotrophic and antigonadotrophic compounds, an idea which has been suggested by Hoffman and Reiter (1966). There have also been recent reports of stimulatory effects of the pineal on the release of prolactin (Relkin, Alachi and Kahan, 1972; Reiter, 1973b, 1974b).

(5) Gonadal Regulation of Pineal Function

Although the pineal gland is becoming accepted as a regulator of gonadotrophin secretion, recent evidence has shown that the gonads themselves may, in turn, alter pineal function. The biosynthesis of melatonin in rats has been shown to be increased by oestrogens, and decreased by progesterone and testosterone (Houssay and Barcelo, 1972a, b). Also the rat pineal gland was suggested as a target organ for androgens by Cardinali, Nagle, and Rosner (1974b), who investigated pineal uptake, cytoplasmic binding, and conversion of testosterone to its 5α-reduced metabolites. Nir et al. (1970) reported a stimulation of protein synthesis by the pineal in response to injections of 10 mg of oestradiol-17β in female rats. They postulated that oestrogen caused LH release which in turn stimulated protein synthesis by the pineal gland.

(6) Transport of Pineal Principles

Many workers have presumed that the principles secreted by the pineal gland reached other regions of the brain via the cerebrospinal

fluid, but there has been no evidence to confirm this. Instead Kappers (1971) was adamant that pineal principles were secreted into the blood, and that there was no anatomical evidence for any vascular pathway from the gland to the cerebral ventricular system. Quay (1973) has suggested that pineal substances may be secreted from the choroid plexus of the suprapineal recess into the cerebrospinal fluid, however this would require movement in the reverse direction to that of the normal blood flow in the great cerebral vein.

(7) Afferent Nervous Pathways

In the rat the pathway by which photic stimuli reach the pineal gland involves the inferior accessory optic tract, then the cranial cervical ganglia of the sympathetic nervous system (Moore et al., 1967; Newman-Taylor and Wilson, 1970). Postganglionic fibres from these ganglia reach the pineal gland principally through the nervi conarii (Kenny, 1965).

The habenular commissure which attaches the pineal gland to the brain is not widely regarded as a source of afferent nerve fibres to the gland (Kappers, 1965; Kenny, 1965). These authors have shown that any fibres in the habenular commissures of rats and bats do not synapse within the pineal gland. However, histological studies of bovine and ovine pineal glands revealed nervous connections from the habenular commissure (Anderson, 1965; Jayatilaka, 1970). This latter finding has also been supported by electrophysiological evidence obtained from rats (McClung and Dafny, 1975).

Consequently, the relative importance of the different afferent nervous pathways to the pineal glands of most species has not been fully resolved.

There is evidence that cholinergic mechanisms are involved in the response of the rat pineal gland to darkness (Wartman et al., 1969), while Aro, Karppanen and Klinge (1973) reported that acetylcholinesterase activity in the bovine pineal gland was decreased after puberty.

Acetycholine also increased the uptake of radio-labelled phosphorus by calf pineal slices (Basinska, Sastry and Stancer, 1973). Such findings indicate that the autonomic fibres reaching the pineal gland from the cranial cervical ganglia may be cholinergic as well as adrenergic.

(8) Role of the Pineal Gland in the Seasonality of Reproduction

In his reviews on the pineal gland, Reiter (1973b, 1974a) contended that one of the most important functions of the gland may be to control or modulate seasonal reproductive rhythms in photosensitive animals. He supported this statement with reference to work on the golden hamster and ferret. Seasonal changes in the histological appearance of pinealocytes in the Weddell seal were described by Cuello and Tramezzani (1969). An association between HIOMT levels and the seasonal reproductive cycle of the northern fur seal has been reported (Keyes et al., 1971). A study of the annual changes in the histological appearance of the ewe pineal gland showed that the gland was most active during the winter months (Nešić, 1962), while Forbes (1975) reported that pineal glands were lighter and had a lower mean cell size in ram lambs submitted to sixteen hours lighting per day, than in those on eight hours lighting per day. These studies indicate that in some species the pineal gland is capable of undergoing variations in activity in response to seasonal environmental changes.

It has been claimed however, that pinealectomy had no effect on the incidence of oestrus, ovulation, or LH levels in ewes (Roche et al., 1970a). These workers recorded the dates of commencement and lengths of the breeding season, numbers of ewes ovulating during the anoestrous period, and plasma and pituitary levels of LH in Western, Suffolk, and Hampshire ewes. Although the authors suggested that pinealectomy had no effect on seasonal breeding in ewes, this result may have been due to inadequacies in experimental design and interpretation of results. Herbert (1972) reported that in the ferret, the onset of the breeding season in pinealectomized females

did not differ from that of their controls until the second post-
operative year, suggesting that breeding rhythm is programmed as a
result of environmental photoperiodic stimuli received considerably
before the breeding season.   He commented that the negative findings
of Roche et al. (1970a) may have resulted from failure to continue
the experiment for a sufficiently long period.

(9)   Pineal Function After Puberty

Some authors have suggested that the pineal gland is functional
only up to and during puberty.   Jordan (1911) stated : "if the
pineal body in the sheep subserves an important physiological function,
this is only active during the first eight months of post natal life".
This observation was based on histological evidence.   Subsequently,
it has been shown that a decline in HIOMT activity in the pineal
glands of both sexes of cattle occurred at about the time of puberty
(Hoermann, 1971).   Also the number of pinealocytes, and vascularity
of the gland, were higher in buffalo calves than in adults (Rao and
Saigal, 1971).   In man, after puberty small concretions of calcium
and magnesium phosphates and carbonates (pineal sand) appear in the
tissue, in association with the involution of the gland (Ganong, 1975).
However, the biochemical activity of the gland in the adults of a number
of different species is such that it is hard to rule out the possibility
that the gland has a role in the control of reproduction.

(10)   Pineal Gland and Olfactory Function

A few reports have indicated the existence of a relationship
between the pineal gland and the olfactory system of rats, for
example prolonged olfactory stimulation with isonitrile caused pineal
atrophy in male rats (Miline, Decerverski and Krstic, 1963).   Also
Reiter et al. (1971) found that regression of the reproductive organs
in male rats could be produced by blinding plus anosmia, although
neither treatment on its own had any effect.   Similarly, puberty was

delayed in blinded or anosmic female rats, and was further delayed if
the treatments were combined (Reiter and Ellison, 1970). Blinded,
olfactory bulbectomized rats had elevated serum levels of prolactin
and growth hormone compared to sham-operated controls, although this
effect was reduced if they were subsequently pinealectomized (Shiino,
Arimura, and Rennels, 1974).

These results indicate that the pineal gland along with other
neural systems, such as the olfactory system, may contribute to the
modulation of animal reproductive physiology by changes in
environmental stimuli.

5. THE PURPOSE OF THE PRESENT STUDY

The experiments described in this thesis were undertaken to
study the seasonal changes in reproduction in N.Z. Romney rams
together with neuroendocrine mechanisms causing these changes.

Initial experiments were designed to evaluate seasonal changes
in semen production and plasma hormone levels in rams under normal
grazing conditions, and to make between-breed comparisons in the
extent of seasonality of the parameters studied. Also, in order to
examine neuroendocrine mechanisms involved in reproductive seasonality,
rams with modified pineal or olfactory function were studied in the
same manner as intact rams. Subsequently, an experiment was carried
out to determine the effects of artificial lighting regimes on both
semen production and plasma hormone levels in N.Z. Romney rams. Then
in the final experiments in this thesis an attempt was made to
elucidate the function of the pineal gland of rams as a mediator of
photoperiod-induced changes in reproductive function.

CHAPTER II

MATERIALS AND METHODS

1. ANIMALS

Adult New Zealand Romney rams were used throughout the experiments described in this thesis. In addition, adult Merino and Polled Dorset rams were included in Experiment 3.

2. ANIMAL MANAGEMENT PROCEDURES

(1) On Pasture

Rams in Experiments 3 and 4 grazed pasture dominated by rye-grass and white clover, and were supplemented with meadow hay during a short period of pasture insufficiency. Anthelmintic drenching (15 ml of "Thibenzole", Merck Sharp and Dohme (N.Z.) Limited, containing 264 mg/litre of added selenium) was carried out once per month. All animals were given preventative footrot vaccinations (Glaxo Footrot Vaccine, Glaxo Laboratories (N.Z.) Limited) and clinical cases of footrot were treated by foot paring followed by formalin foot bathing. Insecticide spraying and shearing were carried out on an annual basis.

Apart from occasional mild cases of footrot, and of facial eczema during the late summer months, the rams remained in good health.

(2) Indoors

Rams in Experiments 5, 6, 7.1, 7.2 and 7.3 were housed in well-ventilated light-proof rooms maintained at a constant temperature of $15^{\circ}C$. Each room was illuminated by two 80W fluorescent lights so that a light intensity of approximately 110 lux was received at eye-level by the rams. Lighting was controlled by automatic time-switches which were adjusted once per week to produce the lighting regimes determined

by the experimental designs.

These rams were held in individual crates, and chaffed meadow hay (approximately 1400 g) and sheep nuts (approximately 650 g) were fed at approximately 09.00 h each day. Each week a few grams of a salt mixture containing sodium molybdate and calcium sulphate were fed in order to prevent any potentially toxic elevation of hepatic copper levels (recommended by Hogan, Money and Blayney, 1968). Water was available ad libitum.

Shearing and anthelmintic drenching were performed at the commencement of each study, whereas foot paring was carried out as required.

Monthly body weight data, together with the general health status of the rams (plus haematological data in Experiment 6), indicated that the animals maintained good health throughout the experiments.

3. SURGICAL TECHNIQUES

(1) General Surgical Procedure

Prior to all surgical procedures rams were placed in individual crates for one week and fed chaffed meadow hay, sheep nuts and water as described earlier. Food (but not water) was withheld for the twenty-four hours preceding surgery.

Anaesthesia was induced with intravenous sodium thiamylal ("Surital", Parke Davis (N.Z.) Limited), and maintained with a mixture of approximately 2% halothane vapour ("Fluothane", Imperial Chemical Industries Limited, U.K.) in oxygen. An open-circuit anaesthetic machine was utilized and gases were administered via endotracheal tube.

Surgery was carried out in a well-equipped small animal operating theatre, and full sterile surgical procedures were employed throughout all operations.

Skin incisions were made using electro-cutting equipment and haemostasis was maintained with a combination of blood vessel ligation and electro-coagulation. When closing surgical wounds, subcutaneous tissues were sutured with catgut (chromic, size 00), while a polyester thread ("Mersilene", Ethicon) was used for the skin sutures, which were sealed with a plastic aerosol spray.

When the rams had regained consciousness they were returned to their crates; prophylactic antibiotic therapy consisted of daily injections of both procaine penicillin and dihydrostreptomycin for five days. Skin sutures were removed approximately one week post-operatively.

(2) Olfactory Bulbectomy

Olfactory bulbectomy was carried out according to the method described by Chapman (1965).

A 7-10 cm incision was made in the midline extending rostrally from the highest point of the poll. The skin and periosteum were then reflected laterally and a 23 mm trephine was used to open the frontal sinus at the level of the supra-orbital foramina. The trephine hole was enlarged with rongeurs then the mucosa of the frontal sinus was stripped out. Extensive bleeding usually followed and was controlled with electro-coagulation. A 12 mm trephine was used to make an opening into the cranial cavity on each side of the medial septum of the frontal sinus, just caudal to the angle of the internal plate of the frontal bone. After the dura had been cut, a blunt probe (3 mm diameter) was passed through the incision into the ethmoidal fossa, where it was manipulated to disrupt the neural tissue of the olfactory bulb. The contents of the ethmoidal fossa were then removed by suction and replaced with absorbable gelatin sponge ("Spongostan", Ferrosan, Denmark). Finally an aqueous solution of crystalline penicillin was sluiced over the operation site prior to closure of the skin incision with continuous

mattress sutures.

During preliminary trials with this technique, one animal was autopsied twelve hours following surgery. Examination of sections of the skull indicated that the ethmoidal fossae were almost entirely devoid of undamaged neural tissue. It was thus considered that the procedure would have deprived the rams of olfactory bulb function.

(3) Cranial Cervical Ganglionectomy

Cranial cervical ganglionectomy was carried out following the procedure described by Appleton and Waties (1957).

This technique utilized a lateral approach via a skin incision parallel to the mandible and which extended from just rostral to the base of the ear, to the level of the thyroid cartilage. In this approach blood vessels which pass caudally from the external jugular and internal maxillary veins were ligated, then divided to provide access to the cranial cervical ganglion. The ganglion was located using blunt dissection, freed from the cervical sympathetic and glossopharyngeal nerves, and removed intact.

After closure of the skin incision with continuous mattress sutures, the ram was turned on to its other side, and the operation repeated to complete the bilateral ganglionectomy.

(4) Olfactory Bulbectomy/Cranial Cervical Ganglionectomy

Rams which were subjected to both olfactory bulbectomy and cranial cervical ganglionectomy were operated on using the procedures described above.

(5) Pinealectomy

Pineal glands were removed surgically from rams using a technique based on that described by Roche and Dziuk (1969) for the ewe.

Rams were placed in ventral recumbency on the operating table

with the head restrained laterally, by cords attached to the ears. A flap of skin and subcutaneous tissue, extending medially over the top of the head from each side of the base of the left ear, was reflected after cutting along three sides of a trapezoid (see Figure 2.1). Dehydration of this skin flap during subsequent surgery was prevented by covering it with a saline-soaked cotton gauze swab.

The coronal sutures were revealed by reflecting the periosteum, then a 2 mm centering hole was drilled into the parietal bone, 10 mm caudal to the coronal suture and 15 mm to the left of the midline (see Figure 2.2). This centering hole was used to locate the centre-piece of a 41 mm diameter trephine. Following removal of a circular plate of bone (see Figure 2.3), the exposed dura mater was incised along the left lateral border of the trephine hole, almost as far as the dorsal sagittal sinus. The flap of dura so formed was reflected to expose the occipital lobe of the left cerebral hemisphere (see Figure 2.4).

Careful insertion of a curved, tapered, perspex spatula between the left cerebral hemisphere and the tentorium cerebelli permitted gentle lateral displacement of the lobe. Subdural veins leading to the dorsal sagittal sinus were sealed by electro-coagulation and excess fluid was removed by aspiration. After puncturing the cisterna venae magnae cerebri and removing the excess cerebrospinal fluid which flowed freely at this stage, the pineal gland was located in the midline rostral to the rostral cerebral colliculi (see Figure 2.5). Removal of the pineal gland was accomplished by placing Allis tissue forceps around the gland and then gently withdrawing the forceps (see Figure 2.6).

Absorbable gelatin sponge ("Spongostan", Ferrosan, Denmark) was placed between the cerebral hemispheres and the tentorium cerebelli, and also into the trephine hole, to control haemorrhage.

The operation site was dusted with 2% chlorotetracycline HCl,

Figure 2.1 : Head of ram clipped and marked to show
approximate line of skin incision.

Figure 2.2 : Skull exposed showing centering hole for
trephine. The coronal suture is visible
just rostral to the centering hole.

(Note : In Figures 2.1 and 2.2 the rostral end of the
surgical field is to the bottom of each figure.)

Figure 2.3 : Removal of circular plate of bone from
            the skull after trephining.

Figure 2.4 : Flap of dura mater reflected to expose
            the occipital lobe of the left cerebral
            hemisphere.

(Note : The rostral end of the surgical field is to the
 bottom of Figure 2.3 and to the left of Figure 2.4.)
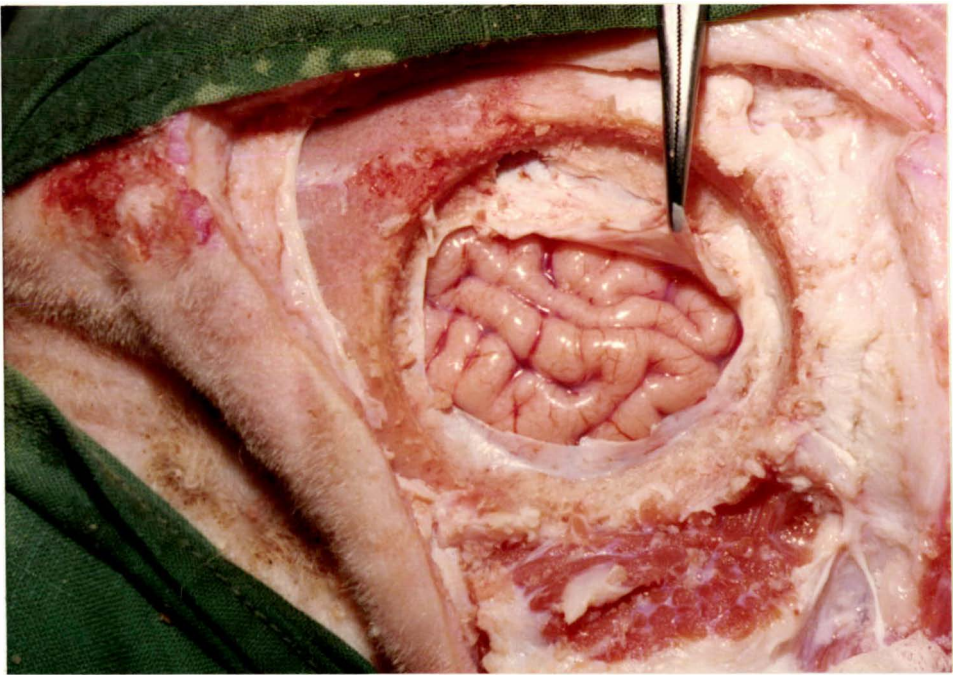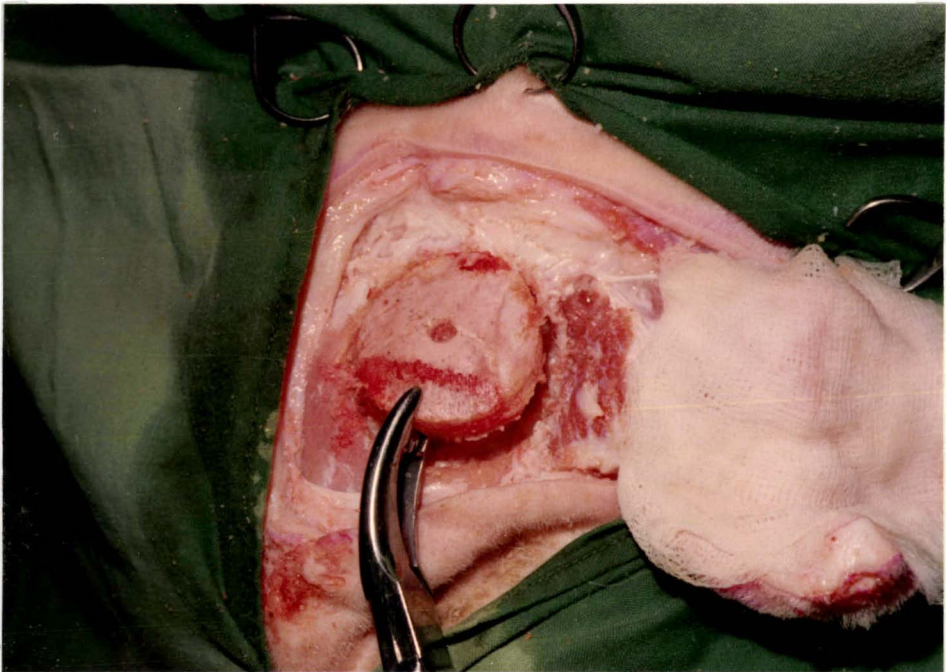
Figure 2.5 : Pineal gland (p) just visible rostral to
the rostral cerebral colliculus (c).

Figure 2.6 : Removal of pineal gland with Allis forceps.

(Note : In Figures 2.5 and 2.6 the rostral end of the
surgical field is to the left of each figure.)
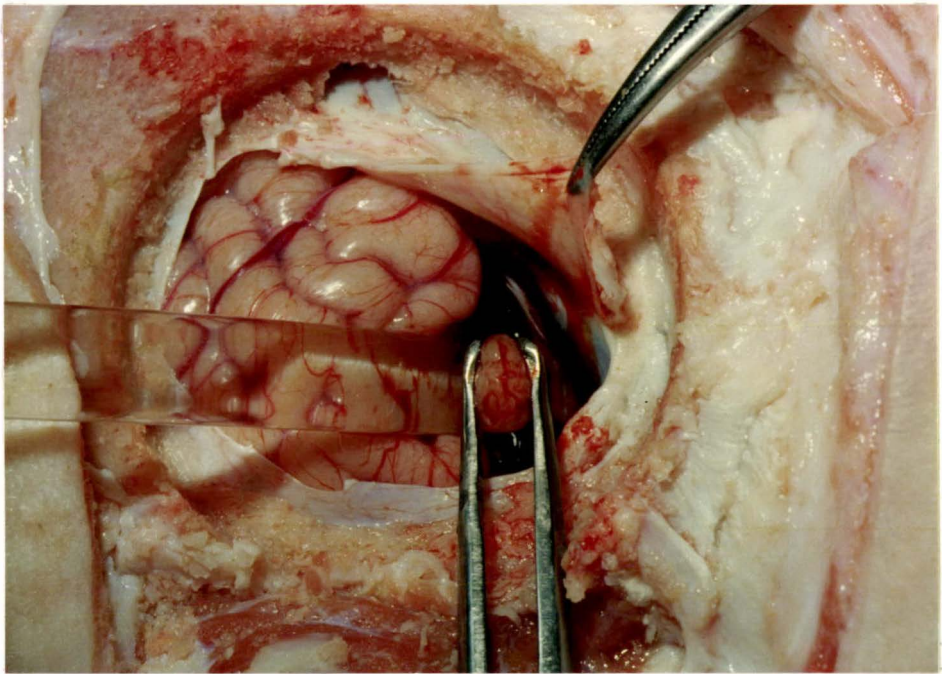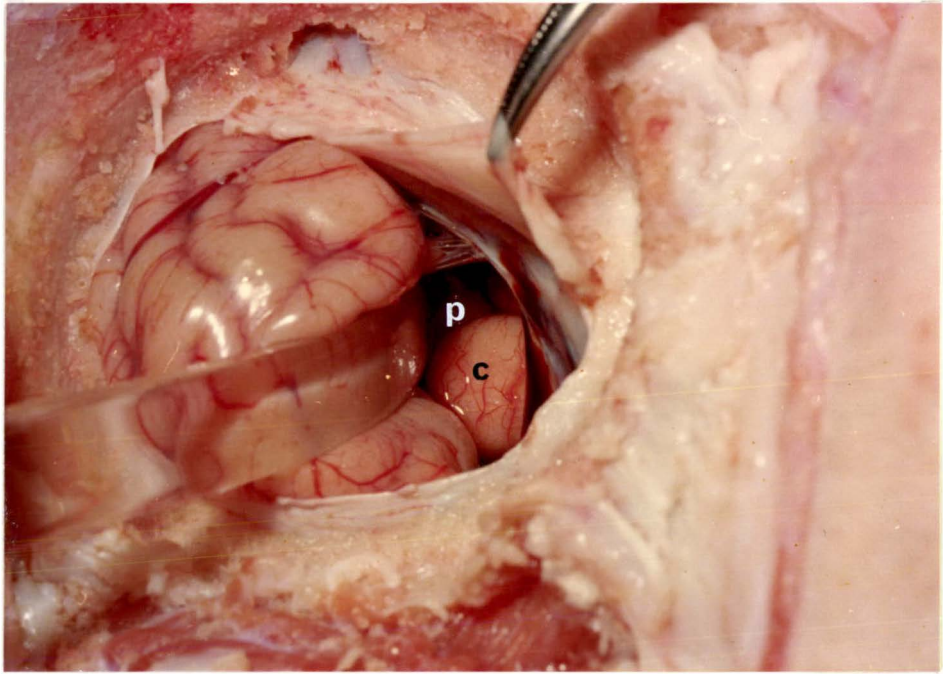
1% benzocaine powder ("Aureomycin powder", Cyanamid Australia Pty
Limited) prior to closure of the skin incision.

Confirmation of the completeness of pinealectomy was made by
macroscopic examination of formalin-fixed brains and histological
examination of brain slices following autopsy.

(6) Sham-pinealectomy

Sham-pinealectomy operations were identical in all respects to
those for pineal gland removal except that after exposure, the pineal
glands were left in situ.

4. SEMEN COLLECTION

Ejaculates were collected from rams at two-weekly intervals.

Initially in Experiments 3 and 4 semen was collected by
artificial vagina whenever possible. The temperature of the artificial
vagina was adjusted to $40-45^{o}$ C, and the inner liner was lubricated with
petroleum jelly. Rams unwilling to mount were ejaculated by electrical
stimulation using a bi-polar rectal probe (de la Nichols and Edgar, 1964).

During the winter months most rams exhibited a decline in libido
which made collection of semen by artificial vagina increasingly
difficult. Thus ejaculates collected after September 1972 in
Experiments 3 and 4, as well as all of those collected in Experiments 5
and 6, were obtained by electro-ejaculation.

During collection semen was protected against temperature
fluctuations, then held in a warm bath at 30° C whilst being appraised.

5. SEMEN APPRAISAL

(1) Ejaculate Volume

Ejaculate volume was measured to the nearest 0.1 ml in

graduated glass centrifuge tubes.

(2) <u>Motility and Percentage Motile Spermatozoa</u>

Motility (scale 0-4; Emmens, 1947) and percentage of motile spermatozoa were estimated by observation of thin films of semen prepared between a microscope slide and a coverslip. A low-power microscope fitted with a warm stage at 37°C was used for these estimations.

(3) <u>Percentages of Unstained and Morphologically Normal Spermatozoa</u>

Congo red-nigrosin smears were prepared for estimation of percentage of unstained spermatozoa (Blackshaw, 1955). One hundred spermatozoa were counted under the oil-immersion microscope and classified as stained or unstained, the number unstained being taken as the percentage. Likewise, one hundred spermatozoa in the same smears were classified as having normal or abnormal morphology, the number with normal morphology being taken as the percentage.

(4) <u>Concentration of Spermatozoa per ml Semen and Number of Spermatozoa per Ejaculate</u>

A 1 in 200 dilution of semen in 1.8% (w/v) sodium chloride-2% (v/v) formalin solution was counted on the red cell grid of a haemocytometer. Multiplication of the total count by 10^{7} gave the concentration of spermatozoa per ml of semen, then multiplication of this concentration by the ejaculate volume (in ml) gave the total number of spermatozoa per ejaculate.

(5) <u>Concentration of Fructose in Semen and Seminal Plasma, and Total Ejaculate Fructose Content</u>

Immediately after collection, a portion of each ejaculate was frozen on dry ice, then stored at -20°C for subsequent determination of

fructose concentration.

Fructose concentration of semen was determined by an automated technique adapted from the method described by Mann (1964). These determinations were performed using an AutoAnalyzer (Technicon Corporation, U.S.A.) set up as shown diagrammatically in Figure 2.7.

After thawing, semen samples were disrupted ultrasonically for approximately twenty seconds, using a one-eighth inch titanium micro-probe at an amplitude setting of 4-5 microns peak to peak. Sonication was carried out to disperse clumps of spermatozoa which otherwise tended to block the sampler tubing. Within the AutoAnalyzer the semen was diluted in 0.03% (w/v) polyoxyethylene lauryl ether (Brij-35) solution then conveyed to the dialyzer where solutes, including fructose, passed into a recipient Brij-35 solution. After leaving the dialyzer, this recipient solution was mixed with 0.1% (w/v) aqueous resorcinol, followed by 30% (w/v) hydrochloric acid, and passed through a $95^{o}C$ heating bath for ten minutes to allow red colour development. After debubbling, the transmittance of the coloured solution was measured at 480 nm.

A standard curve (Figure 2.8) was constructed after assaying serial dilutions of a standard fructose solution (16 mg/ml). Determination of the slope of the curve and subsequent calculation of seminal fructose concentrations, were performed on an IBM 1620 computer.

There was good agreement between fructose concentrations obtained by the above method and those determined simultaneously by the method of Mann (1964). See Table 2.1.

Total fructose content per ejaculate (mg) was calculated by multiplying the seminal fructose concentration by the ejaculate volume. The concentration of fructose in seminal plasma was obtained by dividing the total fructose content by the volume of seminal plasma
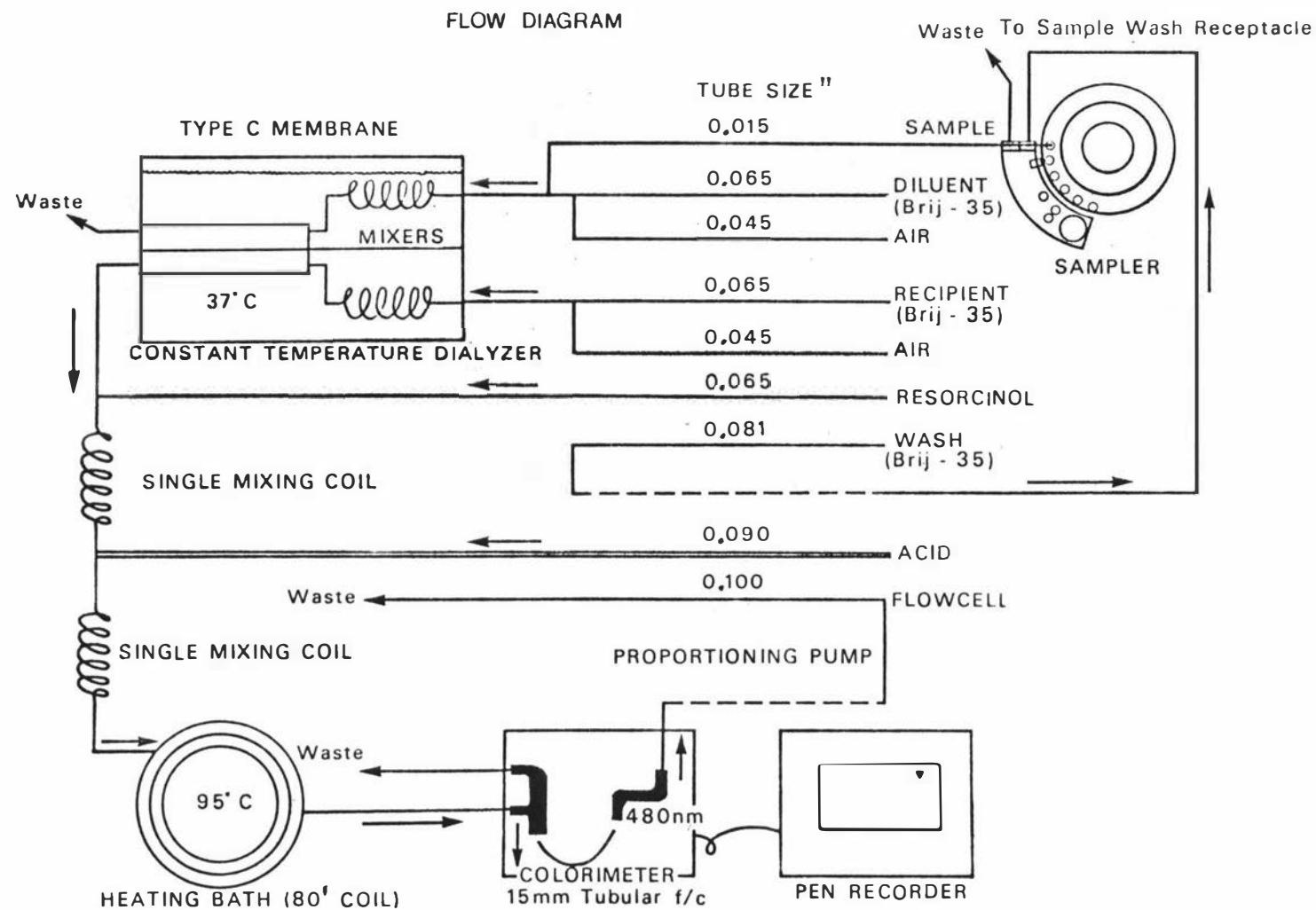
FLOW DIAGRAM

Figure 2.7 : Flow diagram of AutoAnalyzer assay for seminal fructose.
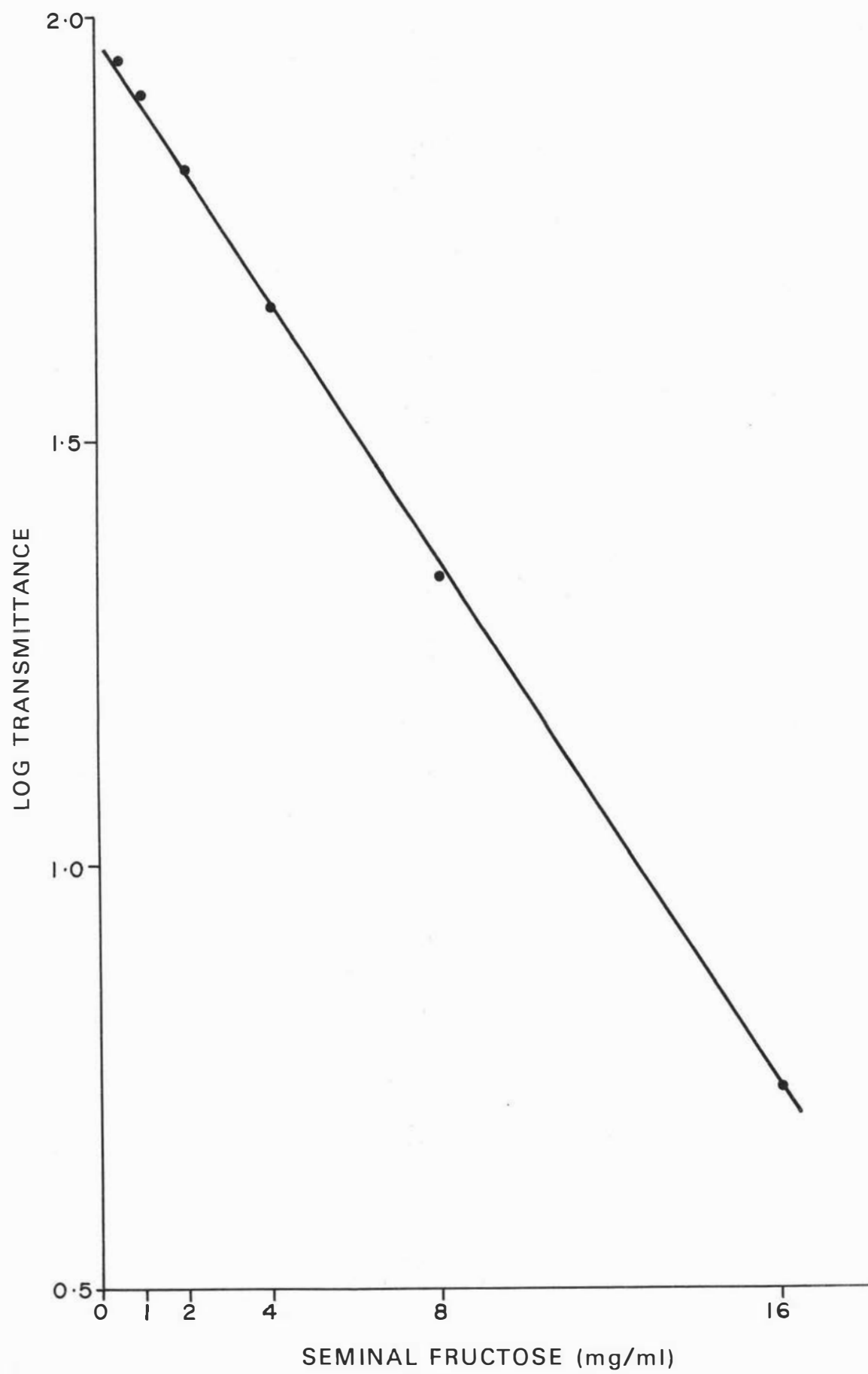
Figure 2.8 : Standard curve for AutoAnalyzer seminal fructose assay.

Table 2.1

Effect of determining fructose concentrations (mg/ml) of five semen samples by the AutoAnalyzer or by the manual method of Mann (1964).

| Sample Number | AutoAnalyzer Method | | | Manual Method | | |
	Estimates		Mean	Estimates		Mean
1	0.61,	0.61	0.61	0.76,	0.76	0.76
2	2.70,	2.66	2.68	2.58,	2.27	2.42
3	4.28,	4.27	4.28	3.64,	3.94	3.79
4	2.58,	2.59	2.58	2.25,	2.40	2.32
5	1.53,	1.53	1.53	1.48,	1.56	1.52
6	2.50,	–	2.50	2.58,	2.49	2.53

in each ejaculate. Seminal plasma volume was determined by subtracting from the ejaculate volume the volume occupied by spermatozoa, assuming that each spermatozoon, occupied a volume of 6.5×10^{-11} ml (cited by Glover, 1956).

6. BLOOD COLLECTION

In Experiments 3 - 6, weekly blood samples were obtained by jugular venepuncture using 10 ml evacuated glass tubes containing 143 USP units of sodium heparin.

Blood was collected between 09.00 h and 10.00 h each Monday, then centrifuged as soon as possible after collection in order to minimize the metabolism of androgens by a 17α-hydroxysteroid dehydrogenase present on ruminant erythrocytes (Lindner, 1965).

Half-hourly blood samples obtained in Experiments 7.2 and 7.3 were collected in the same manner as weekly blood samples.

In Experiment 7.1 blood samples were collected every 20 minutes for 26 hours by means of indwelling jugular cannulae consisting of silicon rubber ("Silastic", Dow Corning Corporation, U.S.A.) or polythene tubing, of approximately 2 mm outside diameter. These cannulae were inserted, while the rams were lightly sedated with xylazine hydrochloride (0.25 ml of 2% (w/v) "Rompun", Bayer, Germany), on the day prior to blood sampling. Both jugular veins of each ram were cannulated to minimize the risk of blood sample collection failure. Cannula patency was maintained by a continuous infusion of heparinised saline at 1 ml/hour; the infusion was interrupted only when each blood sample was collected. Saline infusion and blood sampling were performed from outside the room housing the rams, so that the animals were not aware of the sampling procedure, and so the prescribed photoperiod could be maintained. Again blood samples were centrifuged and the plasma frozen within minutes of collection.

7. AUTOPSY PROCEDURE

Rams in Experiments 3 and 4 were weighed prior to slaughter,
which was performed by cutting the throat.

The testes, epididymides, seminal vesicles, ampullae and
thyroid, pineal and pituitary glands were removed from each animal,
dissected free of any adhering adipose and connective tissue, and
weighed. Samples of testes and pineal glands were fixed in Bouin's
fluid for twenty-four hours before transfer to 70% ethanol to await
histological processing. The epididymides, seminal vesicles and
remaining portions of the pineal glands were frozen on dry ice for
subsequent determination of : epididymal spermatozoal reserves,
seminal vesicular fructose contents, and pineal hydroxyindole-0-methyl
transferase activities, respectively.

In Experiment 6, rams were anaesthetized with intravenous
pentobarbitone sodium ("Nembutal", Abbott Laboratories (N.Z.) Limited).
Immediately afterwards the common carotid arteries were cannulated and
the jugular veins severed so that the head of each ram could be perfused,
first with isotonic saline, then with a 10% (v/v) formalin-0.9% (w/v)
saline solution to fix the brain tissue. Subsequently the brains were
removed and stored in 10% (v/v) formalin-0.9% (w/v) saline prior to
histological processing; also the pituitary glands were removed and
weighed. Thyroid and accessory sex glands, and testes were processed
in the manner described above for Experiments 3 and 4.

(1) Histological Procedures

Fixed samples of : testes from Experiments 3, 4 and 6, pineal
glands from Experiments 3 and 4, and 4 mm thick paramedian sections of
brain tissue from Experiment 6, were embedded in paraffin wax after
automatic processing.

Testicular and pineal gland sections of 5 μm thickness were

stained with Mayer's haemalum and 1% eosin Y (Culling, 1974).

Subsequently seminiferous tubule diameters were measured as the mean

diameter measured from twenty circular tubules (Skinner and van Heerden,

1971), while the density of pineal cells was estimated as the mean

number of cell nuclei measured in five randomly selected microscope

fields of 0.0156 mm^2 area (Roth, Wurtman and Altschule, 1962).

Axons in pineal tissue were demonstrated by silver staining

10 μm thick pineal sections by Bodian's protargol method (Bodian, 1937).

Fifteen μm thick sections of brain were stained with solochrome cyanin

(Page, 1965) and counterstained with cresyl fast violet (Disbrey and

Rack, 1970). These brain sections were then examined to determine the

completeness of removal of pineal tissue from pinealectomized rams, and

the integrity and normal appearance of the pineal glands of sham-

operated rams.

(2) Seminal Vesicular Fructose Content and Concentration

Seminal vesicular fructose content was measured using a method

based on that described by Lindner and Mann (1960). Both seminal

vesicles from each ram were homogenized in a Sorvall omnimixer (Ivan

Sorvall Inc., U.S.A.), together with distilled water to a total volume

of 40 ml. After centrifuging the homogenate, 2 ml of the supernatant

were deproteinised with 1 ml of 0.3 N barium hydroxide followed by 1 ml

of 5% (w/v) zinc sulphate ($ZnSO_4.7H_2O$). Duplicate 0.6 ml aliquots of

the supernatant obtained following centrifugation were placed on to a

column of 0.5 ml of anion-exchange resin (Dowex-1-chloride; 1X8-200,

Sigma Chemical Co, U.S.A.) in a pasteur pipette (5 mm internal diameter).

A 2 ml distilled water eluate was collected from the column for fructose

estimation.

This ion-exchange chromatographic step eliminated phosphofructose

which is a significant constituent of seminal vesicles (Lindner and

Mann, 1960; Mann, 1964). The separation procedure avoided the laborious ethanol extraction step in the method of Lindner and Mann and was based on a similar technique described by Bossi and Greenberg (1972).

The 2 ml eluates were treated with a 2 ml ethanolic solution of 0.1% (w/v) resorcinol and 6 ml of 30% (w/v) hydrochloric acid, then incubated at $90^{\circ}C$ for 10 minutes in a water bath. After cooling, the intensity of the red colour formed was measured in a Spectronic 20 colorimeter (Bausch and Lomb Inc., U.S.A.) at 490 nm. Seminal vesicular fructose content was calculated from a calibration curve prepared with standard solutions of fructose. Seminal vesicular fructose concentrations were calculated by dividing the fructose content (mg) by the combined weight of the seminal vesicles, and was expressed as mg/g wet tissue.

(3) Epididymal Spermatozoal Reserves

Epididymal spermatozoal reserves were determined by direct counts of diluted epididymal homogenates prepared by a technique similar to that of Lino (1972). Individual epididymides were cut into pieces and homogenized in 200 ml of 0.9% (w/v) saline using the 400 ml chamber of a Sorvall omnimixer (Ivan Sorvall, Inc., U.S.A.). After homogenization the total volume was made up to 250 ml and duplicate 50 ml aliquots were filtered through two thicknesses of surgical gauze (8 ply), which was washed with a further 50 ml of saline. Spermatozoal concentrations were determined from duplicate haemocyto- meter counts after an appropriate dilution (up to 1:9) of each suspension. Duplicate estimates were made for each epididymis and all results were utilized (after multiplying by dilution factors) to provide an estimate of the total number of epididymal spermatozoa per ram.

The average values for total epididymal spermatozoal reserves

using this method were 40.02 ± 4.95 (S.E.) x 10^9 from rams in
Experiments 3 and 4, and 64.55 ± 7.78 x 10^9 in Experiment 6. Mean
total testicular weights were 234.04 ± 14.74 and 355.74 ± 25.44 g,
respectively. According to the calculation described by Dott and
Skinner (1967), the theoretical epididymal spermatozoal reserves
for testes of these weights (testicular weight x 0.0122 x 10^9 x 13,
to 15) should have been between 37.12 and 42.82 x 10^9 for Experiments
3 and 4, and between 56.42 and 65.10 x 10^9 for Experiment 6.
These theoretical values were in close agreement with the results
obtained by the method described above.

(4) Hydroxyindole-O-methyl Transferase Assay

The hydroxyindole-O-methyl transferase (HIOMT) activities of
pineal glands from rams in Experiments 3 and 4 were assayed by a
method adapted from the technique described by Axelrod, Wurtman and
Snyder (1965).

After thawing, each pineal sample (representing about one-half
of a pineal gland) was transferred to a conical glass hand homogenizer
containing 500 μl of ice-cold 0.2 M tris(hydroxymethyl)aminomethane-
HCl buffer, pH 8.8 (tris-HCl buffer). When the sample was completely
homogenized, duplicate 200 μl aliquots of homogenate were transferred
to 15 ml glass-stoppered test tubes, together with 50 μl of tris-HCl
buffer containing 300 nmoles of N-acetyl serotonin (Sigma Chemical
Company, U.S.A.), and 50 μl of tris-HCl buffer containing 50 nmoles
of S-adenosyl-L-methionine-^{14}C (0.5 mCi/mmole, The Radiochemical
Centre, Amersham). After mixing then incubation at 37°C for 30
minutes, the reaction was stopped by the addition of 1 ml of 0.2 M
borate buffer, pH 10.0. Melatonin formed during the incubation was
extracted by shaking the reaction mixture vigorously with 6 ml of
toluene-isoamyl alcohol (4:1). After centrifugation a 5 ml aliquot

of the solvent layer was transferred to a glass scintillation vial to which 5 ml of scintillation fluid (toluene containing 6 g 2,5-diphenyl oxazole and 200 mg 1,4-di[2-(5-phenyloxazol)benzene) per litre) was added. Radioactivity was determined in a Packard Tri-Carb Scintillation Spectrometer (Model 3375) and the counts obtained were corrected for quenching using the external standard channels-ratio technique. A control incubation, in which 200 μl of buffer was substituted for the pineal homogenate, was performed concurrently to determine the small amount of S-adenosyl-L-methionine-^{14}C that was extracted by the solvent, and the few counts so obtained were subtracted from those recorded for the samples. Enzyme activity was expressed as DPM/mg wet weight of tissue and DPM/pineal gland.

(a) <u>Discussion of HIOMT Assay Method</u>. The use of tris-HCl buffer at pH 8.8 was based on the finding of Quay (1971) that tris-HCl prevented the reduction in product formation by high levels of substrate which occurred in 0.1 M sodium phosphate buffer, pH 7.9. Concentrations of the N-acetyl serotonin and S-adenosyl-L-methionine-^{14}C used in the present assay were calculated from the levels suggested by Weiss (1968).

Empirical designation of HIOMT activity in terms of DPM/wet weight of tissue and DPM/pineal was used for the sake of simplicity and to avoid the assumptions required in the use of a calculation such as picomoles of melatonin formed per mg tissue per hour, which is the usual definition for one unit of activity. This latter calculation would require the assumption that the specific activity of the S-adenosyl-L-methionine-^{14}C had not altered since its determination by The Radiochemical Centre. As S-adenosyl-L-methionine is an unstable compound (K.G. Oldham, personal communication), such an assumption about its labelled derivatives could be incorrect.

Differences in the weights of pineal tissue assayed in this system were small, thus possible slight variations in the homogenate

volumes would not have been expected to introduce significant errors into the estimations of pineal enzyme activity.

The presence of endogenous substrate (N-acetyl serotonin) in pineal homogenates was tested by adding 50 µl of tris-HCl buffer instead of N-acetyl serotonin to the system. This endogenous substrate accounted for 6 DPM/mg wet weight of tissue, or 10.1 units (as defined above).

Ram pineal glands assayed in this system had HIOMT activities ranging from 32.8 to 236.5 DPM/mg wet wt. tissue, or 59.1 to 426.1 units. Values for pineal HIOMT activity obtained in the present assay encompass the levels determined in yearling sheep (ewes and wethers) by D.J. Kennaway (personal communication) of 73.4 ± 7.4 units, and in ewes by Cardinali, Nagle and Rosner (1974a) who reported mean activities ranging from 50 to 199 units.

8.   HORMONE ASSAYS

(1) Reagents

Phosphate buffered saline (PBS) contained 0.01 M phosphate buffer and 0.14 M sodium chloride, with 0.01% (w/v) sodium merthiolate as a preservative;   pH was adjusted to 7.3.

0.02 M EDTA-PBS and 0.05 M EDTA-PBS were solutions of 0.02 M and 0.05 M ethylene diamine tetra acetic acid, disodium salt (EDTA), respectively in PBS.

PBS-0.1% gelatin was a solution of 0.1% (w/v) gelatin in PBS.

0.01 M EDTA-PBS-0.1% gelatin contained an addition of 0.01 M EDTA in PBS-0.1% gelatin.

PBS-1% EW was prepared by addition of 1% (w/v) egg albumin powder to PBS.

PBS-0.1% BSA and PBS-3% BSA were solutions of 0.1% (w/v) and 3% (w/v) bovine serum albumin, respectively in PBS.

Organic solvents (mainly technical grade) were redistilled before use. Ethanol was purified by refluxing for 8 hours over m-phenylenediamine then redistilled three times.

Scintillation fluid consisted of toluene-Triton X-100 (2:1) containing 3 g 2,5-diphenyl oxazole and 100 mg 1,4-di[2-(5-phenyloxazol)] benzene) per litre.

(2) LH Assay

Ovine-LH was measured by radioimmunoassay following the double-antibody technique of Niswender et al. (1969).

(a) Radioiodination of Ovine-LH With ^{125}I. Radioiodination of ovine-LH was carried out using a modification of the chloramine T method of Greenwood, Hunter and Glover (1963).

One mCi of iodine-125 (100 mCi/ml, The Radiochemical Centre, Amersham) was added to 2 μg of highly purified ovine-LH (LER-1374 A) in 25 μl of 0.5 M phosphate buffer, pH 7.5. Thirty μg of chloramine T in 15 μl of 0.05 M phosphate buffer, pH 7.5, were added to start the reaction. After two minutes the reaction was stopped by the addition of 500 μg of sodium metabisulphite in 50 μl of 0.05 M phosphate buffer, pH 7.5, then 100 μl of a transfer solution consisting of 1% (w/v) potassium iodide, 0.01% (w/v) bromophenol blue and 16% (w/v) sucrose in distilled water, was added. This mixture was then transferred quantitatively to a 1 x 25 cm polyacrylamide gel column (Biogel P60, Biorad Laboratories, U.S.A.) previously equilibrated with a combination of 20 ml of 0.05 M phosphate buffer, pH 7.5 and 1 ml of PBS-1% EW. The reaction container was rinsed with 70 μl of a solution containing 1% (w/v) potassium iodide, 0.01% (w/v) bromophenol blue and 8% (w/v) sucrose in distilled water, which was then also transferred to the column. One ml fractions eluted from the column were collected into tubes containing 1 ml of PBS-1% EW. These fractions were counted for radioactivity then stored frozen until required. Iodinated ovine-LH

was usually most concentrated in fractions three to five, the one containing the highest number of counts being used in the radioimmunoassay.

(b) <u>Preparation of Precipitating Antisera.</u> Antisera raised against rabbit gamma globulin were prepared in sheep by intramuscular injections of 25 mg of rabbit gamma globulin (Fraction II "Pentex", Miles Laboratories Inc., U.S.A.) in an emulsion of 2.5 ml of complete Freund's adjuvant and 2.5 ml 0.9% (w/v) sterile saline. Subsequent booster injections, containing 10 and 25 mg of gamma globulin in an emulsion of incomplete Freund's adjuvant and saline, were given at approximately three weekly intervals.

After titration for capacity to precipitate antibody-bound ^{125}I-LH, sera from individual bleedings were pooled and used at a 1 in 8 dilution with 0.05 M EDTA-PBS for the radioimmunoassays.

(c) <u>Radioimmunoassay Procedure.</u> Polystyrene tubes (11 x 75 mm) were used for all phases of the radioimmunoassay procedure, and both unknown samples and standards were assayed in triplicate.

Standard solutions of ovine-LH (NIH-LH-S18) were prepared in 200 μl of PBS-1% EW, to provide a standard curve corresponding to a range of plasma concentrations from 0 to 8 ng/ml. Hypophysectomized ewe plasma (200 μl) was added to each standard tube, while sample tubes contained 200 μl of unknown plasma. Both standard and sample tubes were then adjusted to contain a volume of 500 μl, by addition of PBS-1% EW. Two hundred μl of rabbit anti-ovine-LH serum (# 15, courtesy of Dr. G.D. Niswender, Colorado State University, U.S.A.), diluted 1 in 80,000, with 0.05 M EDTA-PBS containing non-immune rabbit serum (1 in 400) was added to each tube prior to incubation at $4^{o}C$ for 24 hours. Radioiodinated ovine-LH (approximately 50,000 cpm diluted to 100 μl with PBS-0.1% BSA) was added to each tube and the

incubation was allowed to proceed for a further 24 hours at 4°C.

After adding 200 µl of diluted precipitating antiserum, the mixture was incubated for 72 hours at 4°C, before antibody precipitation was completed by centrifuging at 1900 $\underline{g}$ for 30 minutes at 4°C. The supernatant was then removed by aspiration and the precipitate counted for one minute in a Packard Auto-Gamma Scintillation Spectrometer (Model 5285).

Each assay contained a triplicate of tubes from which the first antibody was omitted, to provide a check on the level of non-specific binding of ^{125}I-LH.

An IBM 1620 computer was used to determine plasma LH concentrations by the method of Burger, Lee and Rennie (1972). With this technique a best-fit expression for the standard curve was calculated, then values for the samples (means $\pm$ standard deviations) were computed. A composite standard curve representing the mean values from seven consecutive assays is shown in Figure 2.9.

(d) <u>Validation of Ovine-LH Radioimmunoassay</u>. The specificity of the ovine-LH antiserum has been determined by Niswender <u>et al.</u> (1969). These authors showed that radioimmunoassay estimates of LH potency were not affected by high levels of follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), growth hormone (GH) or prolactin.

Reduction of the quantity of sample plasma down to 25 µl, did not influence the estimate of its LH concentration so long as the volume of plasma per tube was made up to 200 µl with hypophysectomized ewe plasma (Table 2.2). Thus it was possible to use this assay to measure the LH content of plasma containing up to 64 ng/ml LH. This finding also demonstrated parallelism between the dose-response curves for LH in plasma samples and in standards.

Recovery of hormone added to plasma was not checked since the
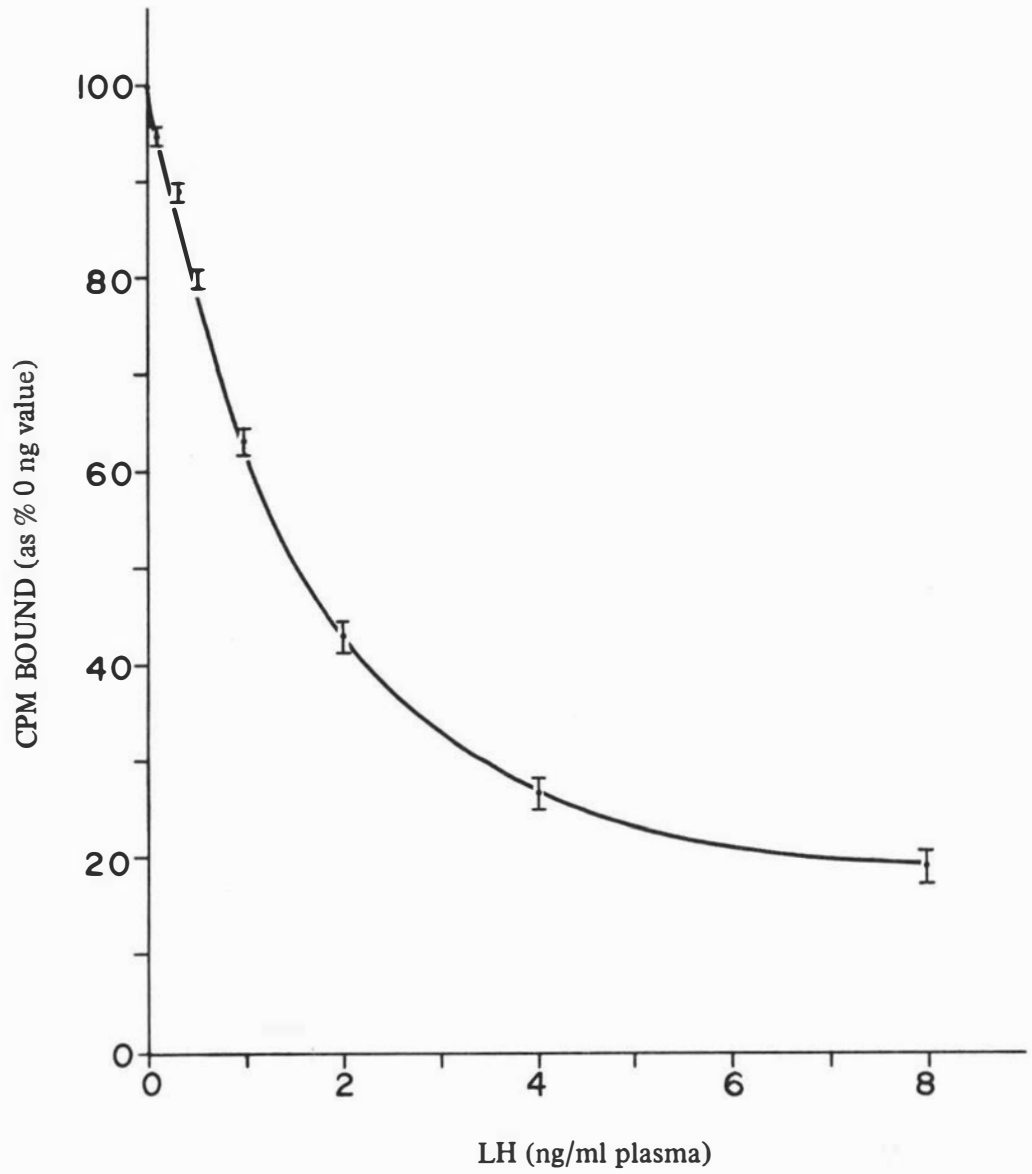
Figure 2.9 : Composite standard curve (mean±S.E. of seven consecutive assays) for ovine LH radioimmunoassay.

Table 2.2

Effect of dilution with hypophysectomized sheep plasma, on estimates of LH concentration : results of five assays.

Plasma LH concentration (ng/ml)*

Undiluted	Diluted 1:1	Diluted 1:3	Diluted 1:7
5.4	5.2	4.9	4.5
4.4	4.3	3.6	3.4
4.8	—	5.0	5.0
4.5	3.9	3.4	3.8
4.0	3.5	3.4	3.8

* Each value represents the mean of a triplicate.

LH standards in the present method were added to tubes containing 200 µl of hypophysectomized ewe plasma, and then processed through the usual procedure. Also the data presented in Table 2.2 indicated that endogenous hormone added to hypophysectomized ewe plasma, was recovered quantitatively.

Non-specific binding, estimated in the absence of unlabelled hormone was always low (less than 16% of the counts bound in the 0 ng/ml standard), and the counts obtained were not subtracted from those recorded for the standards or samples.

Assay sensitivity, defined as the lowest point on the standard curve with a coefficient of variation equal to 50% (Burger, Lee and Rennie, 1972) ranged from 0.04 to 0.11 ng/ml. This range of values corresponded with the lowest plasma LH concentrations with 95% fiducial limits which did not overlap zero.

Reproducibility of assay results was estimated by assaying two plasma samples, four times in each assay, over a number of assays. One sample with a low LH concentration (mean 0.55 ng/ml) had a between-assay coefficient of variation (CV) of 22.5% for four assays, and a within-assay CV of 10.7%. Another sample (mean 4.17 ng/ml) had a between-assay CV of 18.2% in twenty assays, and a within-assay CV of 8.6%.

On the basis of the validation tests performed, it was considered that this assay provided reliable estimates of ovine plasma LH levels.

(3) Prolactin Assay

Ovine prolactin was measured by a double-antibody radioimmunoassay similar to the procedure described above for ovine-LH.

(a) Labelling of Ovine Prolactin With ^{125}I. Radioiodination of ovine prolactin was carried out by the method of Greenwood, Hunter and Glover (1963) with modifications by Fell et al. (1972) and Koprowski

and Tucker (1971), as described by Langley (1974).

One mCi of iodine-125 (100 mCi/ml, The Radiochemical Centre, Amersham) was added to approximately 5 µg of highly purified ovine prolactin (LER-860-2, courtesy of Dr. L.E. Reichert Jr., Emory University, U.S.A.) in 30 µl 0.5 M phosphate buffer, pH 7.5. Twenty µg of chloramine T in 20 µl of 0.05 M phosphate buffer, pH 7.5, were added to start the reaction which was allowed to proceed for 60 seconds. The reaction was stopped by the addition of 20 µg of sodium metabisulphite in 20 µl of 0.05 M phosphate buffer, pH 7.5. Mixing was maintained throughout the period of the reaction and for a further 60 seconds. A transfer solution consisting of 30 µl 1% (w/v) potassium iodide, 0.01% (w/v) bromophenol blue and 16% (w/v) sucrose in 0.05 M phosphate buffer, pH 7.5, plus 30 µl phosphate buffer pH 7.5, and 30 µl hypophysectomized ewe plasma was added. The mixture was then transferred quantitatively to a 0.7 x 18 cm polyacrylamide gel column (Biogel P60, Biorad Laboratories, U.S.A.) which previously had been equilibrated with 15 ml of 0.02 M barbital buffer, pH 8.6, containing 20% (v/v) acetone, and 2 ml of the same buffer containing 5% (w/v) bovine serum albumin. One ml fractions eluted from the column were collected into 1 ml of PBS-0.1% gelatin. Aliquots from these fractions were counted for radioactivity and that containing the greatest number of counts corresponding to radioiodinated prolactin, was further purified on a 1 x 25 cm dextran gel column (Sephadex G100, Pharmacia, Sweden). This column previously had been equilibrated with 25 ml of 0.02 M barbital buffer, pH 8.6 containing 20% (v/v) acetone, and 2 ml of the same buffer containing 5% (w/v) bovine serum albumin. Again 1 ml fractions were collected into 1 ml of PBS-0.1% gelatin and aliquots were counted for gamma radiation. The fraction with the peak level of radioactivity was used in the radioimmunoassay.

(b) <u>Preparation of Prolactin Antiserum.</u> Antiserum (courtesy Prof. D.S. Flux, Massey University) raised against bovine prolactin (NIH-B1) was prepared in rabbits by an initial set of intradermal injections containing a total of 4 mg bovine prolactin in 1 ml of an emulsion of Freund's complete adjuvant and saline. At weekly intervals three further series of injections were made using 2 mg of bovine prolactin in an emulsion of Freund's incomplete adjuvant and saline. One week after the final injections blood was collected from an ear vein and the antiserum subsequently collected after clot contraction.

(c) <u>Radioimmunoassay Procedure.</u> Polystyrene tubes (11 x 75 mm) were used for all phases of the radioimmunoassay procedure, and both standards and unknown samples were assayed in triplicate.

Standard solutions of ovine prolactin (NIH-P-S11) were prepared in 200 µl of PBS-3% BSA, to provide a standard curve corresponding to a range of plasma concentrations from 0 to 256 ng/ml. Hypophysectomized ewe plasma (200 µl) was added to each standard tube, while sample tubes contained 200 µl of unknown plasma. Both standard and sample tubes were then adjusted to contain a volume of 500 µl by addition of 0.01 M EDTA-PBS-0.1% gelatin. Two hundred µl of rabbit anti ovine prolactin serum, diluted 1 in 8,000 with 0.02 M EDTA-PBS containing non-immune rabbit serum (1 in 400), was added to each tube prior to incubation at $4^{o}C$ for 24 hours. Radioiodinated prolactin (approximately 50,000 cpm diluted to 100 µl with 0.01 M EDTA-PBS-0.1% gelatin) was added to each tube and the incubation allowed to proceed for a further 24 hours at $4^{o}C$.* Precipitation of antibody was completed by centrifuging at 1900 g for 30 minutes at $4^{o}C$. The supernatant was removed by aspiration and the precipitate counted for one minute in a Packard Auto-Gamma Scintillation Spectrometer (Model 5285) or an LKB-Wallac Ultrogamma counter (Model 1280).

Non-specific binding and unknown hormone concentrations were

* After adding 200 ul of the precipitating antiserum (as described for the LH assay) diluted 1 in 8, the mixture was incubated for 72 hours at $4^{o}C$.

determined as described for the LH assay. A composite standard curve representing the mean values from six consecutive assays is shown in Figure 2.10.

(d) <u>Validation of Ovine Prolactin Assay.</u> In general, this ovine prolactin assay was validated by conducting tests similar to those described for validation of the LH assay.

Specificity of the prolactin antiserum was indicated by the lack of cross-reaction with the ovine anterior pituitary hormones tested, namely : GH, TSH, LH, FSH and adrenocorticotrophic hormone (ACTH) (see Figure 2.11).

Dilution with hypophysectomized ewe plasma did not affect the estimated prolactin content of two plasma samples (see Table 2.3). Also non-specific binding of ^{125}I-prolactin was low (less than 12% of the counts bound in the 0 ng/ml standard) and thus ignored in the computation of assay results. Assay sensitivity ranged from 1.0 to 2.0 ng/ml and the reproducibility of results was checked by assaying two samples repeatedly in a number of assays. One wether plasma sample (mean 105.4 ng/ml) displayed a between-assay CV of 6.4% in seven assays, and a within-assay CV of 6.2%, based on four estimates per assay. Another wether plasma sample (mean 42.5 ng/ml) displayed a between-assay CV of 10.2% in the seven assays, and a within-assay CV of 9.9%, based on five estimates per assay.

Prolactin levels measured in rams by the assay described above were similar to those reported in the literature (Pelletier, 1973; Chamley <u>et al.</u>, 1974; Forbes <u>et al.</u>, 1975), although both Pelletier and Forbes <u>et al.</u> reported some mean levels higher than 150 ng/ml, which exceeded those obtained in the present studies.

This assay thus seemed to be reliable and accurate.

(4) <u>Testosterone Assays</u>

(a) <u>Protein-binding Assay.</u> Plasma samples from Experiments
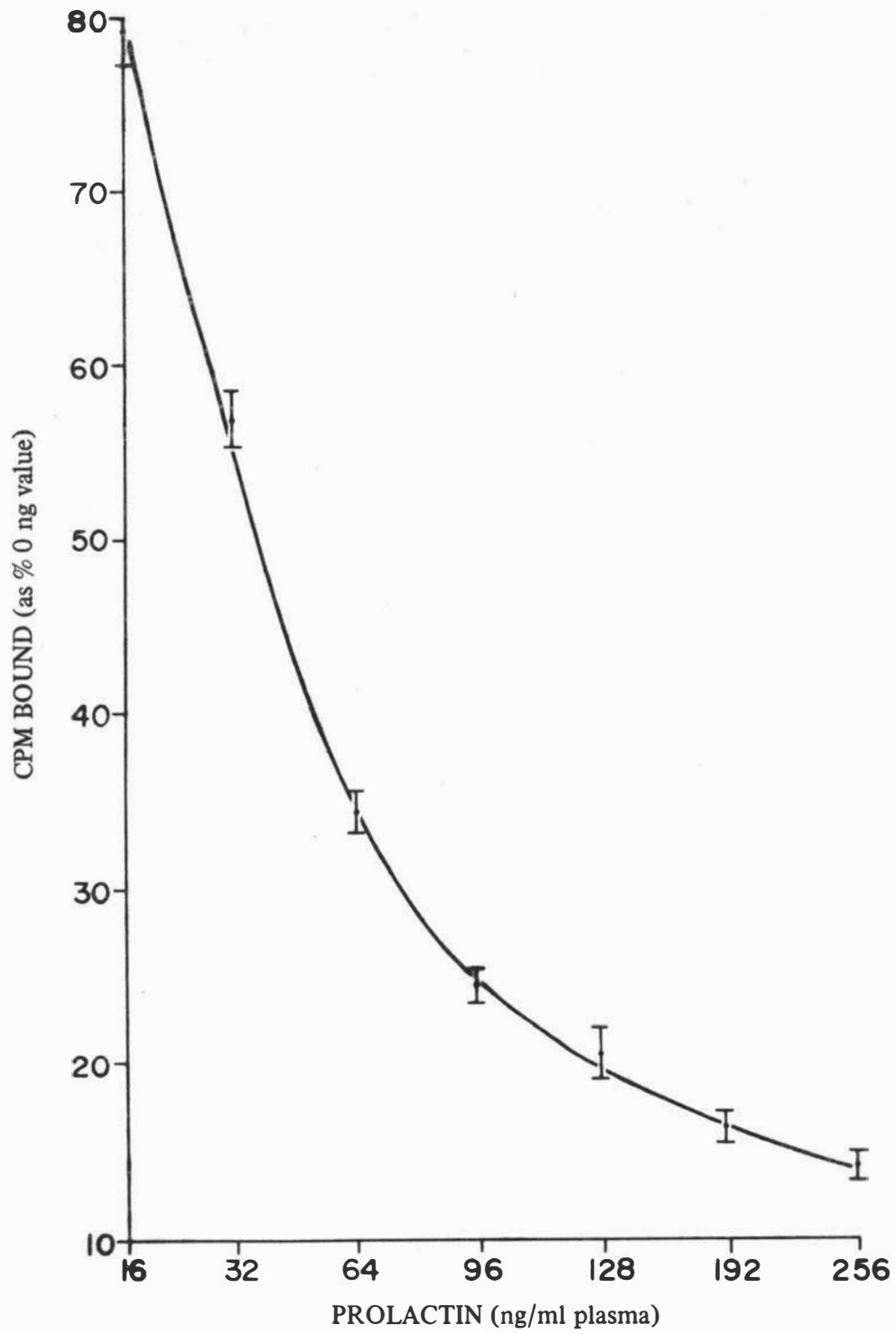
Figure 2.10 : Composite standard curve (mean±S.E. of six consecutive assays) for ovine prolactin radioimmunoassay.

1 TSH, FSH
2 LH, ACTH
3 GH
4 PROLACTIN
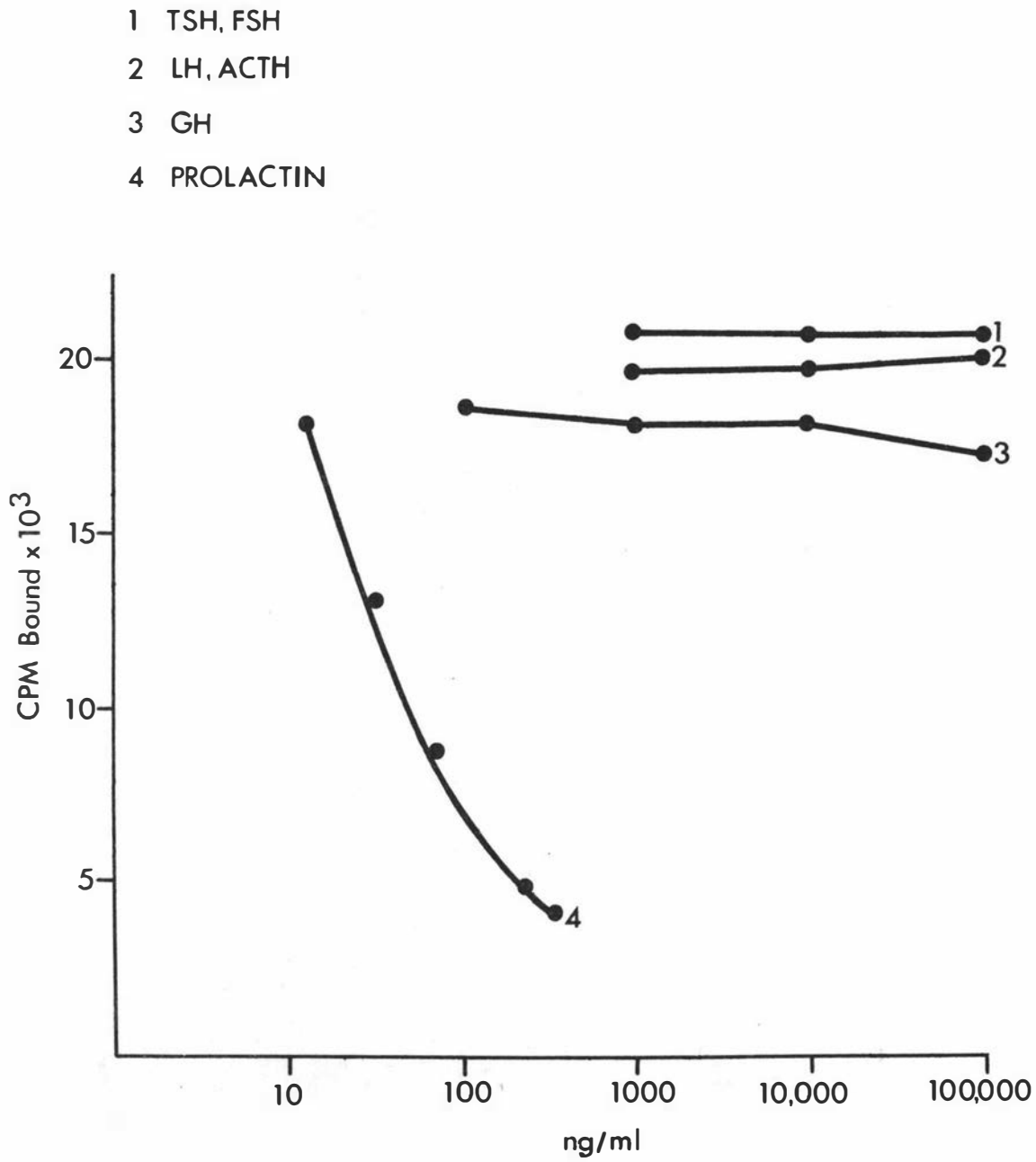
Figure 2.11 : Cross-reaction of anterior pituitary hormones in the ovine prolactin radioimmunoassay.

Table 2.3

Effect of dilution with hypophysectomized sheep plasma, on estimates of prolactin concentration of two plasma samples.

Sample Number	Plasma prolactin concentration (ng/ml)[*]		
	Undiluted	Diluted 1:1	Diluted 1:3
1	56	56	72
	57	66	80
2	80	72	40
	85	84	92
	73	74	64

* Each value represents the mean of a triplicate.

3, 4 and 5 were assayed for testosterone content using a competitive protein-binding assay based on the method described by Anderson (1970).

(i) <u>Extraction Procedure</u>. Duplicate 1 ml plasma samples were alkalinized with 0.1 ml 1 N NaOH, then extracted in 16 x 100 mm glass test tubes by vortex mixing for 20 seconds with 5 ml of toluene-hexane (1:4). After centrifuging the tubes to break any emulsion formed, the aqueous layer was frozen. The solvent layer was then decanted into glass test tubes and evaporated to dryness under air in a water-bath at 45°C. Dried extracts were redissolved in 0.6 ml of 0.15 M sodium chloride containing 4% (v/v) ethanol (ethanol-saline), and stored at 4°C overnight. Extraction efficiency was 84%, as judged by the recovery of tritiated testosterone after extraction and redissolution.

(ii) <u>Assay Procedure</u>. One-half ml aliquots of the redissolved plasma extracts were transferred to 10 x 75 mm glass tubes. Then to each tube was added 1 ml of a solution containing 1% (v/v) human late pregnancy plasma and approximately 45,000 cpm of tritiated testosterone (1α ,2α -^{3}H-testosterone, 56 Ci/mmole, The Radiochemical Centre, Amersham), in equal volumes of 0.09 M phosphate buffer, pH 7.4, and 0.15 M sodium chloride. After mixing, the solution was incubated at 45°C for 10 minutes and then placed in a 1°C ice-water bath for a further 20 minutes. Separation of free and protein-bound steroid was accomplished by adding 40 mg of Florisil (60-100 mesh, Floridin Co., Sweden) and shaking the tubes for 70 seconds in a mechanical shaker. After standing for 20 minutes in the ice-water bath, 0.5 ml aliquots of supernatant were transferred to glass scintillation vials, and 8 ml of scintillation fluid added. Levels of protein-bound tritiated testosterone were counted in a Packard Tri-Carb Liquid Scintillation Spectrometer (Model 2002 or Model 3375).

Duplicate 1 ml samples of pooled wether plasma, containing

additions of 0, 1, 2, 5, 10 and 20 ng testosterone (Mann Research Laboratories, U.S.A.), were processed in the same manner as unknown plasma samples. Standard curves were obtained by plotting cpm bound versus testosterone concentration in these samples of wether plasma. Testosterone concentrations of samples were read directly from the standard curve. A composite standard curve, representing the mean from twelve consecutive assays, is shown in Figure 2.12.

(iii) <u>Validation of Testosterone Assay</u>. Comprehensive studies of the binding of various sex steroids to the sex hormone binding globulin (SHBG) of human plasma have been reported by Kato and Horton (1968), Mayes and Nugent (1968), Maeda <u>et al</u>. (1969), Murphy (1969), Rosenfield, Eberlein and Bongiovanni (1969), Anderson (1970), Horgan and Riley (1974) and Thomas, Gordon and Smid (1974). These reports indicated that the presence of a 17β-hydroxyl group was an essential requirement for this binding, and that the naturally-occurring steroids which displayed highest affinity to SHBG were 5α-dihydrotestosterone, testosterone, 5α-androstane-3β(or α),17β-diol, 5-androstene-3, 17β-diol, 4-androstene-3β(or α),17β-diol, and oestradiol-17β, in that order. With the exception of oestradiol-17β, which would have been eliminated by the alkaline extraction procedure in the present assay (Engel <u>et al</u>., 1950), all the other steroids named above are potent androgens (Murphy, 1969). The evidence available has indicated that testosterone is the principle androgen in ram plasma and that other androgens are present at low concentrations only (Lindner, 1961; Attal, 1970). Also, studies on male human plasma have shown that androgens likely to interfere with this testosterone assay are present at very low concentrations (Ito and Horton, 1970; Tremblay <u>et al</u>., 1970; Gupta, McCafferty and Rager, 1972; Ganjam <u>et al</u>., 1973), or as sulphates (Dessypris and Adlercruetz, 1972). Thus, although on theoretical grounds the present assay is not specific for testosterone,
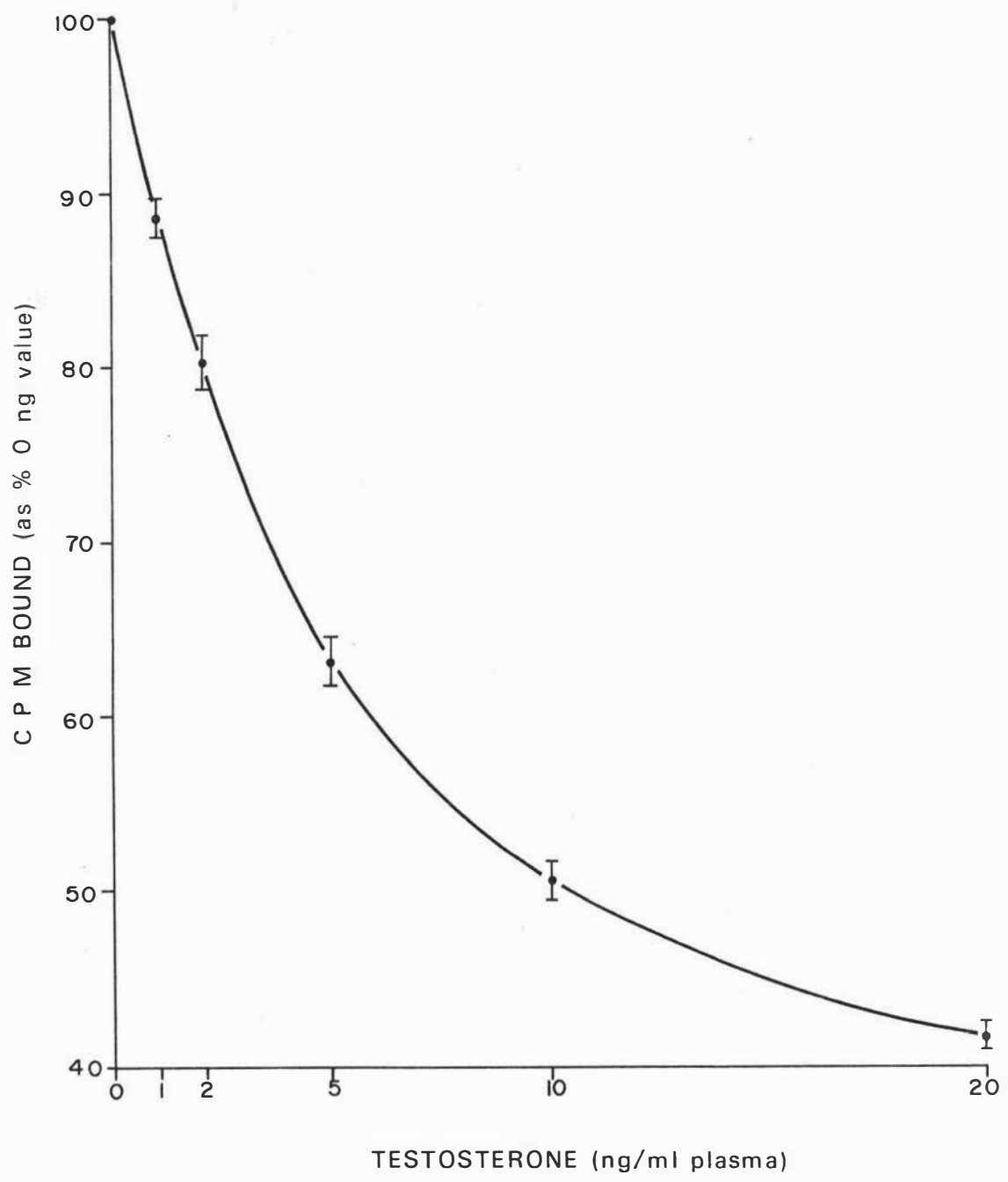
Figure 2.12 : Composite standard curve (mean±S.E. of 12 consecutive assays)
for testosterone protein-binding assay.

only slight over-estimations of testosterone concentrations in ram plasma are likely to have occurred.

Parallelism of the standard curve with a curve produced by assaying varying quantities of androgen, extracted from varying volumes of unknown plasma, was verified by assaying a plasma sample undiluted (10.7±0.8 ng/ml), diluted 1:1 in hypophysectomized ewe plasma (10.2±1.4 ng/ml), and diluted 1:3 (7.8±2.1 ng/ml), (n = 6). Likewise a further plasma sample undiluted (2.4±0.2 ng/ml) and diluted 1:1 in hypophysectomized ewe plasma (2.5±0.2 ng/ml), (n = 6), confirmed parallelism over the useful range of the standard curve.

Recovery of standard testosterone added to wether plasma was quantitative; this result was expected since the standards were added to wether plasma and processed in the same manner as the unknown samples.

Distilled water "blanks" were extracted and assayed by the usual procedure. Also samples of ethanol-saline were processed through the post-extraction steps as a further set of "blanks". Results obtained for both sets of blanks were indistinguishable from the zero standard in wether plasma. This agreement of assay results indicated that the assay system detected only those androgens of testicular origin, and also that the wether plasma did not contain detectable levels of androgens.

Assay sensitivity, defined as the lowest testosterone concentration significantly different from zero (Midgley et al., 1971), varied between 0.5 and 0.8 ng/ml.

Between-assay precision was estimated by assaying two plasma samples in each of twelve assays. The between-assay coefficients of variation (CV) were 12.7% and 22.5% for samples with mean testosterone concentrations of 10.9 ng/ml and 1.0 ng/ml, respectively. Within-assay precision was estimated from the variation between duplicates

for unknown plasma samples over two ranges of hormone concentration. Within-assay CV for fifteen samples was 8.6% in the range 4-7 ng/ml, and 21.6% in the range 1-2 ng/ml.

Plasma testosterone concentrations determined in this assay were consistent with published values for testosterone levels in rams, which range from 0 to 28 ng/ml (Attal, 1970; Johnson, Desjardins and Ewing, 1973; Katongole, Naftolin and Short, 1974; Purvis, Illius and Haynes, 1974; Sanford, Palmer and Howland, 1974a; Sanford et al., 1974b; Falvo et al., 1975; Gomes and Joyce, 1975; Bremner et al., 1976; Lee et al., 1976; Lincoln, 1976a).

(b) Radioimmunoassay I. Plasma samples from Experiment 6 were analysed for testosterone content by a radioimmunoassay based on the method of Smith and Hafs (1973). This assay was developed so that testosterone measurements could be made on smaller volumes of plasma, thereby increasing the number of replicates that could be performed on any one plasma sample. Also radioimmunoassays theoretically offered a higher degree of specificity than protein-binding assays.

(i) Extraction Procedure. Triplicate 100 μl plasma samples were alkalinized with one drop of 0.5 N sodium hydroxide, then extracted with 2 ml of toluene-hexane (1:2) by vortex mixing for 30 seconds in 16 x 100 mm glass test tubes. After freezing the aqueous layer, the solvent was decanted into 10 x 75 mm glass test tubes and evaporated to dryness under air in a water-bath at 45°C. The walls of the tubes were rinsed with 300 μl dichloromethane and the extract again evaporated to dryness. Recovery of tritiated testosterone extracted by this procedure was approximately 90%.

Aliquots of androgen-free wether plasma, containing appropriate additions of testosterone (Mann Research Laboratories, U.S.A.), were extracted in the same manner as the samples to provide a standard

curve over the range 0 to 25 ng/ml.

(ii) <u>Radioimmunoassay Procedure</u>. Two hundred μl of
testosterone antiserum (Pool 667, raised in rabbits against
testosterone-3-(0-carboxymethyl)-oxime-bovine serum albumin, courtesy
Dr. G.D. Niswender, Colorado State University, U.S.A.) diluted 1 in
2,000 in PBS-0.1% gelatin containing non-immune rabbit serum (1 in
200), was added to the bottom of each tube then incubated at room
temperature for 30 minutes. Approximately 15,000 cpm of tritiated
testosterone (1,2,6,7-^{3}H-testosterone, 84 Ci/mmole, The Radiochemical
Centre, Amersham) in 200 μl of PBS-0.1% gelatin were then added and
incubated overnight at 4°C.

Free steroid was precipitated by addition of 300 μl of
dextran-coated charcoal (1% (w/v) dextran T70, Pharmacia, Sweden, and
0.5% (w/v) charcoal, Darco G60, Atlas Chemical Industries, U.K., in
distilled water), incubation at 4°C for ten minutes, then centrifuga-
tion (1900 $\underline{g}$) for ten minutes at 4°C. One-half ml aliquots of
supernatant were transferred to glass scintillation vials, and
following addition of 8 ml of scintillation fluid, the radioactivity
of the bound testosterone was counted in a Packard Tri-Carb
Scintillation Spectrometer (Model 3375).

Calculation of testosterone concentrations in samples was
performed graphically as described for the protein-binding assay. A
composite standard curve showing the mean values from seventeen
consecutive assays is shown in Figure 2.13.

(iii) <u>Validation of Testosterone Radioimmunoassay</u>. Character-
isation of the antiserum carried out by Ismail, Niswender and Midgley
(1972) showed that 5α-dihydrostestosterone was the only steroid likely
to interfere (77% cross reaction) in this radioimmunoassay. However,
for reasons already mentioned with respect to the protein-binding
assay, this cross-reaction with 5α-dihydrotestosterone would have only
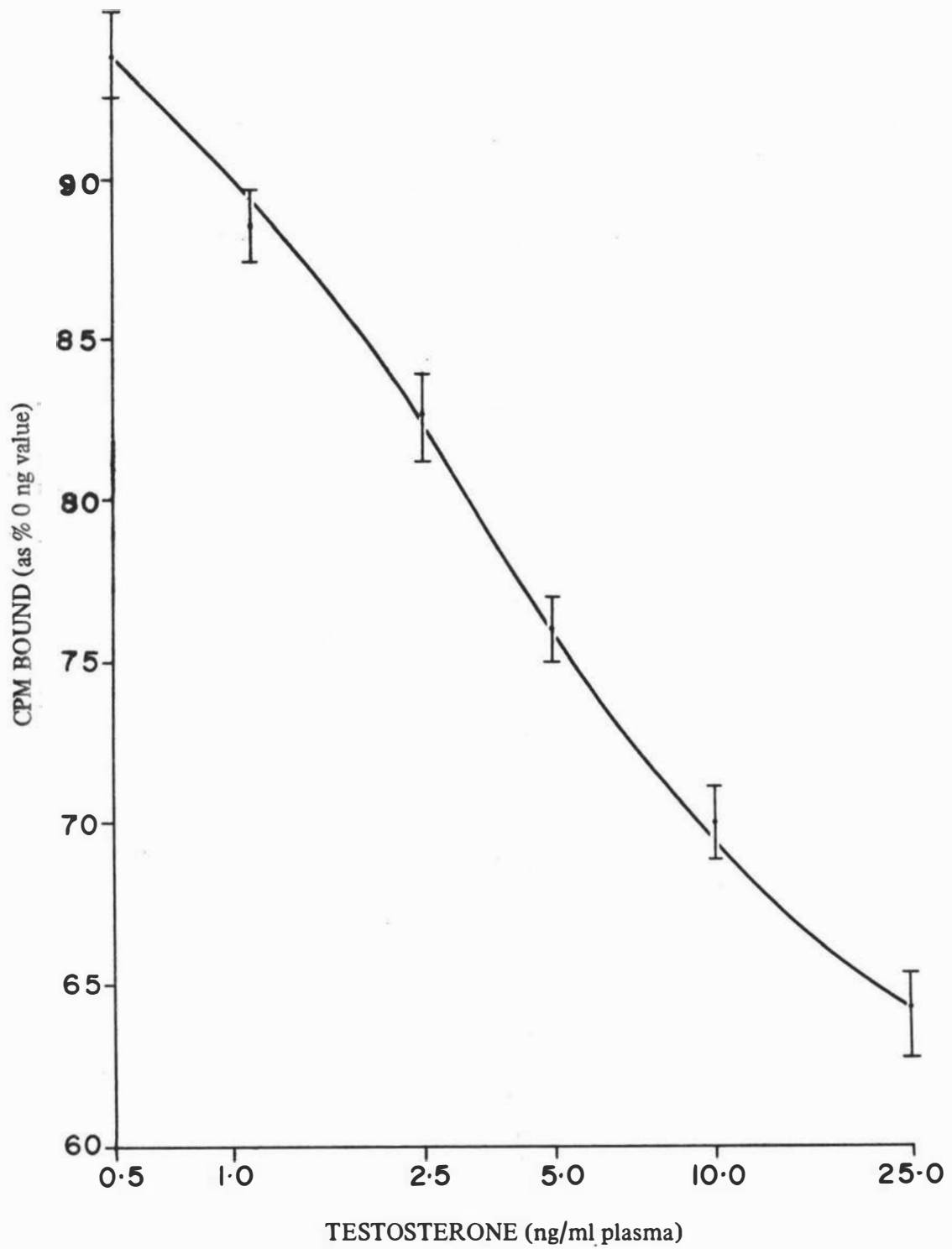
Figure 2.13 : Composite standard curve (mean±S.E. of 17 consecutive assays)
for testosterone radioimmunoassay I.

a minimal effect on the estimation of testosterone levels in ram plasma. Parallelism of the standard curve and a curve obtained by assaying varying quantities of testosterone extracted from a plasma sample was checked by assaying the sample undiluted (10.0 ng/ml), or diluted 1:1 (7.6 ng/ml) and diluted 1:3 (6.0 ng/ml) in wether plasma (n = 3).

The sensitivity of the assay was approximately 0.5 ng/ml. This was a considerable improvement on that of the protein-binding assay for which it was necessary to extract ten times the volume of plasma to give a similar level of sensitivity.

Further evidence for the validity of this radioimmunoassay was obtained from comparisons of hormone levels measured in this and the protein-binding assay. Four samples had values of 0.3 ± 0.1, 2.4 ± 0.2, 7.5 ± 0.5 and 10.7 ± 0.8 ng/ml in the protein-binding assay, and 0.4 ± 0.1, 2.6 ± 0.2, 5.9 ± 0.4 and 10.0 ± 0.7 ng/ml in the radioimmunoassay (n = 6).

Between-assay precision was estimated by repeated assay of plasma samples. Between-assay coefficients of variation (CV) were 38.7% (mean, 1.0 ng/ml), 21.4% (mean, 2.6 ng/ml), 17.7% (mean, 5.9 ng/ml) and 20.0% (mean, 10.0 ng/ml) for four samples assayed in 5, 7, 6 and 8 assays, respectively. The pooled within-assay CV determined from two estimates per assay of the same samples were 26.2%, 39.4%, 19.7% and 18.9%, respectively.

(c) <u>Radioimmunoassay II</u>. Plasma samples from Experiment 7 were assayed for testosterone content by a separate radioimmunoassay based on the method described above, but with modifications, including some described by Terqui and Thimonier (1974) in their progesterone assay.

(i) <u>Extraction Procedure</u>. Single 500 μl plasma samples were extracted with 9 ml of toluene-hexane (1:2) in 16 x 125 mm screw-capped glass culture tubes by shaking for ten minutes in a laboratory shaker.

After freezing the aqueous layer, the solvent was decanted into 16 x 100 mm glass test tubes and evaporated to dryness under air in a water-bath at 40°C. The walls of each tube were rinsed with 1 ml of dichloromethane and the extracts again dried under air. Recovery of tritiated testosterone added to plasma prior to extraction was above 95%.

(ii) <u>Radioimmunoassay Procedure</u>. When dry, the extracts were redissolved in 500 µl of ethanol and three 100 µl aliquots transferred to separate 11 x 75 mm polystyrene test tubes. Testosterone (Mann Research Laboratories, U.S.A.), at appropriate concentrations in 100 µl ethanol, was also added to polystyrene tubes to provide a triplicated series of standards, containing 0 to 5,000 pg testosterone/tube.

After evaporating the ethanol, approximately 25,000 cpm 1,2,6, 7-^{3}H-testosterone (84 Ci/mmole, The Radiochemical Centre, Amersham) in 200 µl PBS-0.1% gelatin and testosterone antiserum (1 in 2,000 with 1 in 200 non-immune rabbit serum, as for the previous testosterone radioimmunoassay system) also in 200 µl PBS-0.1% gelatin, were added to each tube. The mixture was incubated at 40°C for 30 minutes and then at 4°C for two hours.

The methods of separating free from bound steroids by dextran-coated charcoal, and subsequent counting of radioactivity in 0.5 ml aliquots of supernatant, were the same as in the previous testosterone radioimmunoassay, except that the counts bound were determined on a Beckman (Model LS-350) liquid scintillation counter. Computer estimation of assay results was made by the technique described earlier for the protein hormone radioimmunoassays. A composite standard curve showing the mean from twelve consecutive assays is shown in Figure 2.14.

(iii) <u>Validation of Testosterone Radioimmunoassay</u>. As the antiserum used in this assay was identical to that used in the previous
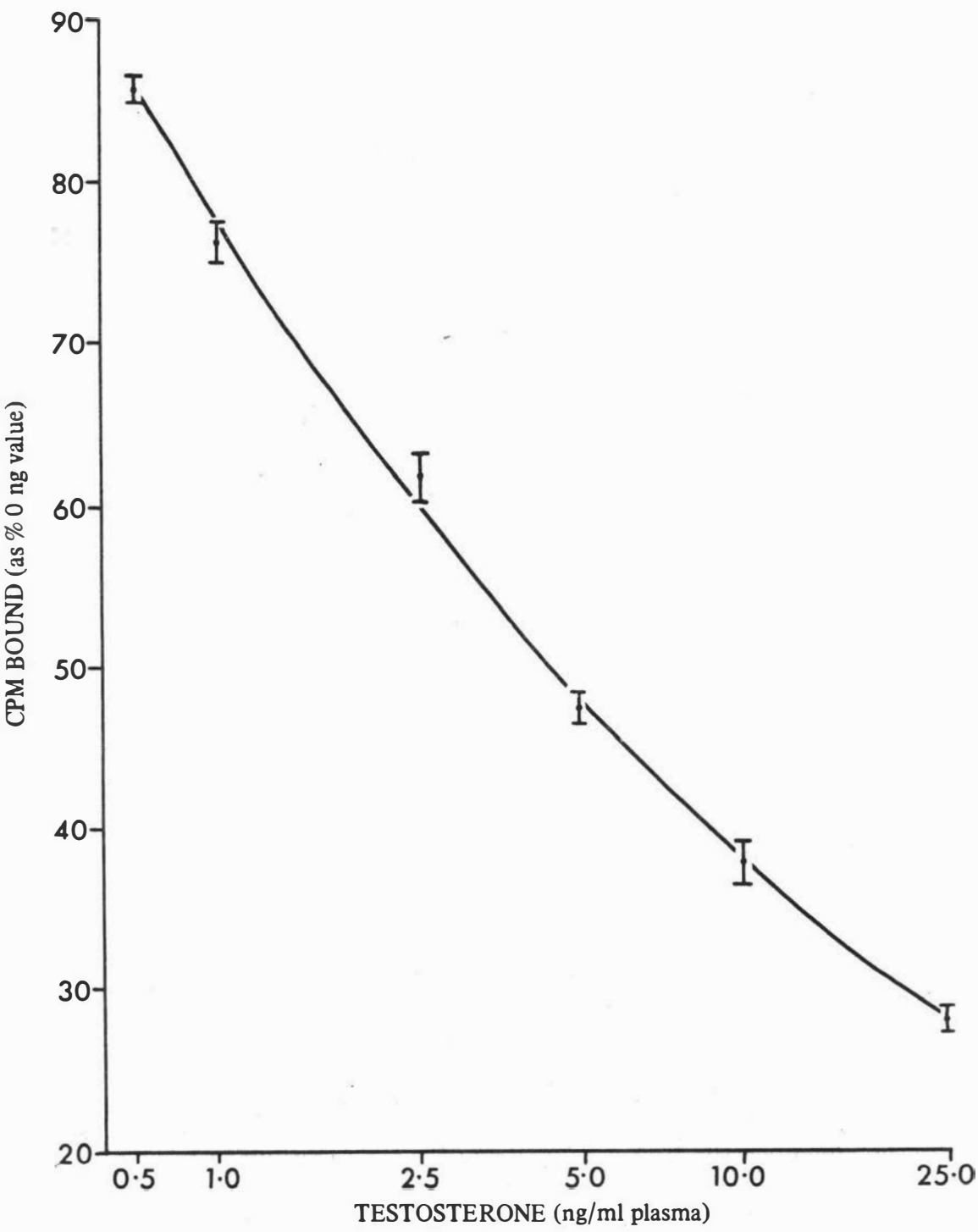
Figure 2.14 : Composite standard curve (mean±S.E. of 12 consecutive assays)
for testosterone radioimmunoassay II.

testosterone radioimmunoassay, the remarks concerning the specificity
of that assay also apply in this case.

Parallelism of the standard curve and curves obtained by
assaying varying quantities of plasma, was checked with two samples,
undiluted (7.3±0.6, 3.5±0.4 ng/ml), diluted 1:1 (7.5±1.7, 3.2±0.7 ng/
ml) and diluted 1:3 (7.9±1.2, 3.7±0.4 ng/ml), respectively (n = 6).
Assay sensitivity (Burger, Leo and Rennie, 1972) ranged from 0.06 to
0.13 ng/ml which was a considerable improvement on the two testosterone
assays used previously in this thesis.

By comparing results obtained in this assay with those from
the previous assay, it was shown that the present method provided
reliable estimates for plasma testosterone concentration.   Three
plasma samples which had testosterone concentrations of 0.4±0.1,
2.6±0.2, 5.9±0.4 ng/ml when assayed by Radioimmunoassay I, were
estimated by the present assay to have testosterone concentrations of
0.19±0.02, 3.52±0.38 and 7.05±0.29 ng/ml, respectively (n = 16).

Assay precision was evaluated by assaying two samples twice
in each of nine assays.   Between-assay coefficients of variation (CV)
were 33.4% and 12.4%, and within-assay CV were 5.8% and 16.6%, for
samples with mean testosterone concentrations of 0.19 and 7.05 ng/
ml, respectively.

Therefore it was considered that this testosterone assay was
a decided improvement on the two earlier ones, both in terms of
sensitivity and precision (lower CV), and also because of the greatly
increased number of samples which could be assayed each day (65 vs
25 and 30 for the protein-binding assay and first radioimmunoassay,
respectively).

(5) Cortisol Assay

Plasma cortisol concentrations were determined by Dr. D.C.
Thurley at Wallaceville Animal Research Centre, Upper Hutt, using

the competitive protein-binding assay of Bassett and Hinks (1969).
This assay was used without modification as outlined below.

Two hundred μl aliquots of ram plasma were deproteinized by
precipitation with 400 μl ethanol, followed by centrifugation.
Duplicate 200 μl aliquots of supernatant were dried and incubated with
500 μl of a solution containing cortisol-binding globulin (CBG) and
tritiated corticosterone ($1\alpha, 2\alpha-^3$H-corticosterone) for 15 minutes in
a water-bath at 45°C, then cooled in iced water for 20 minutes.
Duplicate 200 μl aliquots of standard cortisol solutions in ethanol
were dried and incubated in the same manner, to provide a standard
curve covering the range 0 - 10 ng.   Steroid-free dog plasma
(approximately 0.6% v/v in phosphate buffer) was used as a source of
CBG.   Separation of protein-bound and free labelled steroid was
achieved by transferring the incubation mixture to a small column of
dextran gel (Sephadex G25, Pharmacia, Sweden) and collecting the
initial 1.5 ml of radioactive eluate into scintillation fluid.

Concentrations of cortisol in plasma samples were obtained by
comparing the protein-bound radioactivity (expressed as a percentage
of the protein-bound radioactivity in the 0 ng cortisol standard)
with the standard curve.   According to the authors, the only compound
likely to interfere in this assay, corticosterone, would contribute
little to the values for cortisol concentration obtained from sheep
plasma.

9.   EXPERIMENTAL DESIGN AND ANALYSIS

Details of the experimental designs and of the analyses of
variance used for each experiment are given with the description of
the statistical analyses in the appropriate chapters.

(1)   Analyses of Variance

The analysis of variance applied to each of the variables measured repeatedly in Experiments 3, 4 and 5, could be represented by the following model :

$$Y_{ijkl} = \mu + \alpha_i + \beta_j + \gamma'_{jk} + (\alpha\beta)_{ij} + (\alpha\gamma')_{ijk} + \xi_{ijkl}$$

Where each individual observation (hormone level) or mean of several observations (semen character), made on each animal in each time period or season, was assumed to be the sum of a number of parameters :
μ - the general population mean, and the deviations due to the following fixed effects;

α_i - the 'main effect' of the ith breed or treatment group (i = 3,4),

β_j - the 'main effect' of the jth period or season (j = 1,3,3),

γ'_{jk} - the 'nested effect' of the kth sampling time within the jth period or season (k = 2, ..., 7),

$(\alpha\beta)_{ij}$ - the effect of the interaction of the ith breed or treatment group and the jth period or season,

$(\alpha\gamma')_{ijk}$ - the effect of the interaction of the ith breed or treatment group and the kth sampling time, within the jth period or season,

ξ_{ijkl} - a random error from a distribution with zero mean and homogeneous variance.

Likewise, the analysis of variance applied to each of the variables measured repeatedly in Experiment 6 could be represented by the following model :

$$Y_{ijklm} = \mu + \alpha_i + \beta_j + \gamma_k + \delta'_{kl} + (\alpha\beta)_{ij} + (\alpha\gamma)_{ik} + (\beta\gamma)_{jk}$$
$$+ (\alpha\beta\gamma)_{ijk} + (\alpha\delta')_{ikl} + (\beta\delta')_{jkl} + (\alpha\beta\delta')_{ijkl} + \xi_{ijklm}$$

Where each individual observation (hormone level) or mean of several observations (semen character), made on each animal in each time period, was assumed to be the sum of a number of parameters :
μ - the general population mean, and the deviations due to the following fixed effects;

α_i - the 'main effect' of the ith lighting regime ($i = 2$),

β_j - the 'main effect' of the jth operation ($j = 2$),

γ_k - the 'main effect' of the kth period ($k = 2,3$),

δ'_{kl} - the 'nested effect' of the lth sampling time within the kth period ($l = 3,4,6,7$),

$(\alpha\beta)_{ij}$ - the effect of the interaction of the ith lighting regime and the jth operation,

$(\alpha\gamma)_{ik}$ - the effect of the interaction of the ith lighting regime and the kth period,

$(\beta\gamma)_{jk}$ - the effect of the interaction of the jth operation and the kth period,

$(\alpha\beta\gamma)_{ijk}$ - the effect of the interaction of the ith lighting regime and the jth operation within the kth period,

$(\alpha\delta')_{ikl}$ - the effect of the interaction of the ith lighting regime and the lth sampling time, within the kth period,

$(\beta\delta')_{jkl}$ - the effect of the interaction of the jth operation and the lth sampling time, within the kth period,

$(\alpha\beta\delta')_{ijkl}$ - the effect of the interaction of the ith lighting regime and the jth operation with the 'nested effect' of the lth sampling time, within the kth period,

ξ_{ijklm} - a random error from a distribution with zero mean and homogeneous variance.

The variance was estimated as the mean square due to variation between rams within breeds or treatment groups, by sampling times. A further error variance, between repeated observations or individual rams within each sampling time, which could be estimated for semen characters was not included in the analyses of variance.

All main effects, the nested effects and the interactions were examined by testing the significance of contrasts for individual degrees of freedom, based on orthogonal coefficients (Cochran and

Cox, 1960) which were taken from the tables of Fisher and Yates (1963) or constructed to test specific hypotheses. Individual coefficients were then weighted for differences in : the numbers of rams within breeds or treatment groups, and subdivisions within periods or seasons. Contrasts were constructed a priori and the number of comparisons for any effect did not, by reason of their construction, exceed the number of degrees of freedom for the corresponding mean square.

Where the contrasts were successive terms of a polynomial relationship, the mean squares for individual degrees of freedom were not calculated beyond the third power. Consequently some of the tables showing summaries of analyses of variance contain discrepancies between the number of degrees of freedom available and the number of single degrees of freedom contrasts computed. In these tables "non significant contrasts" refer only to the contrasts which were computed. Contrast numbers in the text refer only to contrasts which are summarized in the tables relevant to each particular results section.

Levels of significance in all analyses are denoted thus :

*	$P < 0.05$
**	$P < 0.01$
***	$P < 0.001$.

(2) Missing Data

Values for missing data were calculated, where necessary by the method described by Cochran and Cox (1960).

(3) Transformations

Prior to their submission as data for statistical analyses, all hormone estimates, except those in Experiments 7.1, 7.2 and 7.3, were transformed to logarithms using the formula :

log hormone concentration $= 100 \log_{10} (x + 1.1)$

where x = plasma hormone concentration in ng/ml.

This transformation was established on empirical grounds by the finding of a linear relationship between the estimated mean and its standard error for subgroups of hormone data.

(4) Computations

All calculations of missing data, transformations of hormone data, and analyses of variance were carried out on an IBM 1620 computer.

The programs used in these analyses included some written by the author and others forming part of a statistical analysis package implemented at Massey University in 1963 by Dr. F.R.M. Cockrem, and variously modified and extended since that date by Prof. R.E. Munford.

CHAPTER III

SEASONALITY OF SEMEN PRODUCTION AND PLASMA HORMONE LEVELS IN
NEW ZEALAND ROMNEY, MERINO, AND POLLED DORSET RAMS AT PASTURE

1. INTRODUCTION

The work described in this chapter was carried out to measure
the extent of the seasonal changes in reproductive characteristics of
rams under local farming conditions. The N.Z. Romney breed was
chosen as this is the predominant breed in New Zealand and, like most
breeds of British origin, has a distinct breeding season. For
purposes of comparison the Merino and Polled Dorset breeds were chosen
since these generally are accepted as having extended breeding seasons
(Belschner, 1972). Hafez (1952) reported that Merino and Dorset
sheep are capable of breeding all year round in some locations.

As pointed out in Chapter I there have been few studies on the
seasonal changes of semen characteristics of rams, and even fewer on
concomitant changes in plasma levels of reproductive hormones. There
have been no reports of any attempt to compare the seasonal changes in
semen characteristics and hormone levels, in different breeds of sheep.

2. MATERIALS AND METHODS

(1) Experimental procedure

Animals studied in Experiment 3 were six N.Z. Romney, five
Merino and four Polled Dorset rams, which were maintained under local
farming conditions. Semen collection and appraisal, and blood
collection were carried out from February 1972 until June 1973, as
described in Chapter II. Blood plasma samples from every fourth week
were assayed for concentrations of LH, testosterone and prolactin.

In June 1973 the rams were slaughtered and subjected to the autopsy procedures described in Chapter II.

(2) <u>Statistical Analyses</u>

(a) <u>Semen data.</u> The time-course of the experiment was divided into ten equal periods, each period representing one-eighth of a year. Within-period data for each semen parameter were pooled to provide a single estimate for every ram. The periods were grouped into three seasons, demarcated by the equinoxes as shown in Figure 3.1, and the orthogonal coefficients used to partition within- and between-seasons effects in the analyses of variance are shown in Table 3.1.

(b) <u>LH and Testosterone Data.</u> Seventeen four-weekly LH and testosterone estimates from each ram were grouped into three seasons as shown in Figure 3.1, the points of division being the solstices. Orthogonal coefficients used to partition the effects of seasons in the analyses of variance are shown in Table 3.2.

(c) <u>Prolactin Data.</u> Sixteen monthly prolactin estimates were grouped into seasons in a similar manner to the semen data, with the points of division again being the equinoxes as shown in Figure 3.1. Orthogonal coefficients used to partition the effects of seasons in the analysis of variance are shown in Table 3.3.

(d) <u>Rationale For Division of Study Period Into Seasons.</u> To perform meaningful contrasts with semen data, it was necessary to divide the time-course of the experiment at the equinoxes so that the period in which ejaculates were obtained by artificial vagina was confined to one season (Season 1). On the other hand, plasma levels of LH and testosterone were expected to show elevations during the breeding season in the autumn, hence it was considered that division of the study period at the solstices would provide the most useful grouping of periods for performing contrasts in the case of those
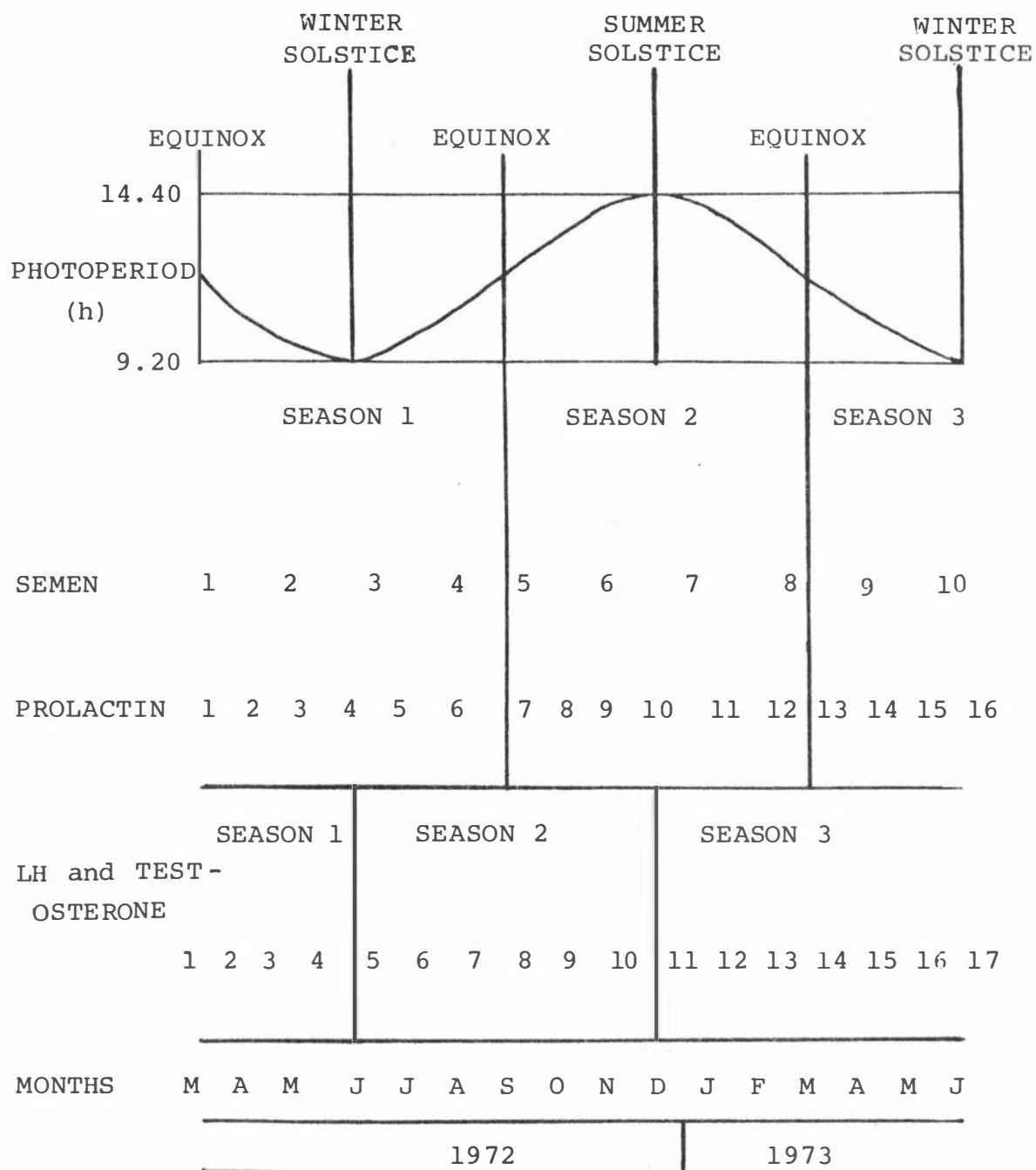
Figure 3.1 : Diagram to show how the time-course of Experiments 3 and 4 was subdivided into sampling periods and seasons for data analyses.

Table 3.1

Orthogonal coefficients* used in partitioning seasonal effects for semen data from Experiments 3 and 4.

Contrast	Season 1				Season 2				Season 3	
Time Periods –	1	2	3	4	5	6	7	8	9	10
Season 1 – Linear	−3	−1	+1	+3	0	0	0	0	0	0
" " – Quadratic	+1	−1	−1	+1	0	0	0	0	0	0
" " – Cubic	−1	+3	−3	+1	0	0	0	0	0	0
Season 2 – Linear	0	0	0	0	−3	−1	+1	+3	0	0
" " – Quadratic	0	0	0	0	+1	−1	−1	+1	0	0
" " – Cubic	0	0	0	0	−1	+3	−3	+1	0	0
Season 3 – Linear	0	0	0	0	0	0	0	0	−1	+1
Season 1 vs Seasons 2 & 3	+3	+3	+3	+3	−2	−2	−2	−2	−2	−2
Season 2 vs Season 3	0	0	0	0	+1	+1	+1	+1	−2	−2

*Coefficients were weighted for unequal group sizes prior to their use in the analyses of variance.

Table 3.2

Orthogonal coefficients[*] used in partitioning seasonal effects for plasma LH and testosterone data from Experiments 3 and 4.

	Season 1				Season 2						Season 3						
Time Periods —	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Contrast																	
Season 1 — Linear	−3	−1	+1	+3	0	0	0	0	0	0	0	0	0	0	0	0	0
" " — Quadratic	+1	−1	−1	+1	0	0	0	0	0	0	0	0	0	0	0	0	0
" " — Cubic	−1	+3	−3	+1	0	0	0	0	0	0	0	0	0	0	0	0	0
Season 2 — Linear	0	0	0	0	−5	−3	−1	+1	+3	+5	0	0	0	0	0	0	0
" " — Quadratic	0	0	0	0	+5	−1	−4	−4	−1	+5	0	0	0	0	0	0	0
" " — Cubic	0	0	0	0	−5	+7	+4	−4	−7	+5	0	0	0	0	0	0	0
Season 3 — Linear	0	0	0	0	0	0	0	0	0	0	−3	−2	−1	0	+1	+2	+3
" " — Quadratic	0	0	0	0	0	0	0	0	0	0	+5	0	−3	−4	−3	0	+5
" " — Cubic	0	0	0	0	0	0	0	0	0	0	−1	+1	+1	0	−1	−1	+1
Season 1 vs Seasons 2 & 3	13	13	13	13	−4	−4	−4	−4	−4	−4	−4	−4	−4	−4	−4	−4	−4
Season 3 vs Season 2	0	0	0	0	−7	−7	−7	−7	−7	−7	+6	+6	+6	+6	+6	+6	+6

[*]Coefficients were weighted for unequal group sizes prior to their use in the analyses of variance.

Table 3.3

Orthogonal coefficients[*] used in partitioning seasonal effects for plasma prolactin data from Experiments 3 and 4.

		Season 1						Season 2						Season 3			
Time Periods –	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Contrast																
Season 1 – Linear	−5	−3	−1	+1	+3	+5	0	0	0	0	0	0	0	0	0	0
" " – Quadratic	+5	−1	−4	−4	−1	+5	0	0	0	0	0	0	0	0	0	0
" " – Cubic	−5	+7	+4	−4	−7	+5	0	0	0	0	0	0	0	0	0	0
Season 2 – Linear	0	0	0	0	0	0	−5	−3	−1	+1	+3	+5	0	0	0	0
" " – Quadratic	0	0	0	0	0	0	+5	−1	−4	−4	−1	+5	0	0	0	0
" " – Cubic	0	0	0	0	0	0	−5	+7	+4	−4	−7	+5	0	0	0	0
Season 3 – Linear	0	0	0	0	0	0	0	0	0	0	0	0	−3	−1	+1	+3
" " – Quadratic	0	0	0	0	0	0	0	0	0	0	0	0	+1	−1	−1	+1
" " – Cubic	0	0	0	0	0	0	0	0	0	0	0	0	−1	+3	−3	+1
Season 1 vs Season 2	+1	+1	+1	+1	+1	+1	−1	−1	−1	−1	−1	−1	0	0	0	0
Season 3 vs Seasons 1 & 2	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	+3	+3	+3	+3

*Coefficients were weighted for unequal group sizes prior to their use in the analyses of variance.

hormones. The direct relationship between daily photoperiod and plasma prolactin levels reported by Pelletier (1973), provided the basis for division of the study period at the equinoxes, so that seasons of short daily photoperiods could be compared with seasons of long daily photoperiods.

(e) Breed Contrasts. Comparisons between the breeds were made using the orthogonal coefficients shown below, weighted for disproportionate group numbers.

Contrast	N.Z. Romney	Merino	Polled Dorset
N.Z. Romney vs Merino & Polled Dorset	+2	-1	-1
Merino vs Polled Dorset	0	+1	-1

3. RESULTS

(1) Semen Data

See Tables 3.4 to 3.10 and Figures 3.2 and 3.3.

The only semen characteristics for which a regular seasonal pattern of change was recorded were ejaculate volume and the three parameters related to seminal fructose levels, all of which reached peak values during the autumn months. Apart from these, motility indices, percentages of motile spermatozoa and percentages of morphologically normal spermatozoa, declined during March and April, 1972, and showed little further change. On the other hand, numbers of spermatozoa per ejaculate declined during the first two months of the study but increased slightly early in the following summer (November, 1972) (Contrasts 1 - 9, Tables 3.9 and 3.10).

Abrupt changes in mean values for ejaculate volume, concentration of spermatozoa per ml and total ejaculate fructose content occurred between Season 1 and Season 2, and corresponded with the change in collection method from predominantly artificial vagina to entirely electrical stimulation (Contrast 8, Tables 3.9 and 3.10). Effects of

Table 3.4

Mean motility indices (scale 0-4) and mean percentages of motile spermatozoa recorded from semen collected in Experiment 3.

Motility index

	Period	N.Z. Romney	Merino	Polled Dorset	Mean
Season 1	1	3.3	3.5	3.6	3.5
	2	2.6	3.2	2.9	2.9
	3	2.2	2.8	3.0	2.7
	4	2.2	2.7	2.2	2.4
	Mean	2.6	3.0	2.9	2.9
Season 2	5	2.4	2.4	2.5	2.4
	6	2.2	2.3	2.4	2.3
	7	2.1	2.4	2.3	2.3
	8	2.2	2.7	2.2	2.4
	Mean	2.2	2.4	2.4	2.4
Season 3	9	2.6	2.4	2.3	2.4
	10	2.0	2.3	2.5	2.3
	Mean	2.3	2.4	2.4	2.4
Overall Mean		2.4	2.7	2.6	

% Motile spermatozoa

	Period	N.Z. Romney	Merino	Polled Dorset	Mean
Season 1	1	61.3	69.9	75.0	68.7
	2	49.8	68.5	55.6	58.0
	3	37.5	53.0	57.5	49.3
	4	39.9	49.0	46.7	45.2
	Mean	47.1	60.1	58.7	55.3
Season 2	5	44.9	47.7	51.7	48.1
	6	40.1	47.1	45.4	44.2
	7	43.7	54.0	48.8	48.8
	8	45.6	53.7	40.6	46.6
	Mean	45.6	50.6	46.6	46.9
Season 3	9	51.6	47.8	48.3	49.2
	10	47.4	42.9	57.8	49.4
	Mean	49.5	45.4	53.1	49.3
Overall Mean		46.2	53.4	52.7	

Table 3.5

Mean ejaculate volumes and mean total fructose content of ejaculates collected in Experiment 3.

	Period	Ejaculate volume (ml) N.Z. Romney	Merino	Polled Dorset	Mean		Period	Total ejaculate fructose content (mg) N.Z. Romney	Merino	Polled Dorset	Mean
Season 1	1	0.98	0.96	0.76	0.90	Season 1	1	5.53	5.65	2.76	4.65
	2	0.72	0.79	0.62	0.71		2	3.18	2.92	2.44	2.85
	3	0.54	0.73	0.65	0.64		3	1.86	2.19	2.68	2.24
	4	0.56	0.61	0.47	0.55		4	1.27	1.32	2.00	1.53
	Mean	0.70	0.77	0.62	0.70		Mean	2.96	3.02	2.47	2.81
Season 2	5	1.10	1.20	1.00	1.10	Season 2	5	3.33	3.70	1.79	2.94
	6	1.12	0.92	1.52	1.19		6	4.26	3.66	4.86	4.26
	7	1.18	0.75	1.31	1.08		7	3.05	3.16	1.56	2.59
	8	1.55	1.96	1.55	1.69		8	11.57	20.04	3.87	11.83
	Mean	1.23	1.20	1.34	1.26		Mean	5.55	7.64	3.02	5.40
Season 3	9	1.56	1.37	1.40	1.44	Season 3	9	9.65	10.83	4.46	8.31
	10	1.28	1.01	1.17	1.15		10	7.35	4.42	3.11	4.96
	Mean	1.42	1.19	1.28	1.29		Mean	8.50	7.62	3.78	6.63
Overall Mean		1.06	1.03	1.04		Overall Mean		5.11	5.79	2.95	

Table 3.6

Mean concentrations of fructose in semen and in seminal plasma of ejaculates collected in Experiment 3.

Seminal fructose concentration (mg/ml)

	Period	N.Z. Romney	Merino	Polled Dorset	Mean
Season 1	1	5.76	6.01	3.63	5.13
	2	4.31	3.98	3.49	3.93
	3	3.47	2.47	4.28	3.41
	4	2.57	2.69	3.52	2.93
	Mean	4.02	3.78	3.73	3.85
Season 2	5	2.68	2.65	1.93	2.42
	6	2.90	3.57	3.15	3.21
	7	2.26	4.44	1.46	2.72
	8	7.24	8.69	2.64	6.19
	Mean	3.77	4.83	2.29	3.63
Season 3	9	6.12	6.61	3.38	5.37
	10	5.31	3.36	2.55	3.74
	Mean	5.71	4.98	2.96	4.55
Overall Mean		4.26	4.45	3.00	

Seminal plasma fructose concentration (mg/ml).

	Period	N.Z. Romney	Merino	Polled Dorset	Mean
Season 1	1	7.26	7.85	4.92	6.68
	2	5.26	5.26	4.90	5.14
	3	4.04	3.29	4.69	4.01
	4	2.99	3.64	4.66	3.76
	Mean	4.88	5.01	4.79	4.89
Season 2	5	3.00	3.23	2.09	2.77
	6	3.62	4.03	3.79	3.81
	7	3.24	5.54	1.85	3.54
	8	8.36	10.04	3.04	7.15
	Mean	4.55	5.71	2.69	4.31
Season 3	9	7.17	8.18	3.89	6.41
	10	6.37	4.18	3.21	4.59
	Mean	6.77	6.18	3.55	5.50
Overall Mean		5.13	5.52	3.70	

Table 3.7

Mean concentrations of spermatozoa/ml and mean numbers of spermatozoa/ejaculate in semen collected in Experiment 3.

Spermatozoa/ml ($\times 10^9$)

	Period	N.Z. Romney	Merino	Polled Dorset	Mean
	1	3.35	3.91	4.01	3.76
	2	3.08	4.22	4.25	3.85
Season 1	3	2.51	3.00	3.02	2.84
	4	2.53	4.60	3.96	3.70
	Mean	2.86	3.93	3.81	3.53
	5	1.40	2.12	1.39	1.64
	6	1.67	1.94	2.73	2.11
Season 2	7	0.86	1.17	2.18	1.40
	8	2.12	2.10	1.66	1.96
	Mean	1.51	1.83	1.99	1.77
Season 3	9	2.03	2.71	1.54	2.09
	10	1.74	1.73	2.89	2.12
	Mean	1.88	2.22	2.21	2.10
Overall Mean		2.13	2.75	2.76	

Spermatozoa/ejaculate ($\times 10^9$)

	Period	N.Z. Romney	Merino	Polled Dorset	Mean
	1	3.09	3.87	3.19	3.38
	2	2.25	3.44	2.72	2.80
Season 1	3	1.30	1.84	2.58	1.91
	4	1.50	2.82	2.05	2.12
	Mean	2.03	2.99	2.63	2.55
	5	1.84	3.03	1.64	2.17
	6	2.79	2.55	4.68	3.34
Season 2	7	1.27	1.28	4.26	2.27
	8	3.36	4.95	3.00	3.77
	Mean	2.31	2.95	3.39	2.88
Season 3	9	4.15	4.06	2.66	3.62
	10	2.60	2.67	3.44	2.90
	Mean	3.37	3.36	3.05	3.26
Overall Mean		2.42	3.05	3.02	

Table 3.8

Mean percentages of unstained and morphologically normal spermatozoa in semen collected in Experiment 3.

		% Unstained spermatozoa						% Morphologically normal spermatozoa			
	Period	N.Z. Romney	Merino	Polled Dorset	Mean		Period	N.Z. Romney	Merino	Polled Dorset	Mean
Season 1	1	76.4	81.9	85.3	8i.2	Season 1	1	88.0	87.8	89.4	88.4
	2	62.7	77.4	77.1	72.4		2	72.0	81.2	85.6	79.6
	3	72.4	82.5	77.3	77.4		3	66.3	80.4	79.8	75.5
	4	70.3	81.3	71.2	74.3		4	67.2	85.6	83.5	78.8
	Mean	70.4	80.8	77.7	76.3		Mean	73.4	83.8	84.6	80.6
Season 2	5	81.9	77.6	79.8	79.8	Season 2	5	70.6	75.8	84.3	76.9
	6	73.5	72.9	78.0	74.8		6	60.1	67.2	64.2	63.8
	7	88.3	77.7	77.9	81.3		7	76.7	61.0	64.1	67.3
	8	75.2	75.9	74.7	75.3		8	53.9	64.9	57.8	58.9
	Mean	79.7	76.0	77.6	77.8		Mean	65.3	67.2	67.6	66.7
Season 3	9	75.7	63.4	76.5	71.9	Season 3	9	68.7	57.8	67.7	64.7
	10	68.9	59.1	75.7	67.9		10	62.0	49.0	68.9	60.0
	Mean	72.3	61.2	76.1	69.9		Mean	65.4	53.4	68.3	62.4
Overall Mean		74.5	75.0	77.4		Overall Mean		68.6	71.1	74.5	

Table 3.9

Experiment 3 : Summary of Analyses of Variance for Semen Data.

Source of Variation	Contrast No.	D.F.	Volume	Motility	% Motile	Sperm./ml	Sperm./ejac.
MAIN EFFECTS							
A. SEASONS		9					
Season 1 – Linear	1	1	6.78*	25.42***	22.29***	1.01	4.73*
" " – Quadratic	2	1	0.25	0.64	0.74	2.23	0.71
" " – Cubic	3	1	0.10	0.45	0.01	6.49*	0.46
Season 2 – Linear	4	1	14.80***	0.10	0.03	0.07	3.37
" " – Quadratic	5	1	7.50**	0.96	0.19	0.02	0.21
" " – Cubic	6	1	4.18*	0.03	1.10	3.06	4.50*
Season 3 – Linear	7	1	4.09*	0.79	0.05	0.02	0.88
Season 1 vs Seasons 2 & 3	8	1	98.68***	34.45***	11.92**	89.31***	2.27
Season 2 vs Season 3	9	1	0.15	0.40	0.37	1.60	0.66
B. BREEDS		2					
N.Z. Romney vs Merino & Polled Dorset	10	1	0.19	5.78*	7.93**	11.18**	3.70
Merino vs Polled Dorset	11	1	0.04	0.05	0.00	0.01	0.00
INTERACTION (AxB)		18					
Season 1 – Cubic x Contrast 10	12	1	0.73	5.18*	4.08*	0.11	0.13
(Seasons 1 vs 2 & 3) x Contrast 11	13	1	8.60**	24.45***	21.18***	11.02**	3.26
Season 2 – Quadratic x Contrast 11	14	1	13.26***	0.25	0.12	4.50*	12.79***
Season 3 – Linear x Contrast 11	15	1	0.07	0.82	0.68	4.65*	1.21
Non significant contrasts		14	0.55	0.62	0.65	0.34	0.38
Residual Mean Square		120	0.14	0.34	205.22	1.17	3.37

(Key, Semen Parameters : Volume = ejaculate volume; Motility = motility index; % Motile = percentage of motile spermatozoa; Sperm./ml = concentration of spermatozoa/ml; Sperm./ejac. = number of spermatozoa/ejaculate.)

Table 3.10

Experiment 3 : Summary of Analyses of Variance for Semen Data.

Source of Variation	Contrast No.	D.F.	Fr. Conc.	Fr. Cont.	S.P. Fr. Conc.	% Unstained	% Normal
MAIN EFFECTS							
A. SEASONS		9					
Season 1 – Linear	1	1	9.82**	6.50*	14.33***	2.02	4.82*
" " – Quadratic	2	1	0.47	0.38	1.10	1.90	5.11*
" " – Cubic	3	1	0.11	0.21	0.01	1.62	1.26
Season 2 – Linear	4	1	21.35***	40.93***	22.70***	0.33	8.72**
" " – Quadratic	5	1	7.89**	22.58***	6.08*	0.00	1.38
" " – Cubic	6	1	4.89*	11.11**	3.85	3.16	1.53
Season 3 – Linear	7	1	5.18*	6.80*	4.87*	0.80	0.37
Season 1 vs Seasons 2 & 3	8	1	0.00	29.11***	0.78	0.24	37.26***
Season 2 vs Season 3	9	1	5.47*	3.01	7.01**	10.25**	1.22
B. BREEDS		2					
N.Z. Romney vs Merino & Polled Dorset	10	1	2.56	1.80	1.59	1.07	2.49
Merino vs Polled Dorset	11	1	11.82***	15.77***	13.18***	0.91	3.59
INTERACTION (AxB)		18					
Season 1 – Cubic x Contrast 10	12	1	0.69	0.15	0.50	4.08*	1.51
(Seasons 1 vs 2 & 3) x Contrast 10	13	1	2.90	2.24	5.09*	77.82***	23.69***
Season 1 – Linear x Contrast 11	14	1	4.52*	1.48	4.89*	2.46	0.00
Season 2 – Linear x Contrast 11	15	1	8.53**	20.07***	8.30**	0.31	1.03
(Seasons 1 vs 2 & 3) x Contrast 11	16	1	7.40**	6.59*	8.24**	4.92*	0.02
(Seasons 2 vs 3) x Contrast 11	17	1	0.08	0.14	0.01	4.66*	3.00
Non significant contrasts		12	1.24	1.98	1.31	0.30	0.72
Residual Mean Square		120	3.74	11.10	4.89	112.48	227.14

(Key, Semen Parameters : Fr. Conc. = fructose concentration of semen; Fr. Cont. = total ejaculate fructose content;
   S.P. Fr. Conc.= fructose concentration of seminal plasma; % Unstained = percentage of unstained spermatozoa;
   % Normal = percentage of morphologically normal spermatozoa)
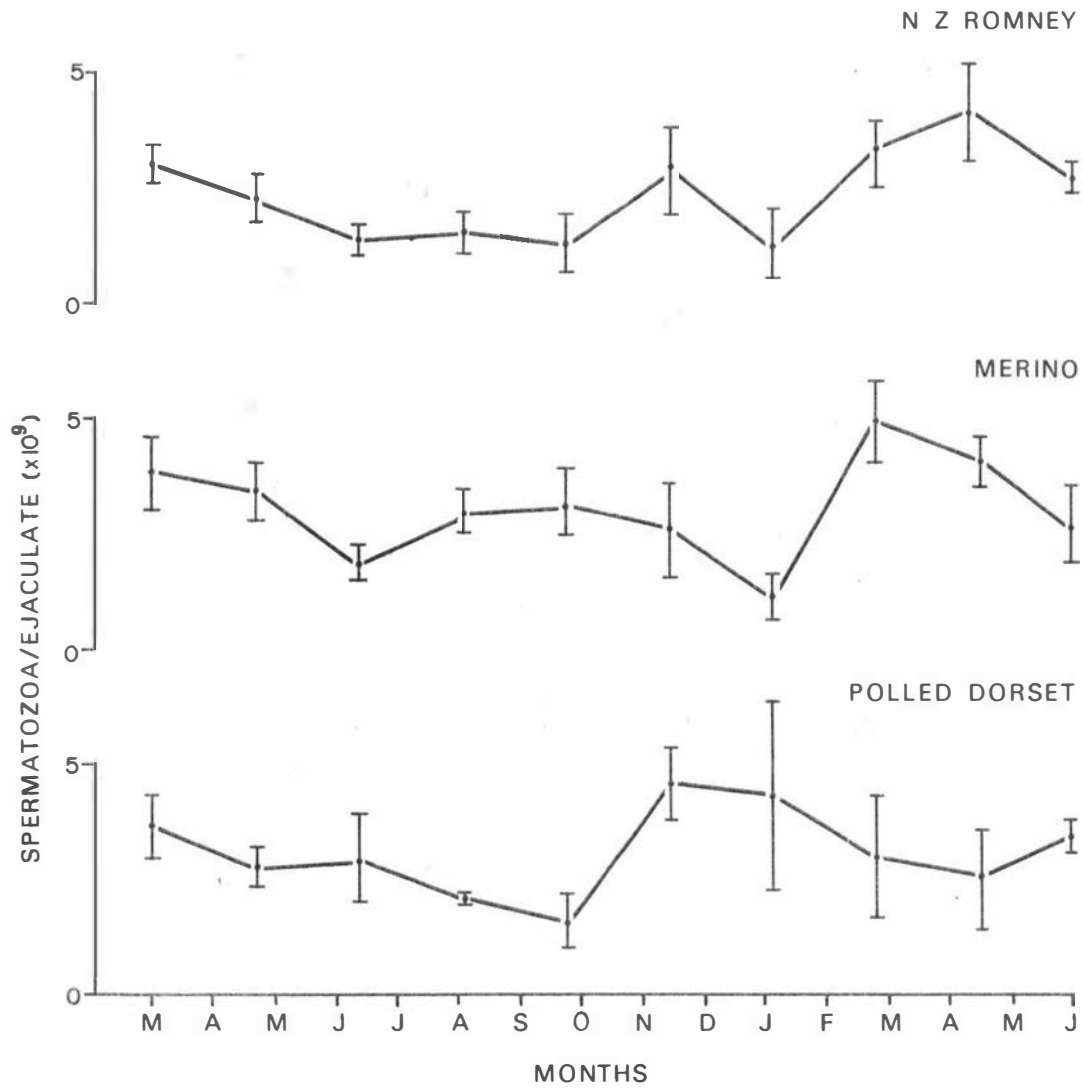
Figure 3.2 : Seasonal variations in numbers of spermatozoa/ejaculate (mean±S.E.) in semen collected from N.Z. Romney, Merino and Polled Dorset rams, between March 1972 and June 1973.
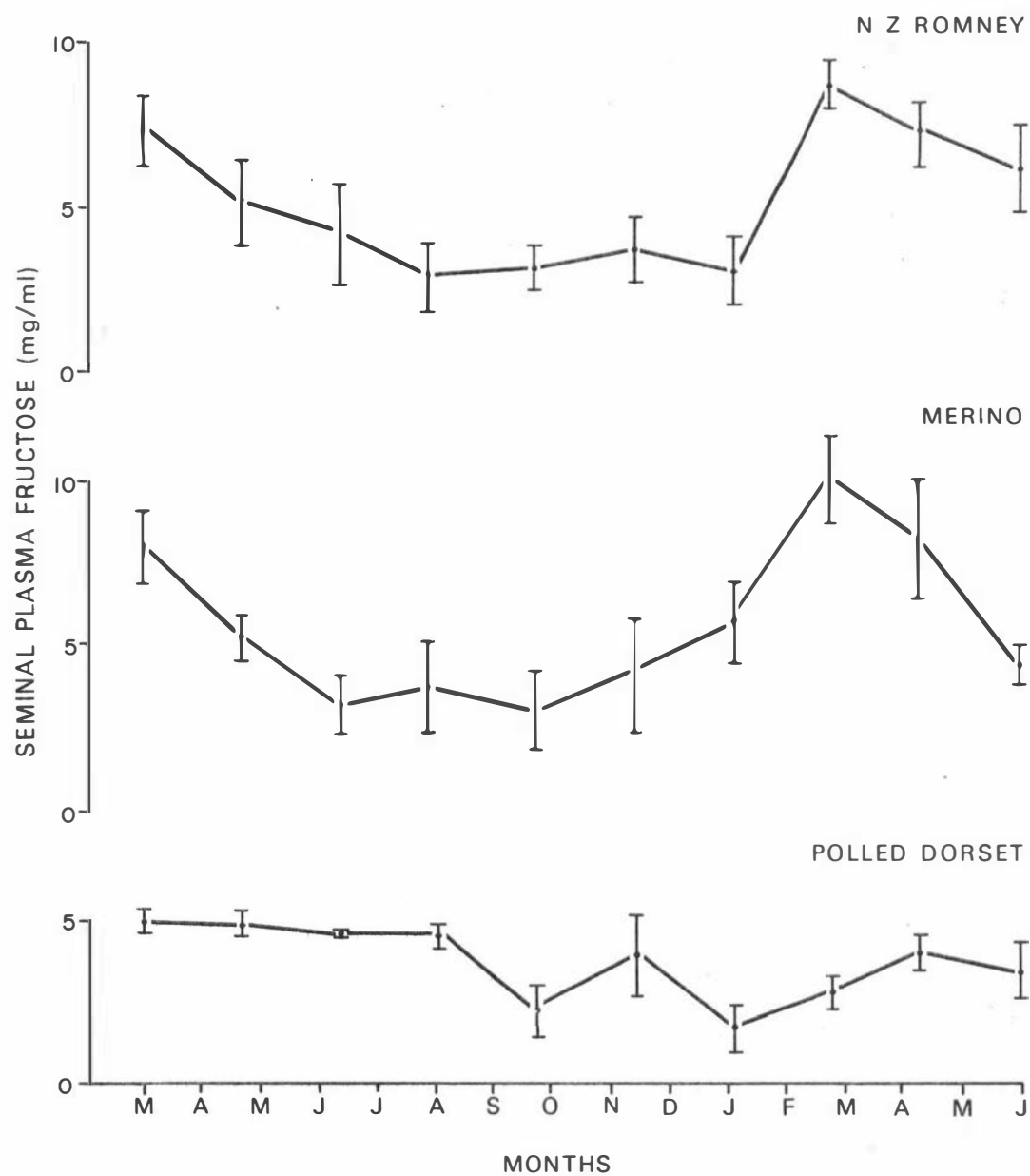
Figure 3.3 : Seasonal variations in seminal plasma fructose concentrations (mean±S.E.) in semen collected from N.Z. Romney, Merino and Polled Dorset rams, between March 1972 and June 1973.

seasonal changes on semen characteristics during Seasons 1 and 2 would have been confounded by the change in collection method, however it was likely that seasonal effects accounted for the higher mean values recorded in Season 1 for: motility index, percentage of motile spermatozoa and percentage of morphologically normal spermatozoa.   In the late autumn months of 1973 (April, May and June), percentages of unstained spermatozoa were lower than in the summer of 1972-1973 (Contrast 9, Table 3.10).

Semen from N.Z. Romney rams had lower overall mean values for motility index, percentage of motile spermatozoa and concentration of spermatozoa per ml than did that from Merino and Polled Dorset rams (Contrast 10, Table 3.9).   This difference between breeds was especially evident in Season 1, as indicated by the interaction of Seasons x Breeds, for these parameters (Contrast 13, Table 3.9). Similar significant interactions were recorded for ejaculate volumes, and percentages of unstained and morphologically normal spermatozoa (Contrast 13, Tables 3.9 and 3.10).

All three measures of seminal fructose levels were higher in semen from Merino rams than in that from Polled Dorsets (Contrast 11, Table 3.10).   This result, in conjunction with the significant interaction contrasts (Contrasts 13, 14 and 15, Table 3.10), reflected the virtual absence of an autumn peak in these parameters for the semen of Polled Dorsets.

During the mid-summer months (December 1972, January 1973) the Polled Dorset rams produced semen containing elevated spermatozoal numbers in comparison to that from Merinos (Contrast 14, Table 3.9). At the same time Merino rams had lower ejaculate volumes than the Polled Dorsets (Contrast 14, Table 3.9).   Of the three breeds, Merinos showed the greatest decline in percentages of unstained spermatozoa during the course of the experiment (Contrasts 13 and 17,

Table 3.10).

(2) Plasma Hormone Data

(a) L.H. See Tables 3.11, 3.12 and Figure 3.4.

Although changes in plasma LH levels were less marked than for testosterone, there was a seasonal pattern with maximal levels being recorded during the summer months (November to March), and lower levels during the midwinter months (April to August). Peak mean levels were 0.88 ± 0.31 ng LH per ml plasma while midwinter levels often were below the limit of sensitivity of the LH assay (0.04-0.11 ng/ml).

With the orthogonal coefficients used for making contrasts in the Seasons x Breeds interaction, no significant differences were detected. However, the Merinos did appear to have much lower peak plasma LH levels than the N.Z. Romney or Polled Dorset rams.

(b) Testosterone. See Tables 3.11, 3.12 and Figure 3.5.

All three breeds exhibited a very marked peak of plasma testosterone levels during January, February and March, 1973, while minimum levels occurred from May to November in both years. Declining plasma testosterone levels from March 1972 indicated the probable presence of a peak in the previous January-February period, similar to that recorded in 1973. This decline in plasma testosterone levels during the autumn of 1972 appeared to be more pronounced in Merino and Polled Dorset rams than in the N.Z. Romneys. Consequently, in Season 1, mean plasma testosterone levels were higher in the N.Z. Romneys than in the other two breeds (Seasons x Breeds interaction contrasts 11 and 14).

(c) Prolactin. See Tables 3.13, 3.14 and Figure 3.6.

Plasma prolactin levels showed a well-defined seasonal pattern with elevated levels during the summer months (November to April) and depressed levels during winter (May to September) (Contrasts 1-5,7,8 10,11). Maximum levels were recorded in November 1972 and minimum

Table 3.11

Mean plasma LH and testosterone concentrations recorded from rams in Experiment 3.
(Values presented are $100 \log_{10}(x + 1.1)$, where x is hormone concentration in ng/ml.)

| | | Luteinizing Hormone | | | | | | Testosterone | | | |
	Period	N.Z. Romney	Merino	Polled Dorset	Mean		Period	N.Z. Romney	Merino	Polled Dorset	Mean
Season 1	1	14.7	21.8	15.3	17.3	Season 1	1	77.4	68.0	59.6	68.3
	2	12.6	5.0	5.2	7.6		2	79.1	35.6	24.2	46.3
	3	10.1	14.4	6.5	10.3		3	30.8	21.3	22.8	25.0
	4	7.5	4.1	4.1	5.1		4	53.0	15.4	31.9	33.4
	Mean	11.2	11.3	7.8	10.1		Mean	60.1	35.1	34.6	33.4
Season 2	5	13.9	7.0	19.1	13.3	Season 2	5	45.9	22.8	56.1	41.6
	6	15.1	11.6	5.0	10.6		6	46.8	14.9	45.4	35.7
	7	11.5	12.1	10.1	11.2		7	54.0	49.5	33.1	45.5
	8	13.4	19.8	21.0	18.1		8	42.6	54.5	45.9	47.7
	9	23.3	16.0	21.9	20.4		9	44.9	57.5	70.9	57.8
	10	9.9	8.1	15.8	11.3		10	37.6	43.8	58.5	46.6
	Mean	14.5	12.4	15.5	14.1		Mean	45.3	40.5	51.6	45.8
Season 3	11	29.0	16.4	30.1	25.2	Season 3	11	65.9	103.6	95.8	88.4
	12	24.6	10.6	17.7	17.6		12	93.0	90.3	114.3	99.2
	13	20.0	9.3	19.4	16.2		13	82.3	85.7	87.4	85.1
	14	17.8	11.4	16.5	15.2		14	77.2	87.8	101.2	88.7
	15	6.4	6.3	5.3	6.0		15	38.2	13.9	35.7	29.3
	16	7.1	4.1	4.1	5.1		16	30.5	13.8	18.0	20.8
	17	4.9	4.6	5.4	5.0		17	18.2	20.7	8.6	15.8
	Mean	15.7	9.0	14.1	12.9		Mean	57.9	59.4	65.9	61.0
Overall Mean		14.2	10.7	13.1		Overall Mean		54.0	47.0	53.5	

Table 3.12

Experiment 3 : Summary of Analyses of Variance for LH and Testosterone Data.

Source of Variation	Contrast No.	D.F.	LH	Testosterone
			Variance Ratios	
MAIN EFFECTS				
A. SEASONS		16		
Season 1 – Linear	1	1	7.22**	18.77***
" " – Quadratic	2	1	0.70	5.52*
Season 2 – Cubic	3	1	8.41**	2.55
Season 3 – Linear	4	1	42.56***	156.36***
" " – Quadratic	5	1	0.80	8.80**
" " – Cubic	6	1	0.15	15.00***
Season 1 vs Seasons 2 & 3	7	1	4.52*	8.35**
Season 3 vs Season 2	8	1	0.66	17.75***
Non significant contrasts		3	1.59	1.62
B. BREEDS		2		
N.Z. Romney vs Merino & Polled Dorset	9	1	2.98	1.36
Merino vs Polled Dorset	10	1	1.03	0.08
INTERACTION (AxB)		32		
Season 1 – Cubic x Contrast 9	11	1	2.78	9.83**
Season 2 – Linear x Contrast 9	12	1	0.39	4.31*
Season 3 – Linear x Contrast 9	13	1	1.62	6.50*
(Seasons 1 vs 2 & 3) x Contrast 9	14	1	0.07	14.05**
Non significant contrasts		18	0.85	0.73
Residual Mean Square		204	112.34	617.48
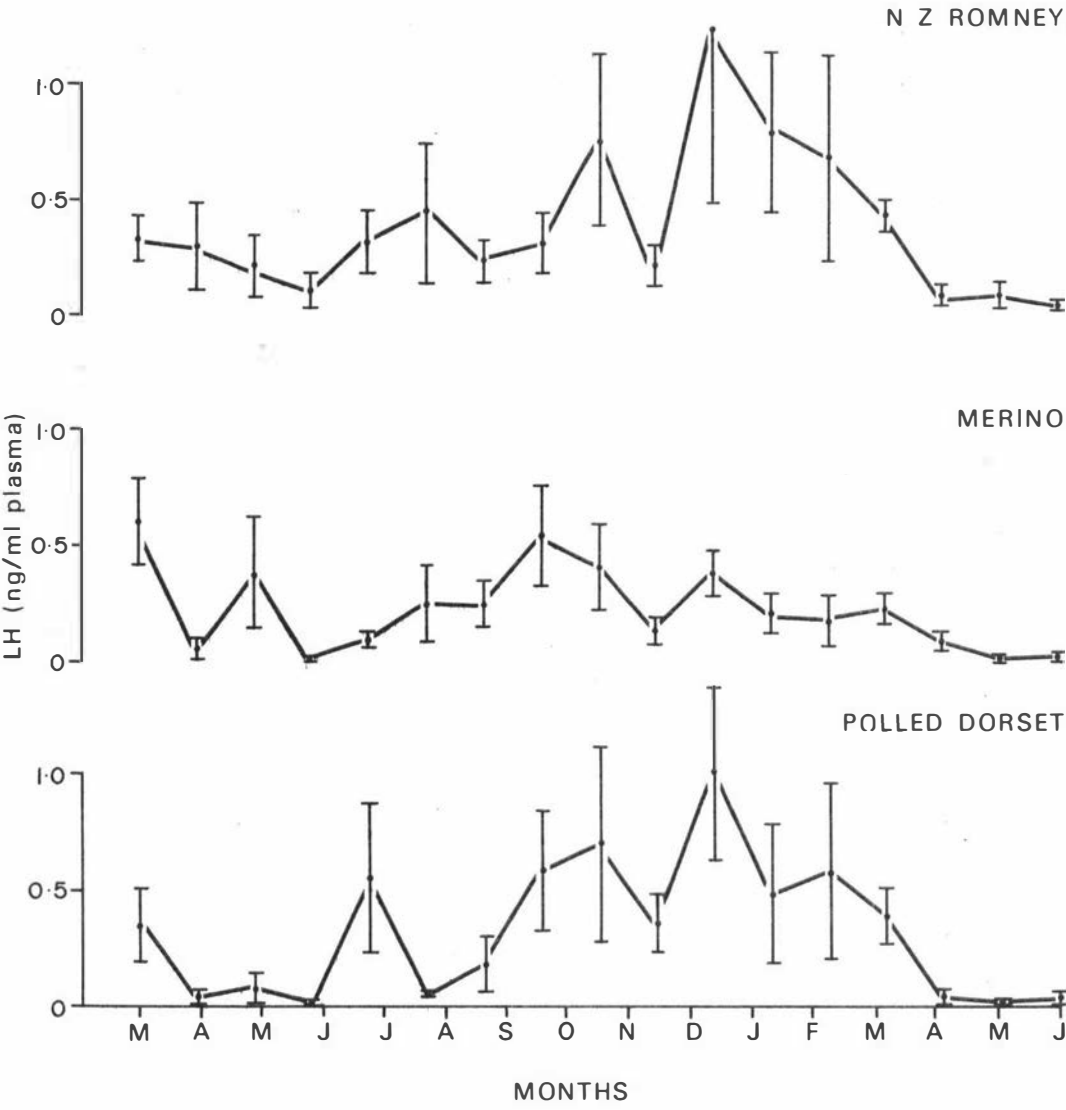
Figure 3.4 : Seasonal variations in plasma LH concentrations
(mean±S.E.) recorded from N.Z. Romney, Merino and Polled Dorset
rams, between March 1972 and June 1973.
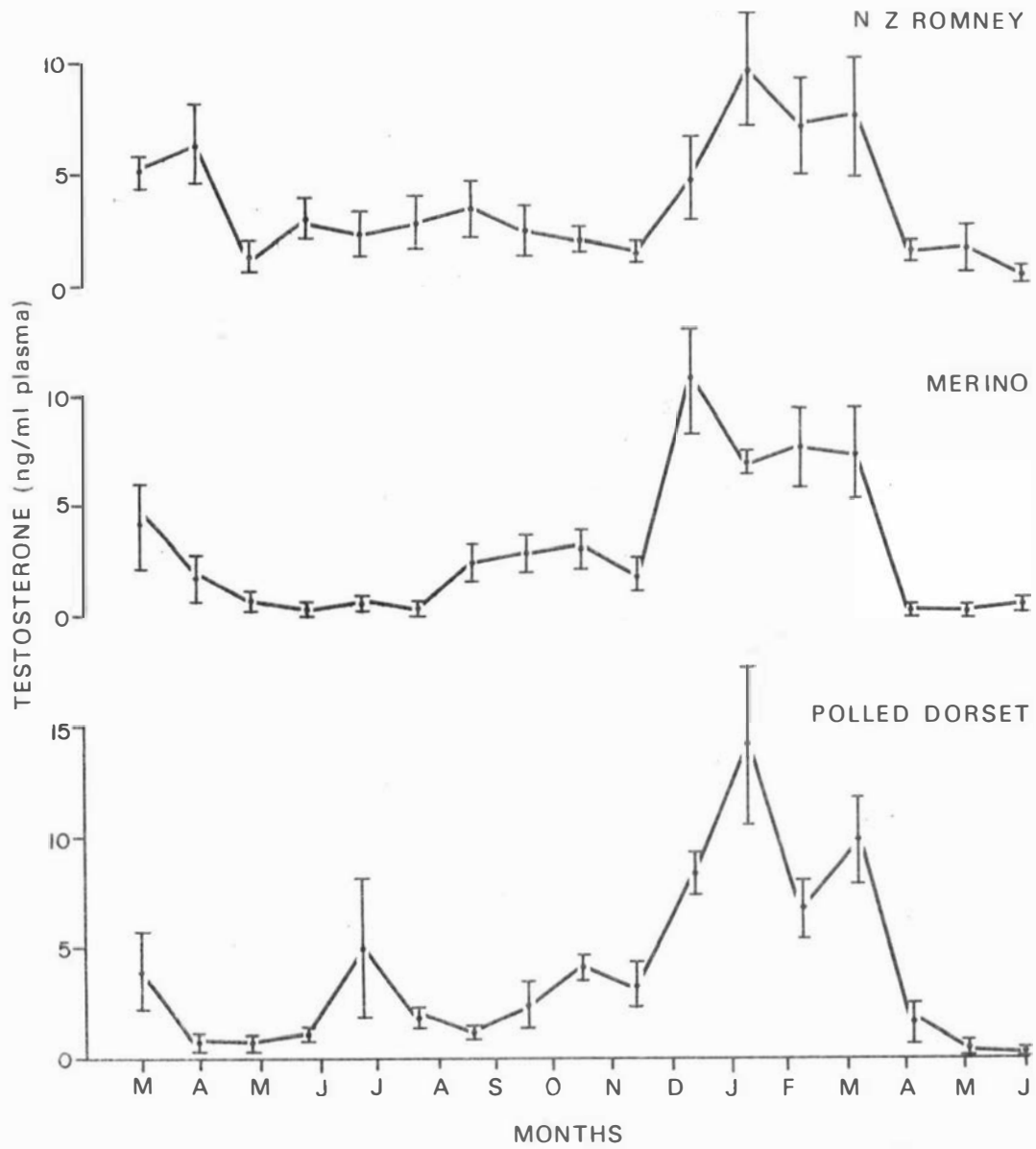
Figure 3.5 : Seasonal variations in plasma testosterone concentrations (mean±S.E.) recorded from N.Z. Romney, Merino and Polled Dorset rams, between March 1972 and June 1973.

Table 3.13

Mean plasma prolactin concentrations recorded from rams in Experiment 3.
(Values presented are 100 $\log_{10}(x + 1.1)$, where x is hormone concentration in ng/ml.)

	Period	N.Z. Romney	Merino	Polled Dorset	Mean
	1	115.9	221.1	82.6	101.2
	2	155.8	154.2	154.2	173.7
Season 1	3	42.1	111.6	30.9	61.5
	4	97.3	130.6	38.3	88.7
	5	103.0	131.2	94.6	109.6
	6	92.3	171.9	128.9	131.0
	Mean	101.1	163.4	88.2	95.2
	7	141.4	176.3	138.3	152.0
	8	143.1	166.6	168.4	159.4
Season 2	9	213.3	212.9	219.8	215.2
	10	173.2	182.6	199.8	185.2
	11	185.0	198.8	212.3	198.7
	12	134.7	193.6	180.6	169.6
	Mean	165.1	188.5	186.5	180.0
	13	164.0	220.5	203.2	195.9
Season 3	14	123.1	170.1	147.4	146.9
	15	59.7	157.5	63.9	93.7
	16	89.0	153.4	66.7	103.0
	Mean	108.9	175.4	120.3	134.9
	Overall Mean	127.1	175.8	133.1	

Table 3.14

Experiment 3 : Summary of Analysis of Variance for Prolactin Data.

Source of Variation	Contrast No.	D.F.	Variance Ratio
MAIN EFFECTS			
A. SEASONS		15	
Season 1 – Linear	1	1	5.90*
" " – Quadratic	2	1	56.87***
" " – Cubic	3	1	5.00*
Season 2 – Linear	4	1	4.08*
" " – Quadratic	5	1	13.51***
" " – Cubic	6	1	0.31
Season 3 – Linear	7	1	50.49***
" " – Quadratic	8	1	7.81**
" " – Cubic	9	1	2.03
Season 1 vs Season 2	10	1	107.36***
Season 3 vs Seasons 1 & 2	11	1	5.35*
B. BREEDS		2	
N.Z. Romney vs Merino & Polled Dorset	12	1	27.03***
Merino vs Polled Dorset	13	1	40.79***
INTERACTION (AxB)		30	
Season 1 – Cubic x Contrast 12	14	1	21.97***
" " – Linear x Contrast 13	15	1	5.75*
Season 3 – Linear x Contrast 13	16	1	5.34*
(Season 1 vs Season 2) x Contrast 13	17	1	22.43***
Non significant contrasts		18	0.99
Residual Mean Square		192	1590.23
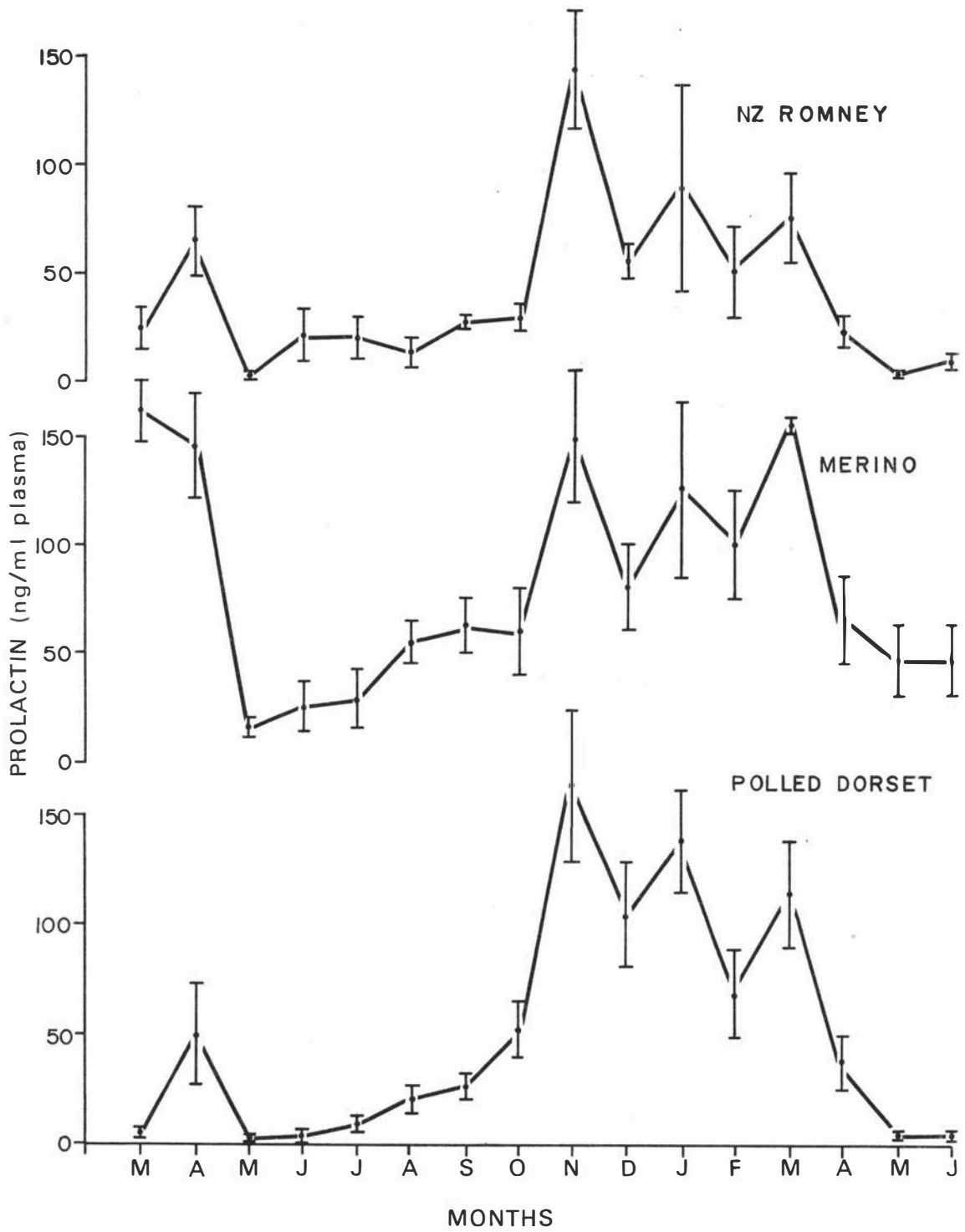
Figure 3.6 : Seasonal variations in plasma prolactin concentrations (mean±S.E.) recorded from N.Z. Romney, Merino and Polled Dorset rams, between March 1972 and June 1973.

levels in May 1972 (151.4+16.6 and 6.3+1.6 ng/ml, respectively).

Generally, the lowest prolactin levels were recorded from the N.Z. Romneys, and the highest from the Merinos, with the Polled Dorsets intermediate (overall means 41.0, 83.1, and 51.0 ng/ml, respectively).

In March and April, 1972, both the N.Z. Romney and Polled Dorset groups had low levels of plasma prolactin in comparison with the Merinos (Contrasts 14, 15 and 17). Further, in May 1973, the Polled Dorsets displayed a sudden fall in plasma prolactin to near baseline levels, while the Merinos exhibited a more moderate fall (Contrast 16). (Note : the N.Z. Romneys displayed a decrease in plasma prolactin levels similar to that recorded from the Polled Dorsets, but this decline was not tested separately by the orthogonal contrasts made in the analysis of variance).

(3) Autopsy Data

See Tables 3.15, 3.16 and 3.17.

These data were obtained in June 1973, after the breeding season, when plasma LH and testosterone were at minimum levels.

The only significant difference between the breed groups was the recording of lower weights of ampullae from the Merinos than from rams of the other two breeds.

(4) Meteorological Data

See Figure 3.7.

Both daily photoperiod and temperature displayed similar sinusoidal patterns throughout the year. Total rainfall also showed a seasonal pattern, but in contrast to that for photoperiod and temperature, had maximum values during the winter months and minimum values during the summer months. Changes in mean relative humidity (not shown) were less marked and corresponded with the changes in total rainfall.

Table 3.15

Data (means[*] ± S.E.) collected following autopsy of rams utilized in Experiment 3.

	Body weights (Kg)	Testicular weights (g)	Seminiferous tubule diameters (μm)	Epididymal weights (g)	Epididymal spermatozoal reserves ($\times 10^9$)	Ampullar weights (g)	Seminal vesicular weights (g)
N.Z. Romney	71.4±3.4	188.9±14.0	158.4± 5.5	42.6±2.5	36.75±13.35	3.73±0.30	7.82±0.78
Merino	66.4±4.6	252.7±33.3	160.4± 8.8	44.7±3.6	23.67± 8.37	2.47±0.11	6.39±0.62
Polled Dorset	64.5±5.0	264.6±60.7	163.6±14.6	50.9±8.3	49.00±14.07	3.72±0.39	7.71±1.28

	Seminal vesicular fructose Total content (mg)	Concentration (mg/g)	Thyroid weights (g)	Pituitary weights (mg)	Pineal weights (mg)	Hydroxyindole-O-methyl transferase activity (DPM/mg pineal)	(DPM/pineal)	Pineal cell nuclear densities (No./std. grid)
N.Z. Romney	36.2± 7.5	443.2± 55.6	5.52± 0.53	702.2±56.3	69.1± 8.4	125.0±15.4	8276±1076	388.3±22.6
Merino	17.3± 8.3	245.2± 95.7	6.25± 0.63	837.8±58.2	85.5±10.1	132.9±25.3	10701±1326	351.7±16.5
Polled Dorset	40.0±15.1	461.9±120.2	6.04± 1.71	838.6±57.8	98.4±25.7	122.2±41.2	9892±2543	380.2± 9.6

[*] Where data have been obtained from paired organs, means for each group were based on totals per ram.

Table 3.16

Variance ratios for contrasts in the analyses of variance of data presented in Table 3.15. (D.F. = 1,12)
Note : Variance ratios related to pineal gland data are presented separately in Table 3.17.

	Body weights	Testicular weights	Seminiferous tubule diameters	Epididymal weights	Epididymal spermatozoal reserves
Contrast 1	1.28	2.88	0.11	0.89	0.00
Contrast 2	0.11	0.05	0.05	0.79	1.88
Error Mean Square	89.42	6024.83	417.58	105.83	760.42

	Ampullar weights	Seminal vesicular weights	Seminal vesicular fructose Total content	Concentration	Thyroid weights	Pituitary weights
Contrast 1	3.62	0.57	0.42	0.77	0.32	3.91
Contrast 2	8.57*	1.00	2.37	2.79	0.02	0.00
Error Mean Square	0.40	3.75	483.42	37440.83	4.28	16918.33

(Footnote: Contrast 1 : N.Z. Romney vs Merino and Polled Dorset;
             Contrast 2 : Merino vs Polled Dorset).

Table 3.17

Variance ratios for contrasts in the analyses of variance of pineal gland data presented in Table 3.15. (D.F. = 1,12)

	Pineal weights	Hydroxyindole-O-methyl transferase activity		Pineal cell nuclear densities
		(DPM/mg pineal)	(DPM/pineal)	
Contrast 1	1.85	0.01	1.19	0.98
Contrast 2	0.37	0.08	0.12	0.99
Error Mean Square	1006.67	3357.48	12290295.17	1819.42

(Footnote: Contrast 1 : N.Z. Romney vs Merino and Polled Dorset
Contrast 2 : Merino vs Polled Dorset.)
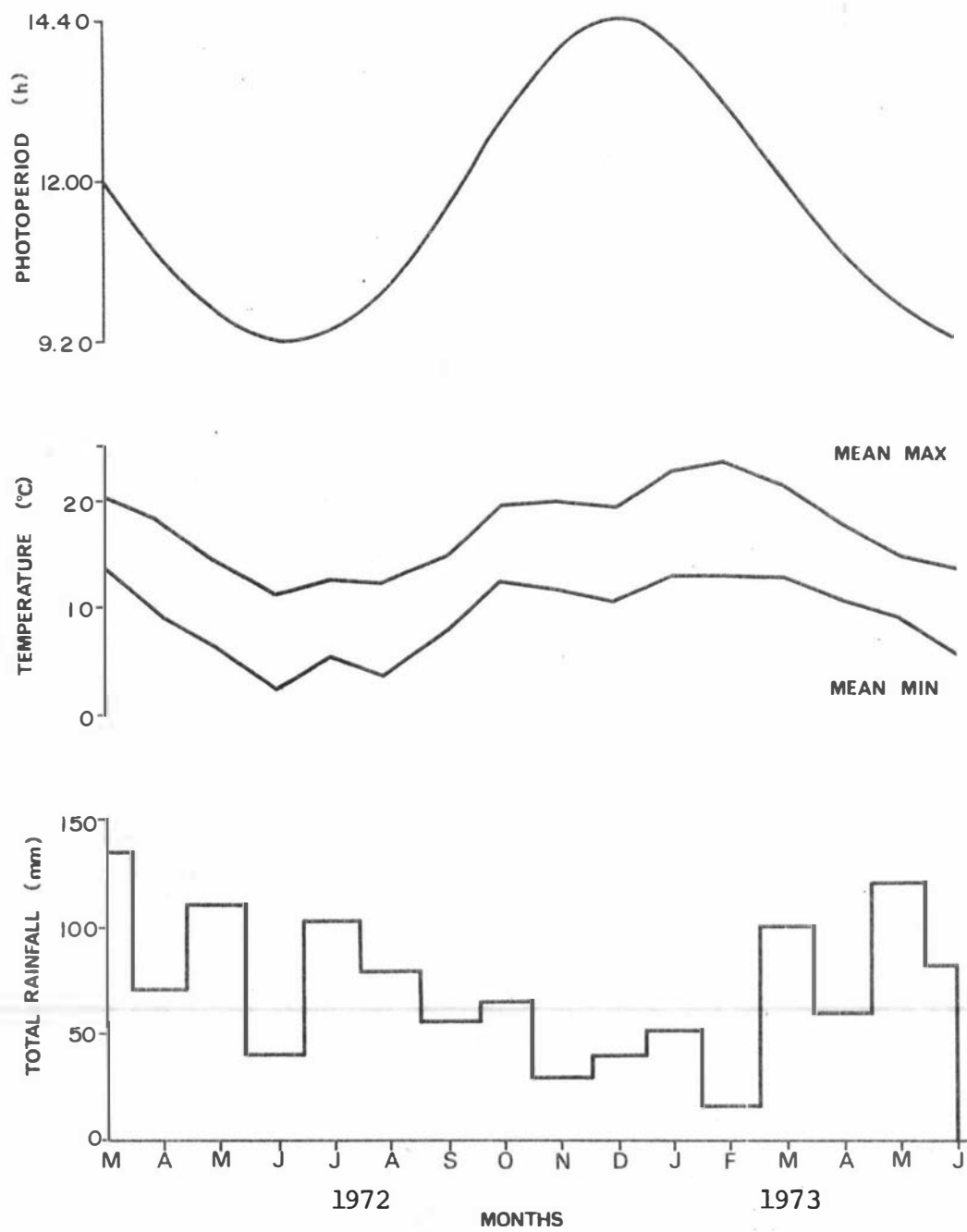
Figure 3.7 : Monthly variations in daily photoperiod (calculated from N.Z. Nautical Almanac, 1972), and temperature and total rainfall (recorded at Massey University), during the time-course of Experiments 3 and 4.

4. DISCUSSION

    (1) <u>Seasonal Changes in Semen Production</u>

Seasonal changes in ram semen production have been reported
by a number of workers (see reviews by Emmens and Robinson, 1962; and
Lodge and Salisbury, 1970). Although the results have tended to be
equivocal, generally higher quality semen has been recorded during
autumn and early winter. Variations between previous reports
mostly can be attributed to factors such as the particular semen
attribute under study, the breed of sheep, and the location of the
experiment.

In the present experiment a well-defined seasonal pattern was
recorded for ejaculate volume and seminal fructose data, these being
the semen parameters which are influenced most by accessory sex gland
function (Mann, 1964). This seasonal pattern consisted of a peak in
values for these characteristics during autumn and low levels during
spring, which supports earlier reports from studies on rams in
California (Capps et al., 1960) and Israel (Amir and Volcani, 1965).

All other semen characteristics studied related to
spermatozoal numbers, their activity, or their morphology, and largely
were indicative of testicular and epididymal function. These other
characteristics did not show any evidence of a seasonal pattern,
except for numbers of spermatozoa per ejaculate, which tended to be
elevated during the autumn months. In contract to these results,
Smyth and Gordon (1967) recorded significant increases during the
autumn months in percentages of live spermatozoa, spermatozoa per ml
and spermatozoa per ejaculate, for Galway, Suffolk and Wicklow rams,
in Ireland. These authors considered that the marked changes in
spermatozoal production were related to seasonal changes in length of
daylight. In contrast workers in Australia reported that seasonal changes in

semen quality were related to excessive heat and undernutrition,
rather than other seasonal factors (Gunn, Sanders and Granger, 1942).
Subsequently other authors have attributed increased frequencies of
spermatozoal abnormalities, or impaired spermatogenesis, to high
summer temperatures (Hafez, Badreldin and Darwish, 1955; Hiroe et al.,
1960). Also, Moule, Braden and Mattner (1966) suggested that a
seasonal increase in the quantity and quality of forage available to
grazing Merino rams accounted for an autumnal peak of seminal fructose
concentrations recorded from these rams. In Experiment 3 no marked
seasonal changes in semen characteristics dependent on testicular or
epididymal activity were recorded, indicating that neither high
summer temperatures nor inadequacies in feed supply, had significant
effects on production of spermatozoa. On the other hand, the
autumnal peak of accessory gland activity, as determined from
ejaculate volume and seminal fructose levels, might have been related
to increased pasture production during the autumn, but more likely
resulted from changes in daily photoperiod. Together, the present
and previous findings, indicate that several environmental factors
can influence reproduction in rams, and that the relative importance
of each factor can be evaluated only in experiments in which
environmental factors can be varied independently, by use of
controlled-climate rooms.

Hafez, Badreldin and Darwish (1955) reviewed a number of
studies and concluded that the degree of seasonality in semen
characteristics depended on the latitude of the place of study, a
higher degree of seasonality being recorded at higher latitudes. It
is thus necessary to consider differences in location when comparing
results reported in the literature. Much of the relevant work done
in Australia, California and Israel was performed at lower latitudes
than the present studies, located near Palmerston North (lat. 40°21'

S), whereas in the United Kingdom and Europe such work has been carried out at higher latitudes.

Amir and Volcani (1965) indicated that even for semen characteristics showing a degree of seasonality, the extent of variations depended on the breed of rams. For example, no seasonal fluctuations in seminal plasma volume, and in seminal fructose and citric acid concentrations were recorded from German Mutton Merino, Corriedale and Dorset Horn rams, and contrasted with the markedly seasonal patterns displayed by Awassi and Border Leicester rams. In Experiment 3 the N.Z. Romneys had lower values for motility indices and spermatozoal concentrations than the other two breeds. This may have been related to the deficiency in spermatogenesis shown by rams of a related breed (Romney Marsh) compared with Merino, Corriedale and Southdown rams, in Brazil (Mies Filho and Ramos, 1956).

The Polled Dorset rams did not display any seasonality in seminal fructose levels, which may have been related to their extended breeding season, yet the Merinos which have a similar breeding pattern had a well-defined autumn peak for this semen parameter. A similar pattern of seminal fructose changes in Merino rams was also reported by Moule, Braden and Mattner (1966). In comparison to the Merinos, the Polled Dorsets in the present study showed less distinct fluctuations in ejaculate volume and spermatozoal numbers, which suggested also that the Merinos had a greater tendency for seasonal changes in semen production than Polled Dorsets.

Fertility per se was never tested in this experiment but indices published by Edgar (1959) and Hulet and Ercanbrack (1962) have provided some guidelines for evaluating semen quality with respect to potential fertility. Spermatozoal concentrations in semen from rams in this experiment were satisfactory or good (over 1×10^9 spermatozoa/ml) according to these guidelines. The rather low values for percentage

of motile (less than 50%) and percentage of abnormal spermatozoa (less than 70-80%) probably reflected differences in subjective assessment of these parameters, between this and other studies.

Subfertility was never expected to occur at any time during the course of Experiment 3. However, changes in potential fertilizing capacity of sperm may have been detectable by use of more stringent in vitro tests of spermatozoal viability. Such tests include spermatozoal motility after incubation at body temperature (Ludwick, Olds and Carpenter, 1948; Buckner, Willett and Bayley, 1965), and spermatozoal viability after freezing (Colas et al., 1972).

Abrupt changes in values for ejaculate volume and spermatozoal concentration coincided with the change in semen collection method to electro-ejaculation, and supported previous evidence that ejaculates collected by this technique have a greater volume and lower spermatozoal concentration than those collected by artificial vagina (Brady and Gildow, 1939; Ortavant, Laplaud and Thibault, 1948; Mattner and Voglmayr, 1962; Salamon and Morrant, 1963; Rathore, 1970). An increase in total ejaculate fructose content also occurred simultaneously with the change in semen collection method, which supported an earlier observation that electro-ejaculation stimulated the accessory glands more than did service into an artificial vagina, and thereby raised seminal fructose content and concentration (Mattner and Voglmayr, 1962). In the present experiment no similar change in the concentration of fructose in semen and seminal plasma occurred indicating that the change in total ejaculate fructose content could be accounted for by the increased ejaculate volume.

Because the change in semen collection method was confounded with seasonal changes, it was not possible to attribute the concomitant fall in indices of motility and percentages of motile and morphologically normal spermatozoa, to collection method alone, if at

all.   Other workers have reported higher indices of motility or
percentages of motile spermatozoa in semen collected by artificial
vagina than in that obtained by electro-ejaculation (Brady and
Gildow, 1939;   Mattner and Voglmayr, 1962;   Salamon and Morrant, 1963),
however it has been pointed out that the changes in spermatozoal
concentration influence the assessment of spermatozoal motility
(Mattner and Voglmayr, 1962).   Therefore it is likely that the
major effect of electrical stimulation is the production of an
increased volume of accessory gland secretion compared with that
found in artificial vagina ejaculates.

   (2) Seasonal Changes in Plasma Hormone Levels

   (a) LH and Testosterone.   Elevated levels of plasma LH in
rams during the autumn months have been reported by a small number
of workers (Pelletier, 1971;   Johnson, Desjardins and Ewing, 1973).
In Experiment 3 peak LH levels were recorded in December (near
midsummer) which corresponded with a recent French report of a peak
during June (Hochereau-de Reviers, Loir and Pelletier, 1976).   Other
workers have failed to demonstrate seasonal changes in plasma LH
levels in rams (Katongole, Naftolin and Short, 1974;   Sanford, Palmer
and Howland, 1974a).

   Autumnal peaks in plasma testosterone concentrations in rams
have been reported by Attal (1970), Johnson, Desjardins and Ewing
(1973), Katongole, Naftolin and Short (1974), and Sanford, Palmer and
Howland (1974a).   However, in Experiment 3 pronounced seasonal
changes in testosterone levels were displayed by all three breeds,
with peak concentrations occurring during January, February and March;
this result was more in agreement with the pattern described by Gomes
and Joyce (1975), who recorded peak plasma testosterone levels from
Southdown, Shropshire and Targhee rams during the midsummer months.

Although a seasonal pattern of LH secretion was recorded in the present study, the period of elevated plasma levels lasted from September to April. This period encompassed the period of elevated testosterone concentrations, but the peak of LH secretion occurred approximately one month earlier than the highest testosterone peak. Moreover, the period of elevated plasma testosterone concentrations (January, February and March) in Experiment 3 preceded, by approximately one month, the corresponding August-November testosterone peaks described in the literature (Attal, 1970; Katongole, Naftolin and Short, 1974; Sanford, Palmer and Howland, 1974a). August-November in the Northern Hemisphere corresponds to February-May in the Southern Hemisphere, yet in the present study plasma testosterone levels had returned to baseline concentrations by May, in agreement with the corresponding results of Gomes and Joyce (1975) in Ohio, U.S.A.

There was less evidence for a seasonal peak of plasma LH levels in the case of Merino rams. Such differences between breeds in the present study could account for the disparities between results in previous papers. However, these differences undoubtedly have been contributed to by differences in localities, and by the small numbers of rams (2-5 per breed) studied in each paper.

The association in timing of peak levels of secretion of LH and testosterone supported the general view that LH stimulates the production of androgens by the testes. This topic and the roles of feedback control and seasonal effects will be discussed later (See 4, General Discussion).

(b) Prolactin. Elevated plasma prolactin levels during the summer months were recorded from rams in Experiment 3 and suggested a seasonal pattern similar to that described for goats (Buttle, 1974) and bulls (Schams and Reinhardt, 1974). In each case the animals

studied were subjected to seasonal changes in both daily photoperiod and temperature, so the relative importance of these environmental factors could not be determined. Pelletier (1973) showed that there was a direct relationship between the length of the daily photoperiod and plasma prolactin levels in rams and wethers subjected to artificial lighting regimes with contrasting seasonal cycles. It thus seems clear that daylight is the major factor controlling plasma prolactin levels in rams. Also, Hart (1975) showed that the provision of additional lighting to goats during the autumn months, prevented the seasonal decline in prolactin response to milking.

In view of the influence of the stress of venepuncture in causing elevated prolactin levels in goats (Hart, 1973) and cows (Johke, 1969; Raud et al., 1971), it is conceivable that the plasma levels obtained in this study were elevated above normal. However the present results, and those of Pelletier (1973), Buttle (1974) and Schams and Reinhardt (1974), were all based on plasma samples obtained by venepuncture. Seasonal or lighting effects on plasma prolactin levels were therefore of sufficient magnitude to be detectable even though sampling procedures may have elevated prolactin levels. Also, very low prolactin levels were measured in plasma samples collected during winter months suggesting that the elevations caused by venepuncture stress were of minor importance, at least at that time of the year. Furthermore, when taking single blood samples at weekly, or less-frequent intervals, it could be assumed that the influence of stress during sampling was constant for all groups throughout each experiment. Nevertheless it would have been more desirable to sample blood remotely by means of indwelling venous cannulae, to eliminate the possibility that any differences between groups of rams merely represented differences in susceptibility to stress.

Although Merino rams had higher mean plasma prolactin levels,

they displayed similar seasonal patterns of prolactin secretion to the N.Z. Romney and Polled Dorset rams, so the significant between-breeds contrasts may have been of doubtful importance.

(3) Autopsy Data

The negative results as regards any consistent pattern of breed differences in the autopsy data was in accord with the lack of major differences in plasma hormone levels or semen characteristics. Also, the data were collected at a time when hormonal and seminal parameters were at their minimum values. This result does not exclude the possibility that breed differences in these data could have occurred during the breeding season.

The lower weights of ampullae from the Merinos were of dubious importance.

(4) General Discussion

No direct attempt was made in this experiment to investigate the interrelationships between the various parameters studied. It was possible, however, to make informal comparisons of the timing of changes in some variables. The relationships between the seasonal patterns of plasma LH and testosterone, and seminal plasma fructose concentrations, are illustrated in Figure 3.8. This graphic comparison indicated that the highest plasma LH levels preceded those of testosterone by approximately one month. In turn, the increase in testosterone levels occurred about one month earlier than the corresponding elevations in seminal plasma fructose concentrations.

The delay between peak plasma LH levels and the consequent peaks in plasma testosterone was unexpectedly long, since testosterone levels usually rose within twenty to forty minutes of an LH surge in rams during a 24-hour sampling period (Katongole, Naftolin and Short,
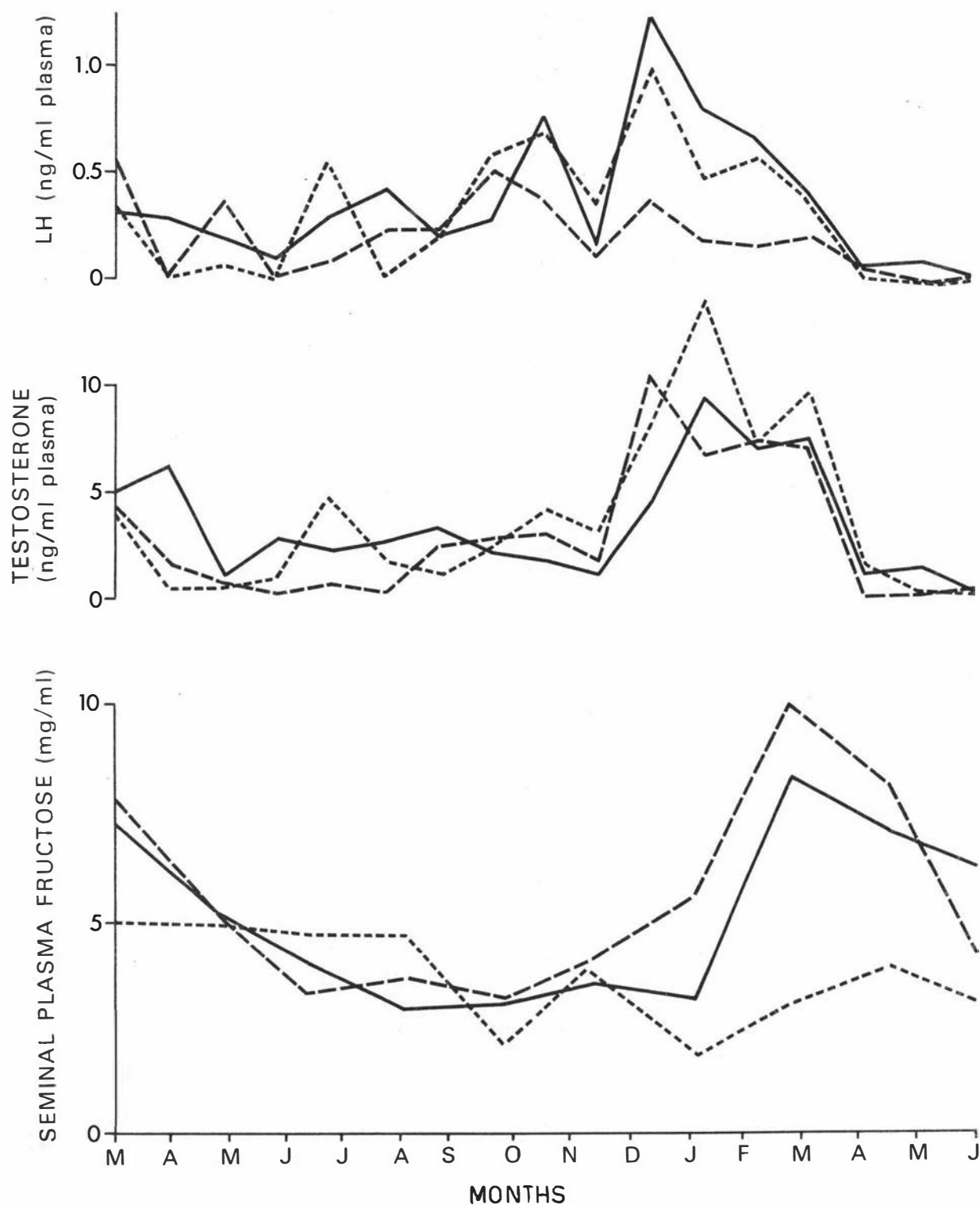
Figure 3.8 : Interrelationships between seasonal variations in plasma LH and testosterone, and in seminal plasma fructose concentrations, for N.Z. Romney (————), Merino (— — — —) and Polled Dorset (---------) rams, between March 1972 and June 1973.

1974; Sanford et al., 1974b). Also, after GnRH administration to rams, plasma testosterone levels rose at the same time as plasma LH (Galloway et al., 1974), or within fifty minutes Bremner et al., 1976; Lee et al., 1976). However, the fact that hormone concentrations were determined on plasma samples collected at four-weekly intervals in Experiment 3, meant that precise statements about their time-relationships could not be made. Furthermore, the present study was an investigation of changes in mean hormone levels throughout the year, not testosterone responses to individual pulses of LH secretion. So, the results presented here indicated that there was a seasonal change in the degree of testicular response to LH stimulation, as well as a seasonal change in LH output. The change in LH output itself might have reflected seasonal changes in hypothalamic or pituitary responsiveness to testosterone feedback, this responsiveness being influenced by other factors, such as daily photoperiod or pineal gland activity. Seasonal changes in testicular responsiveness to LH have been described in the male goat by Racey, Rowe and Chesworth (1975), who suggested that these changes were the result of the long-term effects of the earlier rise in plasma LH levels.

It is possible that a certain duration of stimulation of the testes by raised levels of circulating LH is required to proliferate steroidogenic cells. Thereafter the testosterone response to LH may become progressively greater, and testosterone production may continue at elevated levels, even with reduced LH stimulation. It has been suggested that the frequency of pulses of LH secretion may vary through the year (Katongole, Naftolin and Short, 1974), and could alter the degree of testicular stimulation by affecting the mean LH concentration without necessarily changing the maximal plasma content.

Further experiments will have to be performed to investigate the various aspects of seasonal changes in the controlling mechanisms

for LH and testosterone release in rams. Such experiments include the study of seasonal changes in : the LH and testosterone responses to GnRH injections; the testosterone secretory responses to LH injections; and the feedback effects of testosterone injections on LH output.

Increased levels of fructose in the seminal plasma of castrated rams have been reported during daily (Moule, Braden and Mattner, 1966) or alternate daily (Knight, 1973) treatment with testosterone injections. An eight to eleven day delay from commencement of treatment to seminal plasma fructose response was noted by Moule, Braden and Mattner (1966), and a similar delay can be seen on inspection of Knight's (1973) results. Likewise the rams in Experiment 3 did not show an immediate rise in seminal fructose levels following the seasonal increase in plasma testosterone levels. Also, seminal plasma fructose levels remained elevated after plasma testosterone concentrations had declined. These results indicate that the responses of ram accessory sex glands to androgen stimulation require a certain amount of time to reach their full secretory potential, this time probably being needed to allow full development of active secretory cells. Once maximum secretory activity has been attained, high levels of fructose output may be maintained even by reduced levels of androgens.

In summary, Experiment 3 showed that the seasonal pattern of changes which characterize the breeding season in rams took the form of an elevated output of LH, followed by a subsequent peak in secretion of androgens by the testes. This led, in turn, to increased secretory activity of the accessory sex glands and also (according to Ahmed, 1955) to increased sexual activity.

In field experiments of the kind described in this chapter it is difficult to ascertain which environmental factors were

responsible for the seasonal patterns recorded for semen production
and plasma hormone levels. Variations in daily photoperiod,
temperature, rainfall or nutrition could not be controlled
independently, and hence could not be ruled out as possible
regulators of seasonality in sheep. However several authors, in
discussion of similar field experiments, have implicated photoperiod
as the major environmental factor influencing seasonal reproductive
changes in rams (Smyth and Gordon, 1967; Pelletier, 1971; Katongole,
Naftolin and Short, 1974; Purvis, Illius and Haynes, 1974; Gomes
and Joyce, 1975). Further support for this view has been obtained
from studies in which environmental factors were controlled so that
the effects of photoperiod could be studied independently (Yeates,
1949; Fowler, 1961; Ortavant, Mauleon and Thibault, 1964; Pelletier,
1971; Jackson and Williams, 1973; Pelletier and Ortavant, 1975a,b).
The need to establish the role of photoperiod in producing some of the
results obtained in the present experiment made it obligatory to
perform experiments described later in this thesis, in which rams
were subjected to controlled environments.

Evidence of marked seasonality, shown by the Merino and Polled
Dorset rams particularly with regard to plasma testosterone levels,
was not anticipated as these rams had been expected to be less seasonal
than the N.Z. Romney breed (Hafez, 1952). It seemed, from the data
obtained during this study, that rams of all three breeds probably
were capable of reproduction throughout the whole year. Nevertheless,
a marked seasonal pattern was exhibited by these rams with regard to
plasma hormone levels and accessory sex gland function, and this
pattern might have been reflected by subtle variations in the relative
levels of fertility, which were not detectable in Experiment 3. It
might therefore have been worthwhile to investigate ram fertility
either _in vivo_ by inseminating ewes, or by use of _in vitro_ viability

tests on semen, in the present experiment. Merinos and Polled Dorsets lacked seasonal variations in plasma LH and seminal fructose levels, respectively, which indicated that important breed differences in seasonality may have occurred.

Although the link between plasma prolactin levels and reproduction in rams may be tenuous (see Chapter I), the markedly seasonal pattern of prolactin secretion indicated that if prolactin was gonadotrophic in rams, it could provide a means by which changes in daily photoperiod could modify reproductive activity. Plasma prolactin levels appeared to be more directly influenced by photoperiod than those of LH and testosterone. Thus prolactin release could be influenced by photoperiodic stimuli via the pineal gland, an organ which some authors feel warrants further investigation regarding its possible involvement in reproductive rhythms in mammals (Reiter, 1974a).

A sharp drop occurred in LH levels in plasma collected on 4 December 1972, with a subsequent and less severe fall in testosterone levels. This was contrary to the general seasonal pattern and thus warrants some explanation. In an attempt to elucidate this matter, plasma samples collected both one week prior to and one week following this occasion were assayed; the results revealed that this decline was spread over a few weeks and was not just an isolated event on one particular day. Approximately seven weeks after the decline in plasma LH levels, semen samples displayed a transient drop in quality, which together with the hormone data, suggested that a severe depression of reproductive system function had occurred in early December 1972. No satisfactory explanation can be put forward to account for this event, but it was not considered to be a normal feature of the pattern of seasonal changes in reproductive characteristics which formed the major findings of this experiment.

CHAPTER IV

SEASONALITY OF SEMEN PRODUCTION AND PLASMA HORMONE LEVELS IN RAMS WITH
MODIFIED OLFACTORY AND PINEAL FUNCTION

1. INTRODUCTION

The work described in this chapter was a preliminary
investigation of the neuroendocrine mechanisms mediating the seasonal
changes in semen characteristics and hormone levels observed in the
previous chapter.

The two systems investigated were the pineal gland and the
olfactory system. Both of these systems have been described in
Chapter I as possible modifiers of reproductive and endocrine activity
in rodents, although little research has been published on their
functions in domestic animals. Pinealectomy in ewes failed to have
any effects on the incidence of oestrus or ovulation, or LH levels
(Roche et al., 1970a). On the other hand, Cardinali, Nagle and
Rosner (1974a) reported a significant relationship between pineal
gland activity and stage of the oestrous cycle in ewes. No other
reports have appeared in the literature relating pineal gland
function to reproduction in sheep. Likewise the role of the olfactory
system, on seasonal changes in semen quality and hormone levels, has
not been established for sheep.

The classical approach to such a study would have been to
observe the effects of surgical removal of the organs. However,
difficulties were encountered in initial attempts at surgical removal
of the pineal gland from rams, so instead, the cranial cervical
ganglia were removed. These ganglia are part of an afferent nervous
pathway to the pineal gland in rats and hamsters, and their removal

abolishes the responses of the pineal gland to environmental darkness (Wurtman, Axelrod and Fischer, 1964; Eichler and Moore, 1971). It was assumed that if cranial cervical ganglionectomy produced any changes in the parameters under study in this experiment, these probably could be attributed to altered pineal gland function. Similarly, the olfactory bulbs of rams were removed to investigate the possibility that the resultant impairment of olfactory function may alter the normal seasonal changes in semen characteristics and blood hormone levels.

2. MATERIALS AND METHODS

(1) Animals and Management Procedure

Adult rams of the N.Z. Romney breed were allocated randomly to a control group (six rams), or to three surgically treated groups (four rams each). Rams in the three treated groups were : olfactory bulbectomized (BulbX), cranial cervical ganglionectomized (GanglionX), or olfactory bulbectomized and cranial cervical ganglionectomized (BulbX/GanglionX). The control group (Controls) in this experiment was the group of N.Z. Romney rams studied in Experiment 3 and the two experiments described in this and the previous chapter were run concurrently, the one group of control N.Z. Romney rams providing data for both studies. All the rams grazed together as one flock, under the management procedures described in Chapter II.

The surgical techniques for olfactory bulbectomy and superior cervical ganglionectomy were described in Chapter II. The general health of the operated rams appeared to be identical to that of the Controls yet, one BulbX ram and one GanglionX ram died from unknown causes in the early stages of the experiment. Any data obtained from these rams were excluded from the results. There was no

postmortem evidence that these deaths resulted from the surgical procedures, or their after-effects.

(2) Data Collection

Semen and blood plasma samples, as well as autopsy data were collected in Experiment 4 in the same manner as described for Experiment 3.

(3) Statistical Analyses

Effects of seasons on semen characteristics and plasma hormone levels were studied using the orthogonal coefficients described in Experiment 3 (see Figure 3.1, and Tables 3.1, 3.2 and 3.3). Comparisons between the effects of the surgical treatments were performed using the orthogonal coefficients shown below, weighted for disproportionate numbers of rams in each group.

Contrast	Controls	BulbX	GanglionX	BulbX/ GanglionX
Controls vs BulbX, GanglionX, & BulbX/ GanglionX	+3	−1	−1	−1
BulbX & GanglionX vs BulbX/GanglionX	0	+1	+1	−2
BulbX vs GanglionX	0	+1	−1	0

However, in the case of autopsy data relating to the pineal gland (pineal gland weight, cell nuclear density, and hydroxyindole-O-methyl transferase activity) the following set of orthogonal coefficients was used, again weighted for disproportionate group size :

Contrast	Controls	BulbX	GanglionX	BulbX/ GanglionX
GanglionX, BulbX/ GanglionX vs Controls, BulbX	−1	−1	+1	+1
GanglionX vs BulbX/ GanglionX	0	0	+1	−1
Controls vs BulbX	+1	−1	0	0

This latter set of orthogonal coefficients was specifically devised to investigate the assumption made in the introduction, that cranial cervical ganglionectomy would alter pineal gland function in rams.

3.   RESULTS

(1)   Semen Data

See Tables 4.1 to 4.7 and Figures 4.1 and 4.2.

(Note - Olfactory bulbectomized rams appeared to show normal behavioural responses, with no impairment of ability to serve the artificial vagina.)

Semen from all groups of rams tended to display similar changes in characteristics to those recorded from the N.Z. Romney rams in Experiment 3.   Again ejaculate volume and fructose levels displayed a seasonal increase during autumn, while motility, percentage motile spermatozoa and percentage morphologically normal spermatozoa declined in the first six months and never regained their March 1972 levels.   Estimates of spermatozoal numbers in semen displayed erratic changes, total numbers of spermatozoa per ejaculate being particularly variable, showing a double peak during the summer months (November 1972 to April 1973) and rising again in June 1973.   Apart from a slight fall in May 1972, percentages of unstained spermatozoa showed little evidence of change throughout the experiment (Contrasts 1-7, Table 4.6 and Contrasts 1-9, Table 4.7).

Significant results in Contrast 6 (Table 4.6) for ejaculate volume, spermatozoa per ml, spermatozoa per ejaculate, and in Contrast 8 (Table 4.7) for total ejaculate fructose content, could be attributed to changing the semen collection method from artificial vagina to electro-ejaculation.   On the other hand the significant results for motility indices, and percentages of motile and

Table 4.1

Mean motility indices (scale 0-4) and mean percentages of motile spermatozoa recorded from semen collected in Experiment 4.

Motility index

	Period	Controls	BulbX	GanglionX	BulbX/ GanglionX	Mean
Season 1	1	3.3	3.2	3.2	3.4	3.3
	2	2.6	2.7	3.1	2.6	2.8
	3	2.2	2.4	2.8	3.1	2.6
	4	2.2	2.3	2.5	2.0	2.2
	Mean	2.6	2.6	2.9	2.8	2.7
Season 2	5	2.4	2.1	2.3	2.3	2.3
	6	2.2	2.0	2.1	2.3	2.2
	7	2.1	2.6	1.8	2.4	2.2
	8	2.2	1.8	2.0	2.9	2.2
	Mean	2.2	2.1	2.1	2.5	2.2
Season 3	9	2.6	1.9	2.7	2.7	2.5
	10	2.0	2.3	2.5	2.8	2.4
	Mean	2.3	2.1	2.6	2.8	2.4
Overall Mean		2.4	2.3	2.5	2.6	

% Motile spermatozoa

	Period	Controls	BulbX	GanglionX	BulbX/ GanglionX	Mean
Season 1	1	61.3	65.3	63.1	69.8	64.9
	2	49.8	51.9	61.4	47.1	52.6
	3	37.5	40.0	54.2	57.5	47.3
	4	39.9	42.5	43.9	36.7	40.8
	Mean	47.1	49.9	55.6	52.8	51.4
Season 2	5	44.9	42.5	43.6	44.6	43.9
	6	40.1	32.8	32.8	44.6	37.6
	7	43.7	50.0	45.8	50.6	47.5
	8	45.6	35.8	32.7	62.7	44.2
	Mean	43.6	40.3	38.7	50.6	43.3
Season 3	9	51.6	38.4	59.2	53.7	50.7
	10	47.4	49.4	52.5	57.2	51.6
	Mean	49.5	43.9	55.8	55.4	51.2
Overall Mean		46.2	44.9	48.9	52.4	

Table 4.2

Mean ejaculate volumes and mean total fructose content of ejaculates collected in Experiment 4.

Ejaculate volume (ml)

	Period	Controls	BulbX	GanglionX	BulbX/GanglionX	Mean
Season 1	1	0.98	1.17	0.86	0.92	0.98
	2	0.72	1.26	0.61	0.98	0.89
	3	0.54	1.05	0.57	0.60	0.69
	4	0.56	0.68	0.64	0.93	0.70
	Mean	0.70	1.04	0.67	0.85	0.81
Season 2	5	1.10	0.93	1.37	1.22	1.16
	6	1.12	1.08	1.19	1.80	1.30
	7	1.18	1.23	1.27	1.56	1.31
	8	1.55	1.54	1.88	1.68	1.66
	Mean	1.23	1.19	1.42	1.56	1.35
Season 3	9	1.56	0.94	1.17	1.47	1.28
	10	1.28	1.78	1.41	1.56	1.51
	Mean	1.42	1.36	1.29	1.51	1.39
Overall Mean		1.06	1.17	1.10	1.27	

Total ejaculate fructose content (mg)

	Period	Controls	BulbX	GanglionX	BulbX/GanglionX	Mean
Season 1	1	5.53	5.51	4.62	5.70	5.34
	2	3.18	4.14	1.86	4.48	3.42
	3	1.86	1.83	1.26	1.14	1.52
	4	1.27	0.74	0.93	2.53	1.37
	Mean	2.96	3.05	2.16	3.46	2.91
Season 2	5	3.33	1.34	1.91	2.37	2.24
	6	4.26	1.82	2.00	4.54	3.16
	7	3.05	5.03	2.15	4.35	3.64
	8	11.57	7.75	6.36	12.61	9.57
	Mean	5.55	3.98	3.10	5.96	4.65
Season 3	9	9.65	5.88	5.96	7.04	7.13
	10	7.35	4.26	4.01	4.84	5.12
	Mean	8.50	5.07	4.98	5.94	6.12
Overall Mean		5.11	3.83	3.11	4.96	

Table 4.3

Mean concentrations of fructose in semen and in seminal plasma of ejaculates collected in Experiment 4.

Seminal fructose concentration (mg/ml)

	Period	Controls	BulbX	GanglionX	BulbX/ GanglionX	Mean
Season 1	1	5.76	4.20	5.63	5.97	5.39
	2	4.31	3.64	2.98	4.35	3.82
	3	3.47	1.42	2.19	1.35	2.11
	4	2.57	1.16	1.61	2.02	1.84
	Mean	4.02	2.60	3.10	3.42	3.29
Season 2	5	2.68	1.38	1.43	1.91	1.85
	6	2.90	1.45	1.57	2.41	2.08
	7	2.26	2.19	1.76	2.92	2.28
	8	7.24	5.27	3.89	7.46	5.96
	Mean	3.77	2.57	2.16	3.67	3.04
Season 3	9	6.12	4.84	5.65	4.56	5.29
	10	5.31	2.64	3.34	3.15	3.61
	Mean	5.71	3.74	4.49	3.85	4.45
	Overall Mean	4.26	2.82	3.01	3.61	

Seminal plasma fructose concentration (mg/ml)

	Period	Controls	BulbX	GanglionX	BulbX/ GanglionX	Mean
Season 1	1	7.26	5.75	7.28	6.94	6.81
	2	5.26	4.99	3.85	5.42	4.88
	3	4.04	1.81	2.59	1.58	2,51
	4	2.99	1.42	2.33	2.95	2.42
	Mean	4.88	3.49	4.01	4.22	4.15
Season 2	5	3.00	1.62	1.80	2.14	2.14
	6	3.62	1.83	1.96	3.16	2.64
	7	3.24	6.19	2.04	3.19	3.66
	8	8.36	6.40	4.48	9.15	7.10
	Mean	4.55	4.01	2.57	4.41	3.88
Season 3	9	7.17	5.41	6.63	6.16	6.34
	10	6.37	3.44	4.13	4.38	4.58
	Mean	6.77	4.42	5.38	5.27	5.46
	Overall Mean	5.13	3.89	3.71	4.51	

Table 4.4

Mean concentrations of spermatozoa/ml and mean numbers of spermatozoa/ejaculate in semen collected in Experiment 4.

Spermatozoa/ml ($\times 10^9$)

	Period	Controls	BulbX	GanglionX	BulbX/GanglionX	Mean
Season 1	1	3.35	3.00	3.84	2.47	3.16
	2	3.08	4.19	3.54	3.15	3.49
	3	2.51	2.94	2.40	2.70	2.64
	4	2.53	2.83	4.77	2.57	3.18
	Mean	2.86	3.24	3.63	2.72	3.11
Season 2	5	1.40	1.75	2.47	1.42	1.76
	6	1.67	1.65	2.95	3.52	2.45
	7	0.86	0.68	2.16	1.29	1.25
	8	2.12	2.60	1.92	2.84	2.37
	Mean	1.51	1.67	2.37	2.26	1.95
Season 3	9	2.03	1.52	2.39	3.95	2.47
	10	1.74	3.72	2.86	4.27	3.15
	Mean	1.88	2.62	2.62	4.11	2.81
Overall Mean		2.13	2.49	2.93	2.82	

Spermatozoa/ejaculate ($\times 10^9$)

	Period	Controls	BulbX	GanglionX	BulbX/GanglionX	Mean
Season 1	1	3.09	3.26	3.30	2.21	2.96
	2	2.25	5.55	2.23	3.02	3.26
	3	1.30	3.39	1.38	1.54	1.90
	4	1.50	2.01	3.24	3.77	2.63
	Mean	2.03	3.55	2.53	2.63	2.68
Season 2	5	1.84	1.84	3.64	2.46	2.44
	6	2.79	2.64	3.91	7.53	4.22
	7	1.27	0.80	3.20	2.05	1.83
	8	3.36	4.94	5.29	4.80	4.60
	Mean	2.31	2.55	4.01	4.21	3.27
Season 3	9	4.15	1.43	3.35	5.96	3.72
	10	2.60	7.64	4.03	7.12	5.35
	Mean	3.37	4.53	3.69	6.54	4.53
Overall Mean		2.42	3.35	3.36	4.05	

Table 4.5

Mean percentages of unstained and morphologically normal spermatozoa in semen collected in Experiment 4.

% Unstained spermatozoa

	Period	Controls	BulbX	GanglionX	BulbX/ GanglionX	Mean
Season 1	1	76.4	76.6	75.2	89.0	79.3
	2	62.7	65.7	75.6	60.6	66.2
	3	72.4	68.0	73.3	81.7	73.8
	4	70.3	78.7	70.8	67.3	71.8
	Mean	70.4	72.2	73.7	74.6	72.8
Season 2	5	81.9	66.8	68.1	77.5	73.6
	6	73.5	66.9	73.4	79.4	73.3
	7	88.3	77.5	69.3	72.0	76.8
	8	75.2	67.5	51.8	78.0	68.1
	Mean	79.7	69.7	65.6	76.7	73.0
Season 3	9	75.7	66.5	79.6	73.2	73.8
	10	68.9	67.4	70.5	65.2	68.0
	Mean	72.3	67.0	75.1	69.2	70.9
Overall Mean		74.5	70.2	70.8	74.4	

% Morphologically normal spermatozoa

	Period	Controls	BulbX	GanglionX	BulbX GanglionX	Mean
Season 1	1	88.0	86.5	74.8	85.6	83.7
	2	72.0	75.1	64.9	75.4	71.8
	3	66.3	77.0	67.7	78.0	72.2
	4	67.2	81.8	72.6	80.0	75.4
	Mean	73.4	80.1	70.0	79.8	75.8
Season 2	5	70.6	77.5	60.0	71.7	70.0
	6	60.1	63.0	47.8	67.0	59.5
	7	76.7	63.5	44.8	54.2	59.8
	8	53.9	48.8	25.7	54.5	45.7
	Mean	65.3	63.2	44.6	61.8	58.8
Season 3	9	68.7	55.2	66.7	58.5	62.3
	10	62.0	60.4	60.2	55.4	59.5
	Mean	65.4	57.8	63.4	57.0	60.9
Overall Mean		68.6	68.9	58.5	68.0	

Table 4.6

Experiment 4·: Summary of Analyses of Variance for Semen Data.

Source of Variation	Contrast No.	D.F.	Volume	Motility	% Motile	Sperm./ml	Sperm./ejac.
					Variance Ratios [2]		
MAIN EFFECTS							
A. SEASONS		9					
Season 1 – Linear	1	1	6.35*	32.97***	26.56***	4.33*	1.13
" " – Cubic	2	1	0.11	0.92	0.03	4.16*	2.76
Season 2 – Linear	3	1	11.74***	0.03	0.38	1.70	2.60
" " – Cubic	4	1	1.13	0.08	2.73	1.62	13.27***
Season 3 – Linear	5	1	2.47	0.03	0.37	3.79	4.94*
Season 1 vs Seasons 2 & 3	6	1	74.44***	26.98***	6.15*	9.02**	8.27**
Season 2 vs Season 3	7	1	0.30	2.80	6.52*	5.93*	6.28*
Non significant contrasts		2	1.11	0.18	0.84	0.03	0.49
B. SURGICAL TREATMENTS		3					
C vs BX, GX, BX/GX	8	1	3.53	1.74	0.92	9.80**	9.87**
BX, GX vs BX/GX	9	1	3.45	5.13*	5.47*	0.10	2.57
BX vs GX	10	1	0.38	1.51	1.63	1.62	0.00
INTERACTION (AxB)		27					
Season 3 – Linear x Contrast 8	11	1	5.44*	4.15*	0.91	3.14	7.73**
(Seasons 1 vs 2 & 3) x Contrast 8	12	1	0.15	1.06	1.41	4.62*	0.61
Season 1 – Cubic x Contrast 9	13	1	0.01	4.54*	3.90	0.00	0.02
Season 2 – Quadratic x Contrast 9	14	1	3.52	0.59	0.56	12.72***	2.99
" " – Cubic x Contrast 9	15	1	0.70	0.33	1.47	0.07	4.05*
(Seasons 1 vs 2 & 3) x Contrast 9	16	1	0.64	4.48*	3.12	4.25*	3.98*
(Seasons 2 vs 3) x Contrast 9	17	1	0.11	0.00	0.45	7.01**	1.63
Season 2 – Linear x Contrast 10	18	1	0.04	0.14	0.04	5.58*	0.13
Non significant contrasts		19	0.68	0.78	0.56	0.77	0.79
Residual Mean Square		120	0.15	0.25	173.19	1.30	4.76

(Key 1, Surgical Treatments : C = Controls; BX = BulbX; GX = GanglionX; BX/GX = BulbX/GanglionX)

(Key 2, Semen Parameters : Volume = ejaculate volume; Motility = motility index; % Motile = percentage of motile spermatozoa; Sperm./ml = concentration of spermatozoa/ml; Sperm./ejac. = number of spermatozoa/ejaculate)

Table 4.7

Experiment 4 : Summary of Analyses of Variance for Semen Data.

Source or Variation	Contrast No.	D.F.	Variance Ratios [2]				
			Fr. Conc.	Fr. Cont.	S.P. Fr. Conc.	% Unstained	% Normal
MAIN EFFECTS							
A. SEASONS		9					
Season 1 – Linear	1	1	31.22***	20.19***	37.61***	1.51	1.88
" " – Quadratic	2	1	1.23	1.00	2.08	4.85*	4.45*
" " – Cubic	3	1	0.30	0.08	0.94	4.27*	0.07
Season 2 – Linear	4	1	31.16***	51.24***	36.14***	1.08	17.59***
" " – Quadratic	5	1	13.36***	16.49***	8.97**	1.93	0.06
" " – Cubic	6	1	2.36	4.63*	1.04	1.30	0.85
Season 3 – Linear	7	1	6.33*	5.02*	5.14*	0.55	0.00
Season 1 vs Seasons 2 & 3	8	1	0.27	21.07***	0.23	0.06	42.60***
Season 2 vs Season 3	9	1	10.49**	6.26*	10.95**	0.94	0.33
B. SURGICAL TREATMENTS		3					
C vs BX, GX, BX/GX	10	1	11.91***	7.07**	8.68**	1.91	1.40
BX, GX vs BX/GX	11	1	3.79	9.41**	3.08	2.98	1.95
BX vs GX	12	1	0.33	0.46	0.00	0.03	7.97**
INTERACTION (AxB)		27					
(Seasons 1 vs 2 & 3) x Contrast 10	13	1	0.09	4.17*	0.03	6.98**	3.26
Season 1 – Cubic x Contrast 11	14	1	0.09	0.21	0.22	5.59*	0.13
Non significant contrasts		25	0.43	0.54	0.55	0.82	0.45
Residual Mean Square		120	3.73	7.12	5.00	121.27	237.42

(Key 1, Surgical Treatments : C = Controls; BX = BulbX; GX = GanglionX; BX/GX = BulbX/GanglionX)

(Key 2, Semen Parameters : Fr. Conc. = fructose concentration of semen; Fr. Cont. = total ejaculate fructose content; S.P. Fr. Conc. = fructose concentration of seminal plasma; % Unstained = percentage of unstained spermatozoa; % Normal = percentage of morphologically normal spermatozoa)
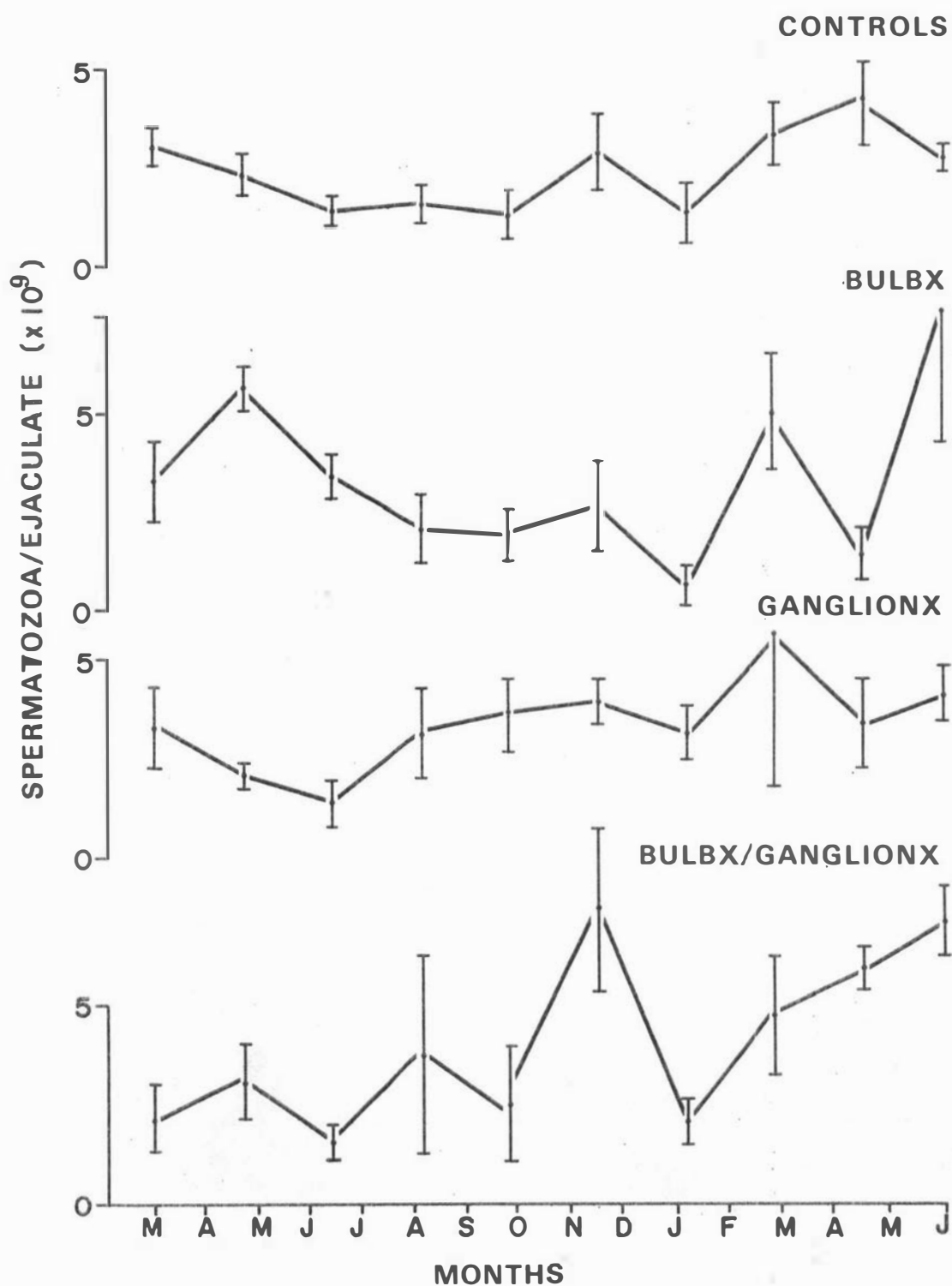
Figure 4.1 : Seasonal variations in numbers of spermatozoa/ejaculate
(mean±S.E.) in semen collected from Controls, BulbX, GanglionX and
BulbX/GanglionX rams, between March 1972 and June 1973.
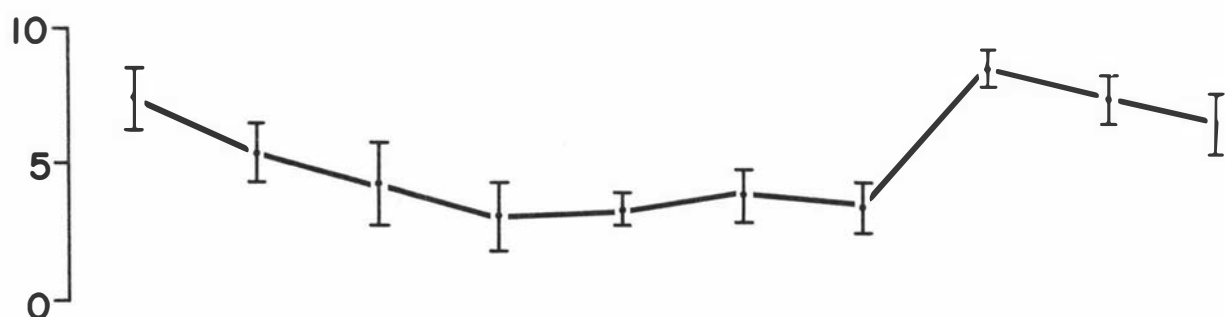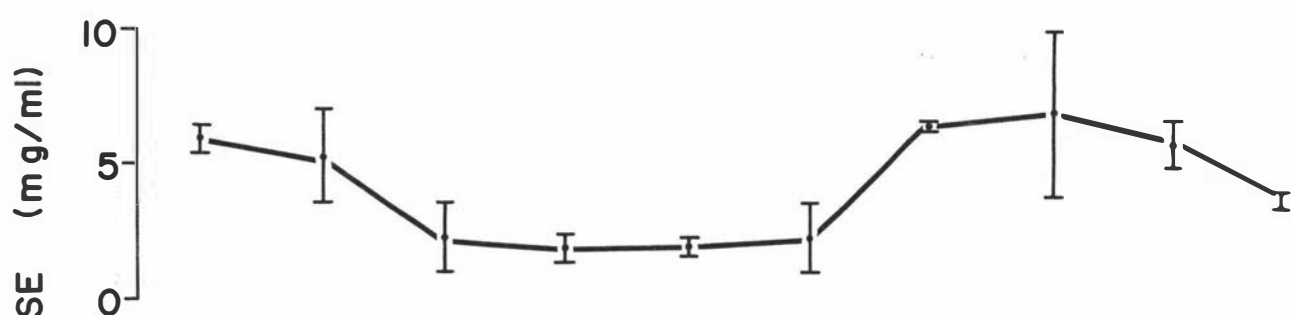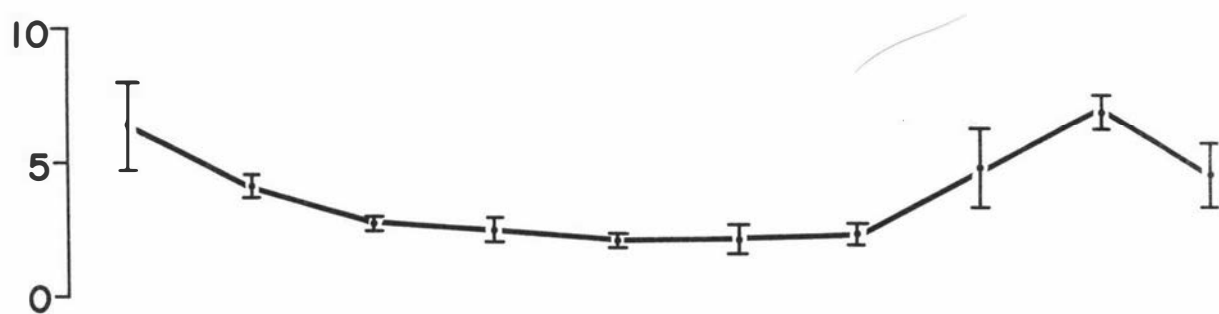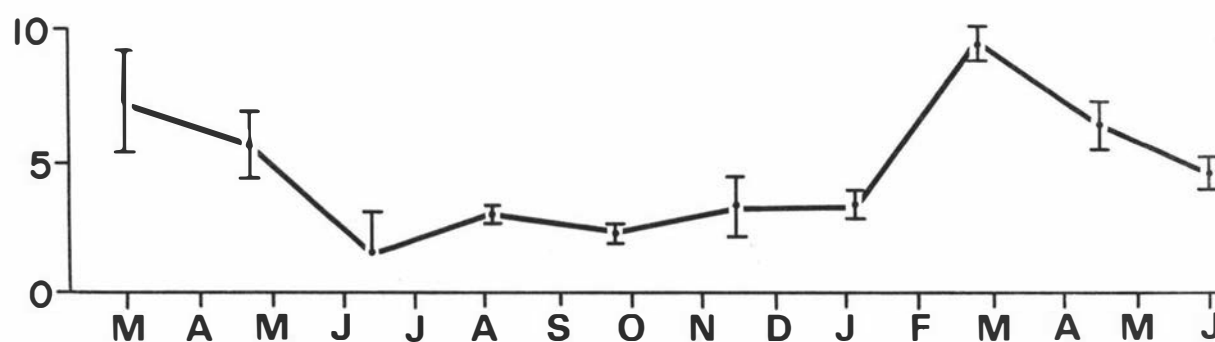
Figure 4.2 : Seasonal variations in seminal plasma fructose concentrations (mean±S.E.) in semen collected from Controls, BulbX, GanglionX and BulbX/ GanglionX rams, between March 1972 and June 1973.

morphologically normal spermatozoa, were the result of their decline from the March 1972 levels, as mentioned above.

During the months from September 1972 to March 1973, semen from the control rams had higher percentages of unstained spermatozoa than that from operated animals (Contrast 13, Table 4.7). Semen from the double-operated group (BulbX/GanglionX) had higher motility indices, percentages of motile spermatozoa, and seminal fructose contents, than that from both the BulbX and GanglionX groups, while the GanglionX group produced semen with the lowest mean percentages of morphologically normal spermatozoa of all four groups (Contrast 9, Table 4.6 and Contrasts 11 and 12, Table 4.7).

Also, semen from the BulbX/GanglionX group displayed much greater oscillations in mean indices of motility (Contrast 13, Table 4.6) and percentages of unstained spermatozoa (Contrast 14, Table 4.7) during Season 1 than did semen from the other groups of rams, especially that from the two single-operation groups.

Ejaculates from the three surgically treated groups had higher mean spermatozoal numbers and lower mean fructose levels than those from the control rams (Contrast 8, Table 4.6 and Contrast 10, Table 4.7). Both BulbX and GanglionX rams produced semen with higher mean concentrations of spermatozoa in the period from March to September 1972, than did the control or BulbX/GanglionX rams (Contrasts 16 and 17, Table 4.6). While the control rams produced semen with declining ejaculate volumes, spermatozoa per ejaculate and indices of motility during April, May and June 1973, that from surgically treated groups displayed an increase in ejaculate volume and spermatozoa per ejaculate, and little change (a slight increase in the case of BulbX/GanglionX rams) in indices of motility, over the same period (Contrast 11, Table 4.6).

(2) <u>Plasma Hormone Levels</u>

(a) <u>LH</u>. See Tables 4.8, 4.9 and Figure 4.3.

The only clear-cut seasonal effects recorded for all groups were a general increase in plasma LH concentrations in the period from July 1972 to December 1972 (Contrast 2), followed by a decline between January and June 1973 (Contrast 3). However, rams from all the surgically treated groups tended to display irregular, rather than seasonal, changes in plasma LH levels. This irregularity was indicated by the fact that complex interaction contrasts (Contrasts 10, 11, 12 and 13) were significant, but contrasts testing linear components of the main effects in Seasons 1 and 2 were non-significant; the latter should have been significant if seasonal changes occurred.

Overall, the control rams had lower mean plasma LH levels than those which had undergone surgery (Contrast 6).

(b) <u>Testosterone</u>. See Tables 4.8, 4.9 and Figure 4.4.

All groups displayed a seasonal pattern of plasma testosterone levels with elevated values in January, February and March 1973 (Contrasts 1 and 3). As in Experiment 3, there was evidence that there had been a peak of testosterone secretion in January and February 1972; this was indicated by the fall in plasma concentrations during the first three months of the experiment.

Contrast 9 was the only contrast, testing the effects of surgical treatments, which was statistically significant. This result indicated that in the period from March until June 1972, testosterone levels fell more markedly in the group of rams subjected to both operations, than the mean result from the two groups of rams which had had only one surgical treatment.

(c) <u>Prolactin</u>. See Tables 4.10, 4.11 and Figure 4.5.

Overall mean plasma prolactin levels, calculated from all four groups of rams, indicated that levels were elevated in the summer months and diminished during the winter (Contrasts 1, 2, 4 and 6).

Table 4.8

Mean plasma LH and testosterone concentrations recorded from rams in Experiment 4.
(Values presented are $100 \log_{10}(x + 1.1)$, where x is hormone concentration in ng/ml.)

		Luteinizing Hormone							Testosterone				
	Period	Controls	BulbX	GanglionX	BulbX/ GanglionX	Mean		Period	Controls	BulbX	GanglionX	BulbX/ GanglionX	Mean
Season 1	1	14.7	15.5	9.8	28.4	17.1	Season 1	1	77.4	64.9	78.7	74.1	73.8
	2	12.6	37.7	20.1	21.1	22.9		2	79.1	67.2	38.1	91.4	68.9
	3	10.1	20.2	24.3	12.9	16.9		3	30.8	35.0	23.3	18.5	26.9
	4	7.5	7.6	9.2	17.9	10.5		4	53.0	34.4	77.4	33.8	49.6
	Mean	11.2	20.2	15.8	20.1	16.8		Mean	60.1	50.4	54.4	54.4	54.8
Season 2	5	13.9	24.7	27.8	24.8	22.8	Season 2	5	45.9	36.3	56.5	45.1	45.9
	6	15.1	30.0	43.1	22.3	27.6		6	46.8	42.7	58.5	48.0	49.0
	7	11.5	32.4	44.9	25.6	28.6		7	54.0	52.8	45.3	37.5	47.4
	8	13.4	38.5	35.8	40.0	31.9		8	42.6	86.3	74.7	71.5	68.8
	9	23.3	29.4	50.7	37.2	35.1		9	44.9	46.5	50.8	62.4	51.1
	10	9.9	14.0	41.1	22.9	22.0		10	37.6	33.7	82.0	43.6	49.2
	Mean	14.5	28.2	40.6	28.8	28.0		Mean	45.3	49.7	61.3	51.3	51.9
Season 3	11	29.0	38.8	35.0	30.1	33.2	Season 3	11	65.9	64.3	85.5	70.6	71.6
	12	24.6	42.5	44.1	37.7	37.2		12	93.0	92.0	102.3	94.2	95.4
	13	20.0	39.6	23.3	22.0	26.2		13	82.3	48.3	34.0	41.6	51.5
	14	17.8	12.9	27.9	26.3	21.2		14	77.2	63.2	86.0	70.3	74.2
	15	6.4	15.7	20.1	31.7	18.5		15	38.2	45.5	38.8	68.0	47.6
	16	7.1	23.5	19.0	12.9	15.6		16	30.5	19.8	34.0	14.7	24.7
	17	4.9	28.7	24.5	25.1	20.8		17	18.2	24.1	36.9	48.3	31.9
	Mean	15.7	28.8	27.7	26.5	24.7		Mean	57.9	51.0	59.7	58.2	56.7
Overall Mean		14.2	26.6	29.4	25.8		Overall Mean		54.0	50.4	53.1	54.9	

Table 4.9

Experiment 4 : Summary of Analyses of Variance for LH and Testosterone Data.

Source of Variation	Contrast No.	D.F.	Variance Ratios	
			LH	Testosterone
MAIN EFFECTS				
A. SEASONS		16		
Season 1 – Linear	1	1	2.27	12.50***
Season 2 – Quadratic	2	1	5.46*	1.81
Season 3 – Linear	3	1	19.25***	47.76***
" " – Cubic	4	1	3.32	3.86*
Season 1 vs Seasons 2 & 3	5	1	18.69**	0.01
Non significant contrasts		6	1.99	1.57
B. SURGICAL TREATMENTS		3		
Controls vs BulbX, GanglionX, BulbX/GanglionX	6	1	50.58***	0.05
BulbX, GanglionX vs BulbX/GanglionX	7	1	0.93	0.00
BulbX vs GanglionX	8	1	0.99	2.43
INTERACTION (AxB)		48		
Season 1 – Linear x Contrast 7	9	1	2.57	5.23*
" " – Quadratic x Contrast 7	10	1	5.05*	1.77
Season 2 – Cubic x Contrast 7	11	1	5.58*	3.84
" " – Cubic x Contrast 8	12	1	6.85*	0.49
(Season 3 vs Season 2) x Contrast 8	13	1	4.15*	0.05
Non significant contrasts		28	0.71	0.81
Residual Mean Square		204	213.06	772.65
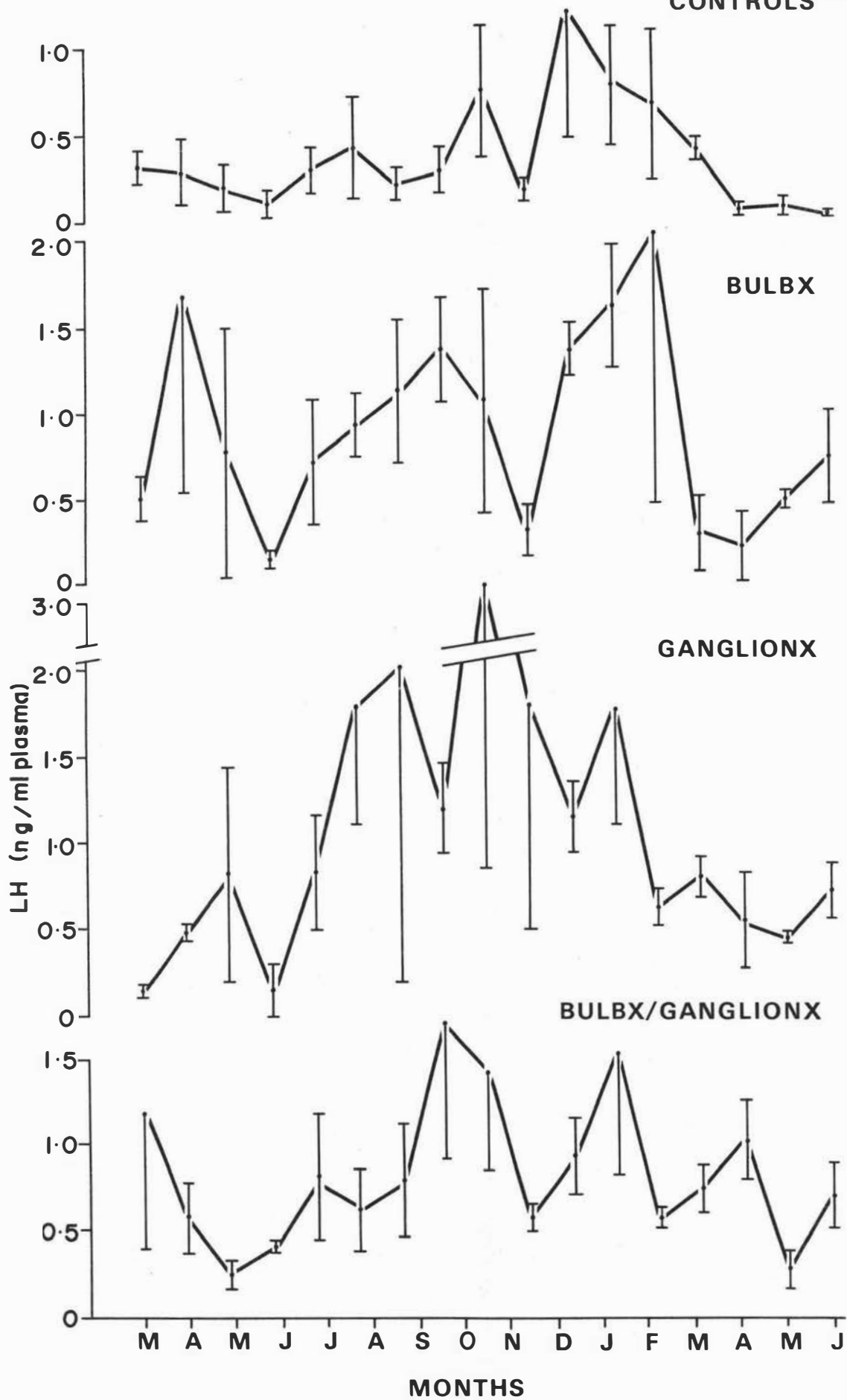
Figure 4.3 : Seasonal variations in plasma LH concentrations (mean±S.E.) recorded from Controls, BulbX, GanglionX and BulbX/GanglionX rams, between March 1972 and June 1973.
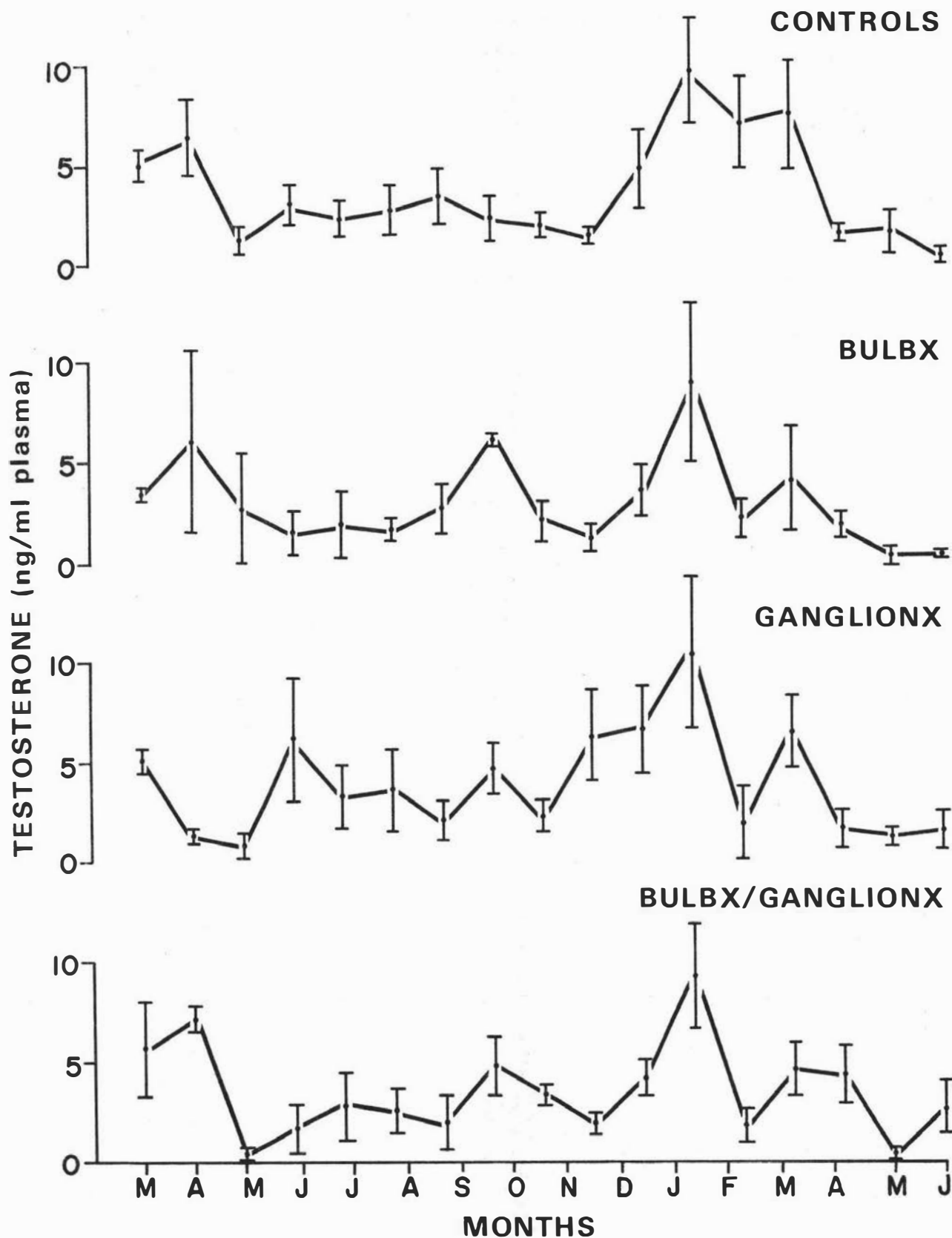
Figure 4.4 : Seasonal variations in plasma testosterone concentrations
(mean±S.E.) recorded from Controls, BulbX, GanglionX and BulbX/GanglionX
rams, between March 1972 and June 1973.

Table 4.10

Mean plasma prolactin concentrations recorded from rams in Experiment 4.
(Values presented are $100 \log_{10}(x + 1.1)$, where x is hormone concentration in ng/ml.)

	Period	Controls	BulbX	GanglionX	BulbX/ GanglionX	Mean
	1	115.9	131.4	118.8	128.6	123.7
	2	155.8	154.6	152.2	144.1	151.7
Season 1	3	42.1	94.2	102.5	55.7	73.6
	4	97.3	81.3	144.7	141.6	116.2
	5	103.0	94.6	170.7	159.0	131.8
	6	92.3	95.6	206.1	182.9	144.2
	Mean	101.1	108.6	149.2	135.3	123.5
	7	141.4	116.9	185.1	197.2	160.1
	8	143.1	126.0	82.6	129.4	120.3
Season 2	9	213.3	215.8	222.1	187.6	209.7
	10	173.2	195.0	207.5	189.2	191.2
	11	185.0	206.8	163.6	151.9	176.8
	12	134.7	142.3	101.2	68.0	111.5
	Mean	165.1	167.1	160.3	153.9	161.6
	13	164.0	156.8	162.7	176.6	165.0
Season 3	14	123.1	105.7	202.0	183.0	153.4
	15	59.7	21.7	154.6	166.6	100.6
	16	89.0	52.6	186.6	158.7	121.7
	Mean	108.9	84.2	176.5	171.2	135.2
Overall Mean		127.1	124.4	160.2	151.2	

Table 4.11

Experiment 4 : Summary of Analysis of Variance for Prolactin Data.

	Contrast No.	D.F.	Variance Ratio
MAIN EFFECTS			
A. SEASONS		15	
Season 1 - Linear	1	1	7.77**
Season 2 - Quadratic	2	1	25.97***
" " - Cubic	3	1	15.39***
Season 3 - Linear	4	1	12.37***
" " - Cubic	5	1	4.92*
Season 3 vs Season 2	6	1	32.26***
Non significant contrasts		5	1.01
B. SURGICAL TREATMENTS		3	
Controls vs BulbX, GanglionX, BulbX/GanglionX	7	1	9.95**
BulbX, GanglionX vs BulbX/GanglionX	8	1	1.54
BulbX vs GanglionX	9	1	15.38***
INTERACTION (AxB)		45	
Season 1 - Linear x Contrast 7	10	1	4.47*
(Season 3 vs Season 2) x Contrast 7	11	1	6.75*
(Seasons 1 vs 2 & 3) x Contrast 8	12	1	6.57*
Season 1 - Linear x Contrast 9	13	1	8.84**
Season 3 - Linear x Contrast 9	14	1	5.00*
(Seasons 1 vs 2 & 3) x Contrast 9	15	1	12.84***
(Season 3 vs Season 2) x Contrast 9	16	1	5.06*
Non significant contrasts		26	0.95
Residual Mean Square		192	1991.71
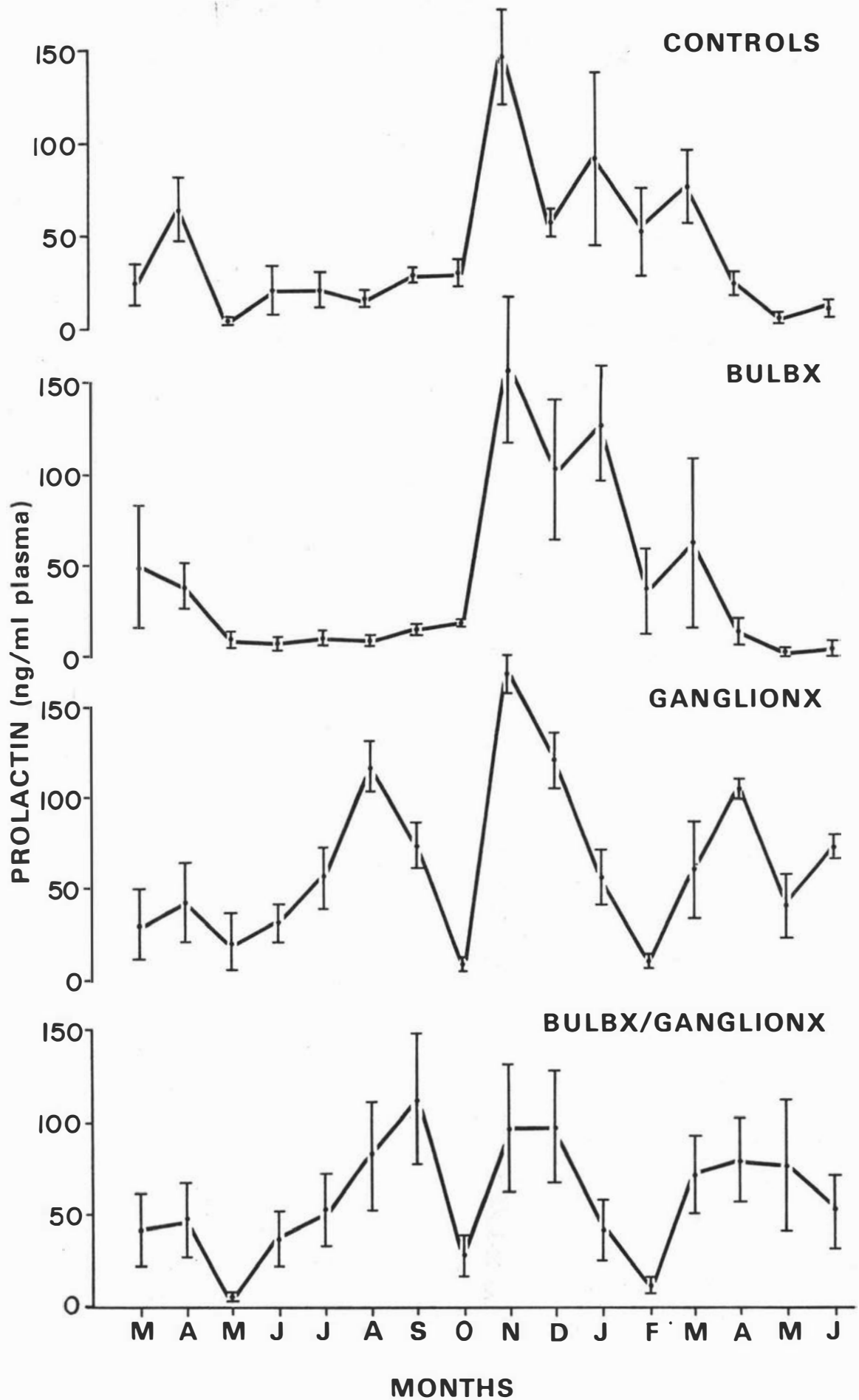
Figure 4.5 : Seasonal variations in plasma prolactin concentrations (mean±S.E.) recorded from Controls, BulbX, GanglionX and BulbX/GanglionX rams, between March 1972 and June 1973.

However, this was a misleading result as this pattern of prolactin secretion was seen only in the control and bulbectomized groups, and not in the GanglionX and BulbX/GanglionX rams. Instead plasma prolactin levels recorded from the latter two groups of rams showed irregular oscillations, with peak levels in August-September 1972, November-December 1972, and March-May 1973, and low levels in October 1972 and February 1973.

The oscillations in plasma prolactin levels in the GanglionX and BulbX/GanglionX groups contributed to the fact that these animals had higher overall mean prolactin levels than the other two groups of rams (Contrasts 7 and 9). They also accounted for most of the significant interaction contrasts (Contrasts 10-16), as well as the significant cubic components of the Seasons main effect (Contrasts 3 and 5).

(3) Autopsy Data

See Tables 4.12 to 4.14 and Figure 4.6.

At autopsy the BulbX/GanglionX group had higher mean testicular and epididymal weights than the BulbX and GanglionX groups. Also, GanglionX rams had higher mean seminiferous tubule diameters than BulbX rams. On the other hand, the control rams had higher mean seminal vesicular fructose contents and concentrations, than did rams in the surgically treated groups.

Autopsy data pertaining to the pineal gland showed that the GanglionX and BulbX/GanglionX groups had significantly lower hydroxyindole-O-methyl transferase activities (Figure 4.6), and higher cell nuclear densities, than the control and BulbX groups. Conversely, subjective histological examination of pineal gland sections did not reveal any substantive evidence for a reduction in the amount of silver-staining material in cranial cervical ganglionectomized rams (i.e. GanglionX and BulbX/GanglionX rams). Thus it was not possible

Table 4.12

Data (means[*] ± S.E.) collected following autopsy of rams utilized in Experiment 4.

	Body weights (Kg)	Testicular weights (g)	Seminiferous tubule diameters (μm)	Epididymal weights (g)	Epididymal spermatozoal reserves ($\times 10^9$)	Ampullar weights (g)	Seminal vesicular weights (g)
Controls	71.4±3.4	188.9±14.0	158.4±5.5	42.6±2.5	36.75±13.35	3.73±0.30	7.82±0.78
BulbX	62.5±1.0	166.2±16.2	143.8±5.8	41.6±6.5	27.75±10.50	3.14±0.10	6.46±0.51
GanglionX	60.2±7.4	226.3±16.9	178.1±9.0	37.8±2.5	43.17± 4.18	2.77±0.26	5.04±0.99
BulbX/ GanglionX	60.9±4.2	287.6±24.0	173.2±4.2	49.9±2.5	60.19± 5.71	3.17±0.36	5.84±0.74

	Seminal vesicular fructose Total content (mg)	Concentration (mg/g)	Thyroid weights (g)	Pituitary weights (mg)	Pineal weights (mg)	Hydroxyindole-O-methyl transferase activity (DPM/mg pineal)	(DPM/pineal)	Pineal cell nuclear densities (No./std. grid)
Controls	36.2±7.5	443.2± 55.6	5.52±0.53	702.2±56.3	69.1± 8.4	125.0±15.4	8276±1076	388.3±22.6
BulbX	11.0±0.2	171.4± 16.5	4.07±0.18	781.0±76.0	67.8±11.8	126.2±16.4	8750±2602	372.7±11.1
GanglionX	16.9±8.4	291.7±130.7	5.18±0.57	777.1±96.6	53.8±13.3	55.4±13.5	3317±1496	480.2±20.2
BulbX/ GanglionX	10.2±2.6	165.8± 29.0	5.56±0.29	777.6±96.3	44.8± 6.4	37.6± 2.9	1698± 285	414.6±22.4

[*] Where data have been obtained from paired organs, means for each group were based on totals per ram.

Table 4.13

Variance ratios for contrasts in the analyses of variance of data presented in Table 4.12. (D.F. = 1,11)
Note : Variance ratios related to pineal gland data are presented separately in Table 4.14.

	Body weights	Testicular weights	Seminiferous tubule diameters	Epididymal weights	Epididymal spermatozoal reserves
Contrast 1	4.23	3.63	1.02	0.02	0.30
Contrast 2	0.00	13.35**	2.17	6.40*	2.42
Contrast 3	0.08	3.17	9.38*	0.49	0.52
Error Mean Square	80.82	1367.09	151.27	35.27	551.27

	Ampullar weights	Seminal vesicular weights	Seminal vesicular fructose Total content	Concentrations	Thyroid weights	Pituitary weights
Contrast 1	3.78	4.96*	9.62*	10.67**	1.21	0.81
Contrast 2	0.22	0.01	1.63	0.50	1.92	0.00
Contrast 3	0.36	0.71	3.14	0.93	1.50	0.00
Error Mean Square	0.45	2.82	199.82	18718.82	1.01	24917.09

(Footnote: Contrast 1 : Controls vs BulbX, GanglionX, BulbX/GanglionX;
Contrast 2 : BulbX, GanglionX vs BulbX/GanglionX;
Contrast 3 : BulbX vs GanglionX).

Table 4.14

Variance ratios for contrasts in the analyses of variance of pineal gland data presented in Table 4.12. (D.F. = 1,11)

	Pineal weights	Hydroxyindole-O-methyl transferase activity		Pineal cell nuclear densities
		(DPM/mg pineal)	(DPM/pineal)	
Contrast 1	3.29	24.95***	20.25***	6.55*
Contrast 2	0.40	0.62	0.79	3.38
Contrast 3	0.01	0.00	0.06	0.17
Error Mean Square	357.55	802.27	5700225.27	2183.55

(Footnote: Contrast 1 : Controls, BulbX vs GanglionX, BulbX/GanglionX;
            Contrast 2 : Controls vs BulbX;
            Contrast 3 : GanglionX vs BulbX/GanglionX).
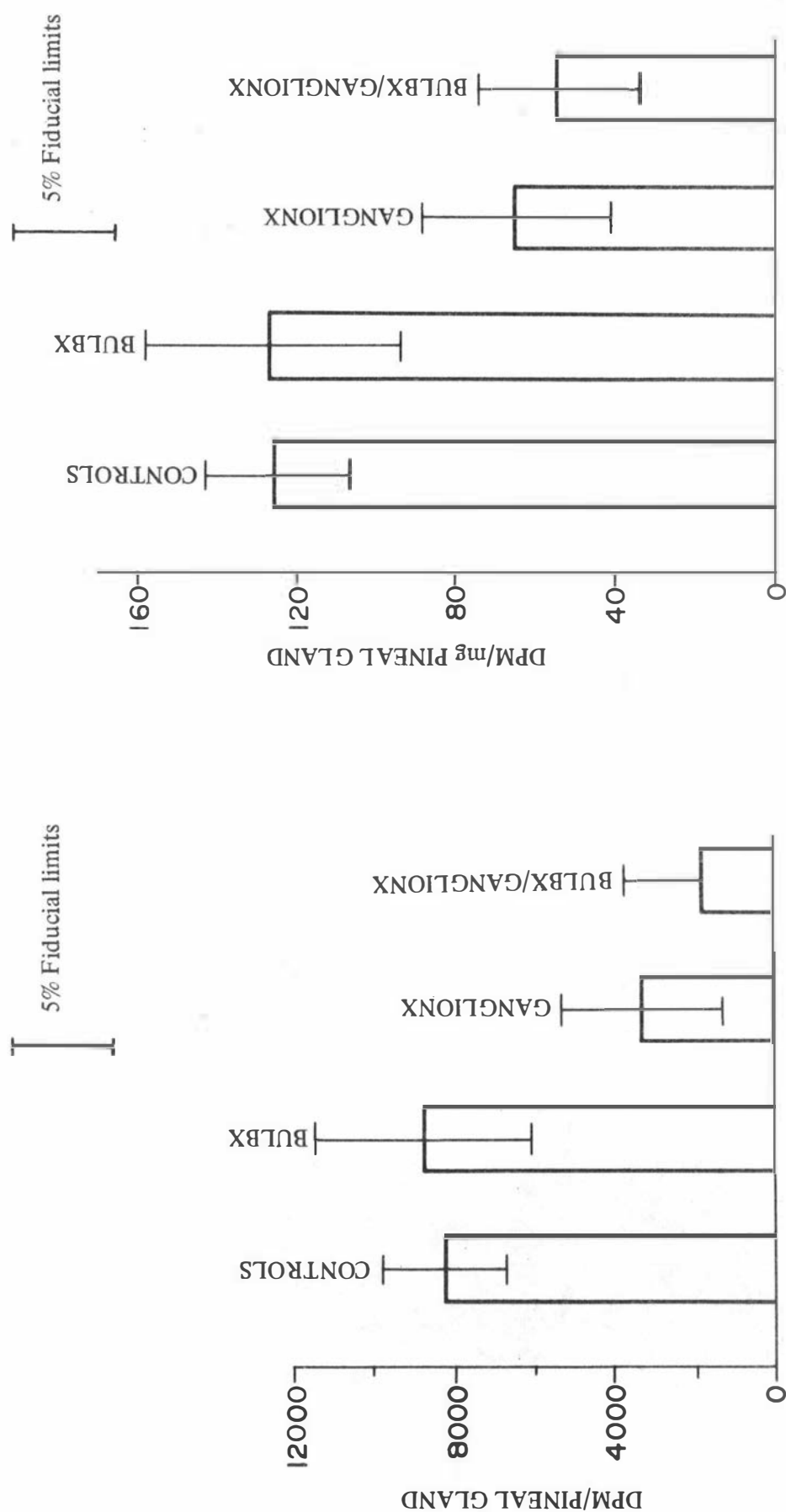
Figure 4.6 : Mean hydroxyindole-O-methyl transferase activity in pineal glands from Controls, BulbX, GanglionX and BulbX/GanglionX rams. Enzyme activity is expressed as DPM/pineal gland and as DPM/mg pineal gland.

to determine any effect of ganglionectomy on the numbers of axons in the pineal glands.

4. DISCUSSION

The seasonal changes in semen characteristics and plasma hormone levels recorded from the control N.Z. Romney rams have been discussed in Chapter III and will not be discussed again. This discussion will be concerned only with results which arose from the surgical treatments.

(1) Semen Data

Although the surgical treatments altered some semen parameters in comparison to the control group, it was difficult to categorize such results as being caused by altered pineal gland function, or altered olfactory function. This difficulty arose because results obtained from the BulbX/GanglionX group did not appear to be different from the Controls by an amount equivalent to the sum of the effects of the two operations singly. Further, it would even have been possible to conclude, in respect of seminal fructose levels, that the effects of the double-operative treatment nearly cancelled out the effects of the two single operations, giving values nearer those of the Controls.

There have been no reports in the literature on the effects of these operations on semen quality, however Whitten (1956) reported that olfactory bulbectomy reduced the volume of secretions from the accessory glands of mice. Reiter (1973b) pointed out that pinealectomy in lower mammals had been notoriously unreliable in producing gonadal responses. He mentioned that anosmia had been designated as a "potentiating factor" which sensitized the neural-gonadal axis of rats to the influence of the pineal gland. This conclusion was based on the finding that anosmia exaggerated the hypertrophy of the gonads and accessory sex organs which followed blinding of male rats (Reiter,

Klein and Donofrio, 1969), and the hypertrophy of the gonads of similarly treated female rats (Reiter and Ellison, 1970). The semen data obtained in the present study did not enable any conclusive comment to be made on such statements.

(2) LH and Testosterone

Previous workers have not been able to demonstrate any effects of olfactory bulbectomy (Mauleon and Signoret, 1964) or pinealectomy (Roche et al., 1970a) on reproductive hormone levels in the ewe. In Experiment 4 all three surgical treatments caused a reduction in the regular seasonal changes in plasma LH and testosterone concentrations, and elevated mean LH levels, compared to those recorded from the unoperated control rams. These differences in the seasonal pattern of plasma LH and testosterone secretion were most evident during the winter months.

Cranial cervical ganglionectomy reversed the stimulatory effects of blinding or darkness on pineal gland activity in rats (Wurtman, Axelrod and Fischer, 1964) and golden hamsters (Eichler and Moore, 1971). Consequently, because pineal activity caused gonadal degeneration in these species (Hoffman and Reiter, 1965; Reiter, 1968), ganglionectomized rams in the present study might have been expected to display higher gonadotrophin levels than the control rams, only during periods of reduced daily photoperiod (increased darkness).

In view of the present paucity of knowledge of olfactory influences on reproduction in domestic animals, it is difficult to account for the effects of olfactory bulbectomy, which tended to be similar to the results of ganglionectomy. Blask and Reiter (1975) found that both blinding and anosmia (singly or combined) significantly lowered plasma LH levels in female rats. Their results were attributed to impaired release of LH, because the combined treatments elevated pituitary levels of this hormone. As the effects of these

surgical treatments were reversed by pinealectomy, the authors concluded that the antigonadotrophic capacity of the pineal gland was potentiated by the two operations. A similar conclusion could not be applied to the effects of olfactory bulbectomy in Experiment 4, because this operation tended to elevate plasma LH levels.

Plasma LH results from the present experiment indicated that the pineal gland in rams may have had an antigonadotrophic function which was inhibited by cranial cervical ganglionectomy or olfactory bulbectomy. Although plasma testosterone data would have been expected to support this conclusion with elevated levels in surgically treated rams, only non-significant trends were observed, and the contrasts tested in the analysis of variance of testosterone data did not reveal any notable differences between the surgically treated and control groups of rams. One significant difference (between the rate of decline of plasma testosterone levels during March to June 1972, for the single operation groups versus the double-operated group) was of doubtful importance because of its low level of significance, and the absence of a similar effect in the same period of 1973.

In summary, the seasonal patterns of secretion of reproductive hormones were diminished by cranial cervical ganglionectomy (by modifying pineal activity) or olfactory bulbectomy (by unknown actions either on pineal function, or on hypothalamic or pituitary function, directly).

(3) Prolactin

Ronnekleiv and McCann (1975) reported that olfactory bulbectomy or cranial cervical ganglionectomy lowered serum prolactin levels in male rats. They concluded that the pineal gland, or its principles, normally stimulated the release of prolactin from untreated control animals, and that this stimulation was induced by olfactory and visual

input to the pineal gland. Unfortunately, all their blood samples were obtained during ether anaesthesia, so the effects of this stress on prolactin release may have been confounded with the surgical treatments. As pointed out by the authors, this objection is particularly valid since the effect of ether stress may have been entirely dependent on the ability of an animal to smell the anaesthetic, and this alone might account for the lower prolactin levels in anosmic rats.

In the present experiment olfactory bulbectomy did not cause any change in prolactin levels. However cranial cervical ganglionectomy induced an elevation of plasma prolactin levels and an almost complete loss of the annual seasonal rhythmic fluctuations in levels of this hormone. It is presumed that the very similar results obtained from rams which had been olfactory bulbectomized as well as ganglionectomized, was the result of cranial cervical ganglionectomy alone.

Goats which had been cranial cervical ganglionectomized in the winter experienced an elevation of their plasma prolactin levels before unoperated control animals and had significantly higher levels for three successive months; however both groups of goats had similar midsummer levels (H. Buttle, personal communication). Although group size was small (n = 3), these preliminary observations from goats were similar to those obtained from rams in Experiment 4.

If the pineal gland is an important regulator of prolactin release in rams, as it is presumed to be in rats and hamsters (Reiter, 1974b), then its major role could be as an inhibitor of prolactin secretion during winter, because pineal activity would be greatest during the period of shortened daily photoperiods (Reiter, 1974a). Although it contrasts with the earlier mentioned conclusions of Ronnekleiv and McCann (1975), the latter hypothesis was supported by the elevated plasma prolactin levels recorded during winter from

cranial cervical ganglionectomized rams. Further, this result

indicated that an intact sympathetic nervous system normally provides

the neural pathways by which the pineal gland receives stimuli

necessary for its inhibitory influence over prolactin release.

Autopsy data which showed that cranial cervical ganglionectomy reduced

pineal activity, provided further support for the above hypothesis.

Thus the results from ganglionectomized rams in the present

study implicated the pineal gland as having a major role in regulating

the seasonal changes in plasma prolactin levels recorded in Experiment

3, and in the photoperiodic control of prolactin release in rams

reported by Pelletier (1973).

(4) Autopsy Data

Comparison of the results for the semen data with those

obtained from autopsy material, helped to clarify the nature of gonadal

responses to the surgical treatments. Towards the end of the

experiment and at autopsy, operated rams, in particular those which

had undergone both operations (BulbX/GanglionX), tended to have higher

values for indices of spermatozoal production than the control rams.

These parameters included spermatozoa per ejaculate, spermatozoal

concentration and motility in semen, testicular and epididymal weights,

and seminiferous tubule diameters. On the other hand, untreated rams

had higher mean values for variables associated with accessory gland

function, for example : seminal fructose levels, seminal vesicular

weights, and seminal vesicular fructose contents and concentrations.

Evidence of a higher level of spermatogenic activity in

surgically treated rams, together with the higher output of LH in these

animals, supported the view that the operations stimulated gonadal

activity by increasing gonadotrophin secretion. This finding also

was consistent with the theory that cranial cervical ganglionectomy

reduced the antigonadotrophic activity of the pineal gland. The

effects of olfactory bulbectomy were unclear, except when combined with cranial cervical ganglionectomy, in which case it appeared to amplify some of the effects of the latter treatment.

Reduced accessory gland function in the operated rams corresponded with lowered seminal vesicular weights and secretion volumes recorded from anosmic male mice by Whitten (1956). Furthermore Sorrentino, Reiter and Schalch (1971) suggested that olfactory bulbectomy and cranial cervical ganglionectomy interfered with the nutritional status and growth hormone secretion in rats. Such effects could reduce accessory gland activity. Plasma testosterone data obtained from rams in Experiment 4 did not show variations which could be used in explaining the different levels of accessory sex gland activity caused by the surgical treatments.

Reduced HIOMT activity of pineal glands from rams which had been cranial cervical ganglionectomized provided a check on the efficiency of the surgery, as well as confirming the assumption that this operation would alter pineal function. Pineal cell nuclear density provided an index of cellular activity as it is reduced when pineal cells are active and have large cytoplasmic volumes, hence lower nuclear densities (Roth, Wurtman and Altschule, 1962). The effect of ganglionectomy on pineal activity also was indicated by the lower pineal weights in the ganglionectomized rams, however the reduction in weights was not statistically significant. With the limitations in the histological staining technique available, it was not possible to assess whether changes in density of pineal axons occurred as a result of ganglionectomy.

It seems reasonable to conclude that since removal of the cranial cervical ganglia reduced pineal activity then, in the ram as in the rat and golden hamster, these ganglia are an integral part of the afferent nerve supply to the pineal gland.

(5) General Discussion

Cranial cervical sympathectomy may have had effects on
structures other than the pineal gland and these must be considered.
The cranial cervical ganglia are involved in the sympathetic
autonomic control of blood vessels in the head, including those
supplying the pituitary gland and the rest of the brain. Removal of
these ganglia has been reported to lead to a fall in cerebral blood
volume in mice (Edvinsson, Owman and West, 1971). Thus it may be
possible to ascribe all the endocrine consequences of ganglionectomy
to alterations in rate of pituitary blood flow. Consequently,
definitive statements about the role of the pineal gland in regulating
reproduction should be made only after observing the effects of
pinealectomy.

The significant results which could be attributed to olfactory
bulbectomy did not provide much insight into the possible role of the
olfactory system in the seasonality of reproduction. Disruption of
the seasonal changes of plasma LH, and to a lesser extent, of plasma
testosterone levels shown by the BulbX group, lent support to the
possibility that olfaction could play a part in controlling the onset
of the breeding season. Furthermore, as destruction of the olfactory
bulbs would have disrupted nervous connections from the vomeronasal
organ (Alberts, 1974; Powers and Winans, 1975), it is not possible to
discount the idea that the alteration in seasonal changes of plasma LH
and testosterone secretion resulted indirectly from the loss of the
vomeronasal component of olfaction. However, the hormone and semen
data was obtained from only three rams, so any conclusions must be
treated with caution.

Nevertheless this experiment established that environmental
factors could influence the reproductive systems of rams via their
pineal glands and olfactory systems. The importance of olfactory

stimuli in seasonal reproductive changes could not be ascertained, nor was it possible to ascertain the mechanisms by which such stimuli could modify reproduction. However on the other hand photoperiodic stimuli, which are known to control annual photoperiodic cycles in domestic animals (Ortavant, Mauleon and Thibault, 1964), may exert their influence on reproduction in rams by reducing pineal activity during the summer months. This effect of daily photoperiod probably is manifested by the increased secretion of gonadotrophins at this time of the year, as was seen for plasma LH levels recorded in Experiment 3. A similar explanation may have accounted for the changes in plasma prolactin levels in unoperated animals, so it would have been informative to have measured FSH as well. Unfortunately, a satisfactory assay for this hormone was not available.

CHAPTER V

EFFECTS OF DIFFERENT LIGHTING REGIMES ON SEMEN PRODUCTION AND PLASMA
HORMONE LEVELS IN RAMS

1. INTRODUCTION

The work described in this chapter was a preliminary attempt to
determine the importance of daily photoperiod as an environmental
factor influencing the seasonal changes in plasma hormone levels and
semen production, described in Chapter III. Similar studies
mentioned in Chapter I have demonstrated that seasonal changes in
reproductive characteristics of rams can be simulated, or substantially
modified, by exposing them to appropriate artificial lighting regimes.
However, none of these studies have involved N.Z. Romney rams, nor
have there been any previous attempts to combine hormonal and semen
production studies.

Some of the experiments described in the literature have
simulated the natural annual cycle of photoperiod changes. The role
of these has been studied by altering the phase of the cycle, usually
in simple phase reversal/non-reversal experiments (Fowler, 1961; Moule,
Braden and Mattner, 1966). On the other hand, "phase-shortening"
experiments, in which changes in daily photoperiod equivalent to the
annual cycle were completed in six months, have been used to investigate
photoperiodic effects on ram semen production (Colas et al., 1972;
Jackson and Williams, 1973) and plasma prolactin and LH levels
(Pelletier, 1973; Pelletier and Ortavant, 1975a,b).

In this experiment the natural seasonal cycle of changes in
daylight were simulated and their effects on reproduction in rams
compared with the effects of a reversed cycle, or a non-changing

photoperiodic regime. The latter regime was included as a check on the persistence of residual rhythms in reproductive parameters, in the absence of changes in photic stimulation.

2. MATERIALS AND METHODS

(1) Animals and Experimental Procedure

Twelve adult N.Z. Romney rams were allocated at random to three experimental groups, each group being subjected to a different lighting regime. Commencing at the autumn equinox 1973, one group continued on the normal seasonal decline in daylight (Normal), another experienced an increasing photoperiodic regime (Reversed), whilst the third group remained on a constant 12 hr light : 12 hr dark (Even) regime.

Information regarding the annual changes in daylight was obtained from the New Zealand Nautical Almanac (1972) using the sunrise and sunset tables for Wanganui (latitude 27 minutes N. of Palmerston North). Daily lighting was taken as the time between sunrise and sunset; this varied in a sinusoidal manner from 9 h 20 min minimum to 14 h 40 min maximum. No allowance was made for twilight.

Collection of blood samples in Experiment 5 commenced in March 1973 and continued until December that year. A programme of two-weekly semen collections commenced at the June solstice and continued until the end of the experiment at the December solstice, 1973. In this experiment autopsies were not performed.

(2) Statistical Analyses

Note. In each case below, contrasts were selected in an attempt to provide the most meaningful evaluation of treatment effects.

(a) Semen Data. Semen data were pooled to give a single mean estimate for each variable for every ram, on each of the six months between the June and December solstices (see Figure 5.1). Orthogonal
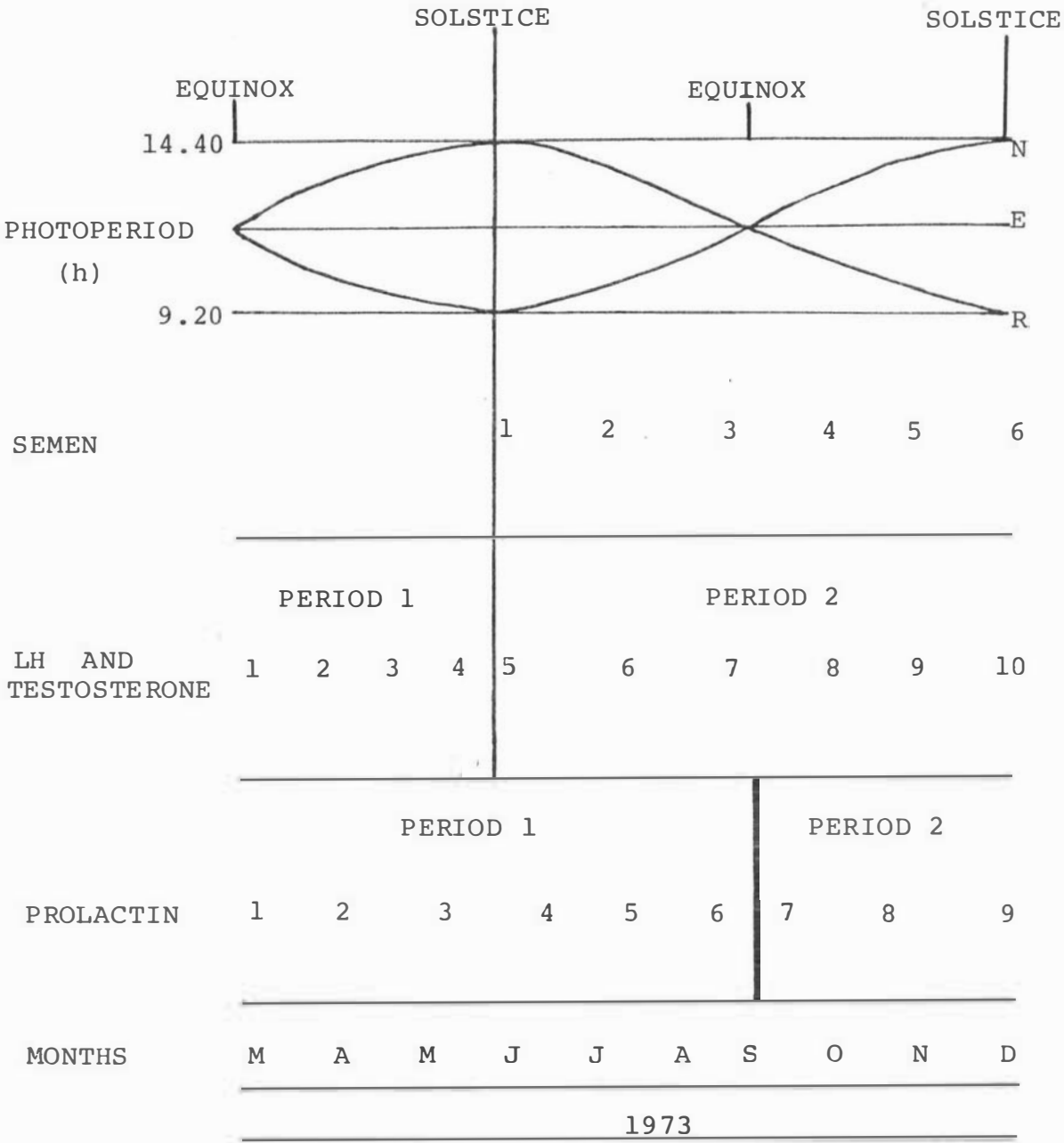
Figure 5.1 : Diagram showing the cyclic changes in daily photoperiod for the lighting regimes used in Experiment 5, and the subdivision of the time-course of the experiment for data analyses.

Table 5.1

Orthogonal coefficients used in partitioning time period effects for semen data in Experiment 5.

Sampling time –	1	2	3	4	5	6
Contrast						
Linear	−5	−3	−1	+1	+3	+5
Quadratic	+5	−1	−4	−4	−1	+5
Cubic	−5	+7	+4	−4	−7	+5

Table 5.2

Orthogonal coefficients used in partitioning time period effects for plasma LH and testosterone data in Experiment 5.

		Period 1				Period 2					
Sampling time –		1	2	3	4	5	6	7	8	9	10
Contrast											
Period 1	– Linear	−3	~1	+1	+3	0	0	0	0	0	0
" "	– Quadratic	+1	−1	−1	+1	0	0	0	0	0	0
" "	– Cubic	−1	+3	−3	+1	0	0	0	0	0	0
Period 2	– Linear	0	0	0	0	−5	−3	−1	+1	+3	+5
" "	– Quadratic	0	0	0	0	+5	−1	−4	−4	−1	+5
" "	– Cubic	0	0	0	0	−5	+7	+4	−4	−7	+5
Period 1 vs Period 2		+3	+3	+3	+3	−2	−2	−2	−2	−2	−2

Table 5.3

Orthogonal coefficients used in partitioning time period effects for plasma prolactin data in Experiment 5.

		Period 1							Period 2		
Sampling time –		1	2	3	4	5	6		7	8	9
Contrast											
Period 1 – Linear		−5	−3	−1	+1	+3	+5		0	0	0
" " – Quadratic		+5	−1	−4	−4	−1	+5		0	0	0
" " – Cubic		−5	+7	+4	−4	−7	+5		0	0	0
Period 2 – Linear		0	0	0	0	0	0		−1	0	+1
" " – Quadratic		0	0	0	0	0	0		+1	−2	+1
Period 1 vs Period 2		+1	+1	+1	+1	+1	+1		−2	−2	−2

coefficients used to partition time period effects in the analyses of variance are shown in Table 5.1.

      (b) <u>LH and Testosterone</u>. The ten four-weekly assay results for mean plasma LH and testosterone concentrations were grouped into two periods, with the June solstice as the dividing point (see Figure 5.1). Orthogonal coefficients used to partition time period effects in the analyses of variance are shown in Table 5.2.

      (c) <u>Prolactin</u>. The nine monthly mean estimates of plasma prolactin concentration were grouped into two periods, with the dividing point at the September equinox (see Figure 5.1). Orthogonal coefficients used to partition time period effects in the analysis of variance are shown in Table 5.3.

      (d) <u>Treatment Contrasts</u>. In the analyses of variance for all semen and hormonal data, comparisons of the effects of the three lighting regimes were performed using the orthogonal coefficients shown below :

	Lighting Regime		
Contrast	Normal	Even	Reversed
Normal <u>vs</u> Reversed	+1	0	-1
Even <u>vs</u> Normal and Reversed	-1	+2	-1

3. RESULTS

(1) <u>Semen Data</u>

See Tables 5.4 to 5.10 and Figure 5.2.

With the exceptions of ejaculate volume and percentage of unstained spermatozoa, all semen characteristics exhibited a general increase in quality over the period of study. The Reversed lighting group contributed most to these increases, and in fact displayed a sharp rise in ejaculate volume at the end of the experiment.

Over the whole period of the experiment, the Even lighting

Table 5.4

Mean motility indices (scale 0-4) and mean percentages of motile
spermatozoa recorded from ejaculates collected in Experiment 5.

Motility index

Lighting Regime

Sampling time	Normal	Reversed	Even	Mean
1	2.1	1.4	1.2	1.6
2	2.3	1.7	1.1	1.7
3	1.7	1.3	0.9	1.3
4	3.2	2.3	2.2	2.6
5	3.6	3.1	2.1	2.9
6	2.9	2.7	2.3	2.6
Mean	2.6	2.1	1.6	

% Motile spermatozoa

Lighting Regime

Sampling time	Normal	Reversed	Even	Mean
1	41.2	34.4	24.4	33.3
2	51.9	28.8	20.8	33.8
3	32.5	28.1	18.8	26.5
4	67.8	45.0	48.1	53.6
5	74.4	56.9	45.0	58.8
6	55.2	61.2	50.2	55.5
Mean	53.8	42.4	34.6	

Table 5.5

Mean ejaculate volumes and mean total fructose contents of
ejaculates collected in Experiment 5.

Ejaculate volume (ml)

Lighting Regime

Sampling time	Normal	Reversed	Even	Mean
1	1.67	1.36	0.75	1.26
2	0.85	0.75	1.15	0.92
3	0.62	0.64	0.99	0.75
4	1.22	0.98	0.79	1.00
5	0.94	1.06	0.79	0.93
6	0.95	1.51	0.75	1.07
Mean	1.04	1.05	0.87	

Total ejaculate fructose content (mg)

Lighting Regime

Sampling time	Normal	Reversed	Even	Mean
1	3.48	1.48	1.12	2.03
2	1.01	0.87	2.51	1.46
3	0.65	0.77	0.85	0.76
4	0.96	2.21	0.51	1.23
5	1.07	6.15	0.51	2.58
6	1.51	6.88	0.35	2.92
Mean	1.45	3.06	0.97	

Table 5.6

Mean concentrations of fructose in semen and in seminal plasma of
ejaculates collected in Experiment 5.

Seminal fructose concentration (mg/ml)

Lighting Regime

Sampling time	Normal	Reversed	Even	Mean
1	1.72	1.20	1.02	1.31
2	1.17	0.98	1.99	1.38
3	0.77	1.01	1.05	0.94
4	0.74	1.54	0.58	0.95
5	1.12	5.09	0.68	2.30
6	1.74	4.42	0.53	2.23
Mean	1.21	2.37	0.98	

Seminal plasma fructose concentration (mg/ml)

Lighting Regime

Sampling time	Normal	Reversed	Even	Mean
1	2.22	1.25	1.06	1.51
2	1.32	1.04	2.02	1.46
3	0.89	1.03	0.74	0.89
4	0.93	2.07	0.87	1.29
5	1.41	6.28	0.76	2.81
6	2.10	5.72	0.61	2.81
Mean	1.48	2.90	1.01	

Table 5.7

Mean concentrations of spermatozoa/ml and mean numbers of spermatozoa/
ejaculate in semen collected in Experiment 5.

Spermatozoa/ml $(x10^9)$

Lighting Regime

Sampling time	Normal	Reversed	Even	Mean
1	1.74	0.63	1.20	1.19
2	1.65	0.95	1.23	1.28
3	1.47	0.87	0.44	0.93
4	2.89	2.65	2.94	2.83
5	3.11	2.65	3.17	2.98
6	2.90	3.22	3.42	3.18
Mean	2.30	1.83	2.07	

Spermatozoa/ejaculate $(x10^9)$

Lighting Regime

Sampling time	Normal	Reversed	Even	Mean
1	4.11	0.85	1.70	2.22
2	1.69	0.60	2.14	1.48
3	1.52	0.60	0.43	0.85
4	4.29	2.74	2.44	3.16
5	3.05	3.05	3.70	3.26
6	2.96	5.78	3.64	4.13
Mean	2.94	2.27	2.34	

Table 5.8

Mean percentages of unstained and morphologically normal spermatozoa in semen collected in Experiment 5.

% Unstained spermatozoa

Lighting Regime

Sampling time	Normal	Reversed	Even	Mean
1	55.0	45.2	30.9	43.7
2	69.5	52.1	48.4	56.7
3	34.0	35.0	24.2	31.1
4	31.5	51.0	43.6	42.0
5	55.1	64.6	56.8	58.8
6	56.6	56.1	57.2	56.6
Mean	50.3	50.7	43.5	

% Morphologically normal spermatozoa

Lighting Regime

Sampling time	Normal	Reversed	Even	Mean
1	50.4	29.8	38.2	39.5
2	56.8	35.4	37.5	43.2
3	47.6	50.0	22.8	40.1
4	79.4	49.2	57.6	62.1
5	73.9	72.6	60.5	69.0
6	57.5	81.0	60.4	66.3
Mean	60.9	53.0	46.2	

Table 5.9

Experiment 5 : Summary of Analyses of Variance for Semen Data.

Source of Variation	Contrast No.	D.F.	Volume	Motility	% Motile	Sperm./ml	Sperm./ejac.
					Variance Ratios		
MAIN EFFECTS							
A. LIGHTING REGIMES		2					
Normal vs Reversed	1	1	0.01	3.23	4.24*	1.20	0.61
Even vs Normal and Reversed	2	1	1.89	6.25*	5.96*	0.00	0.13
B. TIME PERIODS		5					
Linear	3	1	0.29	13.32***	11.64**	18.17***	5.41*
Quadratic	4	1	3.32	0.00	0.00	0.24	1.79
Cubic	5	1	0.75	2.60	2.28	2.08	1.04
INTERACTION (AxB)		10					
Contrast 2 x Contrast 4	6	1	4.14*	0.17	0.15	0.24	0.01
Non significant contrasts		5	1.26	0.08	0.47	0.24	0.83
Residual Mean Square		54	0.30	1.43	630.75	2.67	9.33

(Key, Semen Parameters : Volume = ejaculate volume; Motility = motility index; % Motile = percentage of motile spermatozoa; Sperm./ml = concentration of spermatozoa/ml; Sperm./ejac. = number of spermatozoa/ejaculate) .

Table 5.10

Experiment 5 : Summary of Analyses of Variance for Semen Data.

Source of Variation	Contrast No.	D.F.	Variance Ratios				
			Fr.Conc.	Fr. Cont.	S.P. Fr. Conc.	% Unstained	% Normal
MAIN EFFECTS							
A. LIGHTING REGIMES		2					
Normal vs Reversed	1	1	8.46**	6.23*	9.67**	0.09	1.82
Even vs Normal and Reversed	2	1	6.17*	5.36*	9.64**	2.68	3.23
B. TIME PERIODS		5					
Linear	3	1	5.02*	2.35	8.52**	2.73	12.41***
Quadratic	4	1	3.02	4.64*	4.16*	2.24	0.03
Cubic	5	1	0.09	0.36	0.51	0.09	1.20
INTERACTION (AxB)		10					
Contrast 1 x Contrast 3	6	1	13.53***	16.68***	18.90***	1.86	2.96
Contrast 2 x Contrast 3	7	1	9.48**	5.88*	10.13**	2.16	0.02
Non significant contrasts		4	1.20	0.98	1.15	0.50	0.56
Residual Mean Square		54	1.80	4.94	2.38	382.38	720.50

(Key, Semen Parameters : Fr. Conc. = fructose concentration of semen; Fr. Cont. = total ejaculate fructose content; S.P. Fr. Conc. = fructose concentration of seminal plasma; % Unstained = percentage of unstained spermatozoa; % Normal = percentage of morphologically normal spermatozoa)

Figure 5.2 : Monthly variations in seminal plasma fructose concentrations (mean±S.E.) in semen collected from rams subjected to Normal (N), Even (E) or Reversed (R) lighting regimes. Daily photoperiod changes are illustrated and the timing of the equinox (E') and solstice (S') indicated.

group had the lowest mean spermatozoal motility and percentage of motile spermatozoa, while the Reversed group had a mean percentage of motile spermatozoa intermediate between the other two groups (Contrasts 1 and 2).

The most notable result was the elevation in seminal fructose levels recorded from the Reversed lighting group during the last two months of the experiment (Contrasts 6 and 7).

Although the rams on Even lighting tended to display changes similar to the Normal lighting group for most semen characteristics, their seminal fructose levels fell over the latter part of the study (Contrast 7).

(2) LH and Testosterone

See Tables 5.11, 5.12 and Figures 5.3 and 5.4.

The period from March to June 1973 was characterised by a decline in plasma levels of LH and testosterone. On the other hand, during the second half of the year there was little overall change in plasma LH concentrations, while an increase in plasma testosterone content was almost entirely due to the result from the rams subjected to Reversed lighting (Contrasts 5, 8 and 11). These rams also had higher plasma LH levels in the final six months of the year than did the Normal lighting group (Contrast 10). During October, the Even lighting group displayed a sharp peak in mean plasma LH concentrations (Contrast 12).

(3) Prolactin

See Tables 5.13, 5.14 and Figure 5.5.

During the March-September period the Reversed lighting group were subjected to a summer lighting regime and displayed an elevation of plasma prolactin levels approximately in phase with the change in photoperiod. A similar elevation of prolactin levels, in phase with

Table 5.11

Mean plasma LH and testosterone concentrations recorded from rams in Experiment 5.
(Values presented are $100 \log_{10}(x + 1.1)$, where x is hormone concentration in ng/ml.)

Luteinizing Hormone

		Lighting Regime			
	Sampling time	Normal	Reversed	Even	Mean
Period 1	1	28.5	12.4	14.2	18.4
	2	4.1	6.5	11.9	7.5
	3	15.4	5.1	5.1	8.5
	4	10.1	7.1	14.2	10.5
Mean		14.5	7.8	11.3	11.2
Period 2	5	12.9	7.1	12.1	10.7
	6	13.8	15.2	7.9	12.3
	7	9.4	13.0	15.7	12.7
	8	7.5	15.8	34.2	19.2
	9	8.7	12.5	8.6	9.9
	10	16.5	20.4	13.7	16.9
Mean		11.5	14.0	15.4	13.6
Overall Mean		12.7	11.5	13.8	

Testosterone

		Lighting Regime			
	Sampling time	Normal	Reversed	Even	Mean
Period 1	1	60.2	37.1	18.7	38.7
	2	21.9	5.9	5.1	11.0
	3	21.3	9.5	7.5	12.8
	4	12.1	12.1	11.7	12.0
Mean		28.9	16.1	10.7	18.6
Period 2	5	28.1	5.9	14.9	16.3
	6	15.2	16.3	4.1	11.9
	7	5.1	51.7	27.2	28.0
	8	17.1	63.3	32.4	37.6
	9	20.2	68.5	8.2	32.3
	10	30.5	77.7	21.8	43.3
Mean		19.4	47.2	18.1	28.2
Overall Mean		23.2	34.8	15.2	

Table 5.12

Experiment 5 : Summary of Analyses of Variance for LH and Testosterone Data.

Source of Variation	Contrast No.	D.F.	Variance Ratios	
			LH	Testosterone
MAIN EFFECTS				
A. LIGHTING REGIMES		2		
Normal vs Reversed	1	1	0.38	5.71*
Even vs Normal and Reversed	2	1	0.84	11.07**
B. TIME PERIODS		9		
Period 1 – Linear	3	1	3.97*	8.11**
" " – Quadratic	4	1	6.19*	4.78*
Period 2 – Linear	5	1	1.87	15.60***
" " – Quadratic	6	1	0.23	0.02
Periods 1 vs 2	7	1	1.83	5.63*
Non significant contrasts		2	0.42	0.55
INTERACTION (AxB)		18		
Contrast 1 x Contrast 5	8	1	1.23	14.59***
" " x Contrast 6	9	1	1.12	4.47*
" " x Contrast 7	10	1	5.16*	17.21***
Contrast 2 x Contrast 5	11	1	0.00	4.35*
" " x Contrast 6	12	1	4.79*	0.53
Non significant contrasts		9	1.51	0.49
Residual Mean Square		90	84.63	465.69
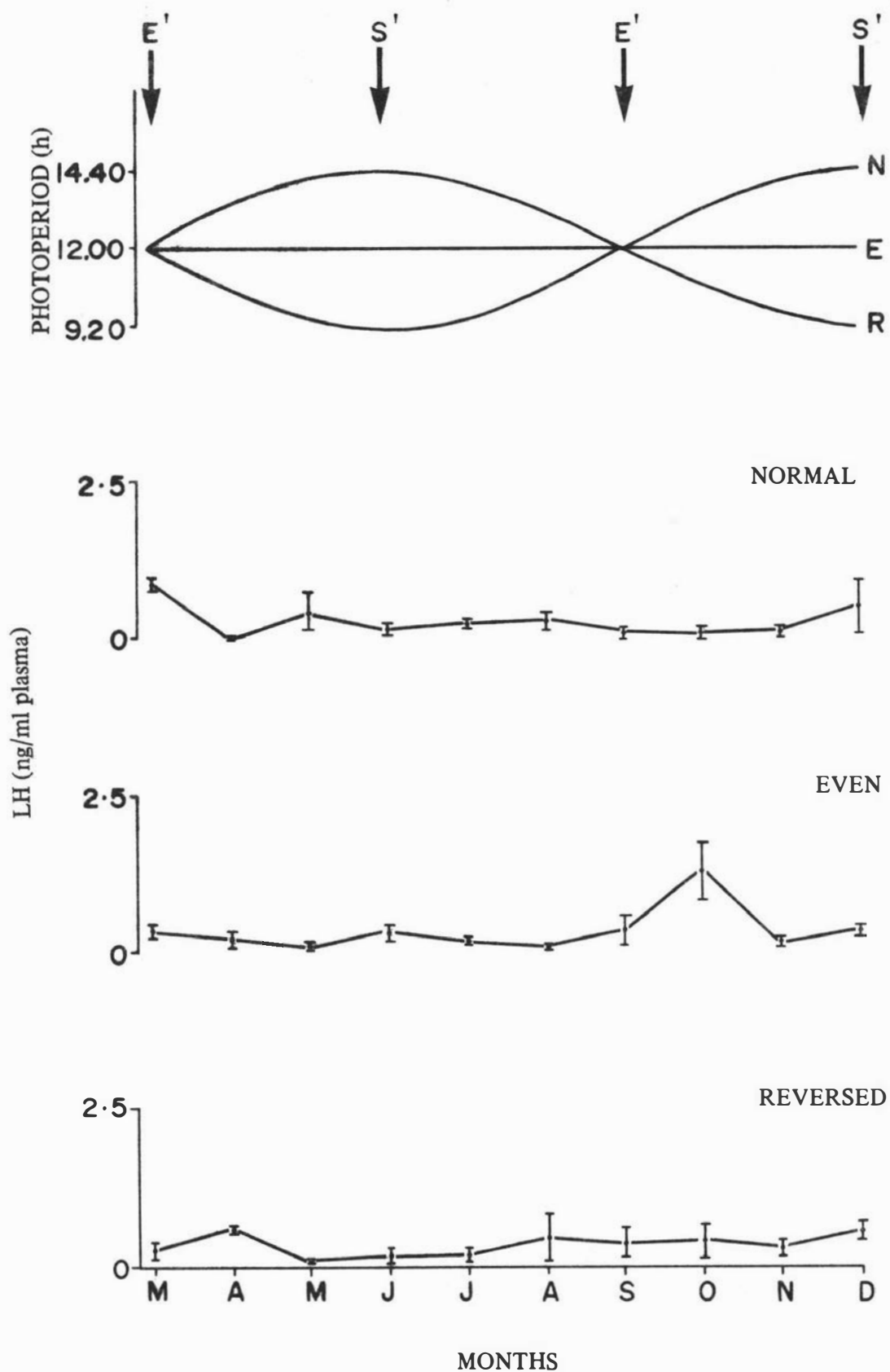
Figure 5.3 : Monthly variations in plasma LH concentrations (mean ±S.E.) recorded from rams subjected to Normal (N), Even (E) or Reversed (R) lighting regimes.   Daily photoperiod changes are illustrated and the timing of the equinoxes (E') and solstices (S') indicated.
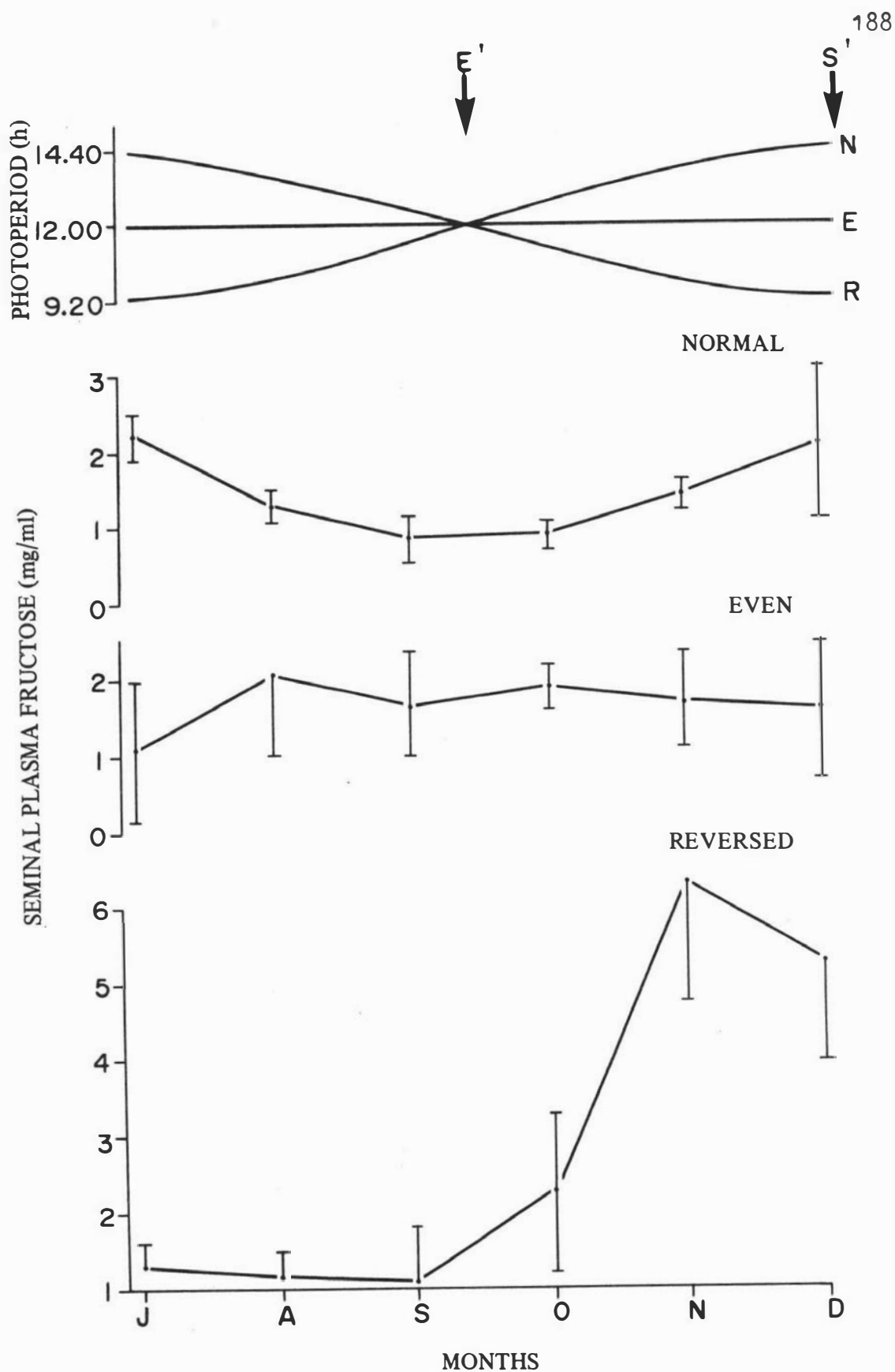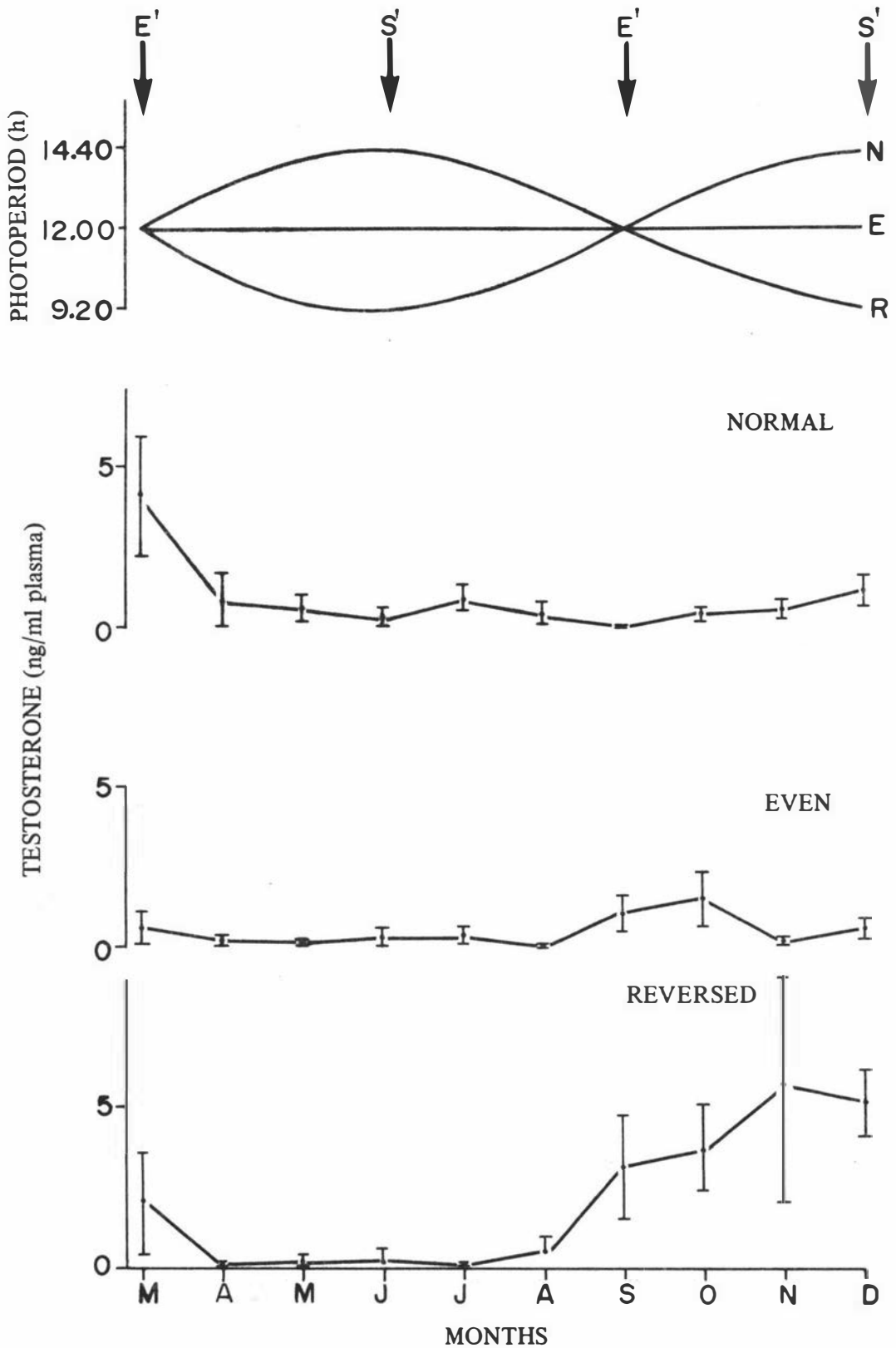
Figure 5.4 : Monthly variations in plasma testosterone concentrat-
ions (mean±S.E.) recorded from rams subjected to Normal (N), Even
(E) or Reversed (R) lighting regimes.   Daily photoperiod changes
are illustrated and the timing of the equinoxes (E') and solstices
(S') indicated.

Table 5.13

Mean plasma prolactin concentrations recorded from rams in Experiment 5.
(Values presented are $100 \log_{10}(x + 1.1)$, where x is hormone concentration in ng/ml.)

Lighting Regime

	Sampling time	Normal	Reversed	Even	Mean
	1	60.0	98.4	105.8	88.1
	2	81.1	140.1	90.3	103.8
Period 1	3	114.1	151.6	129.3	131.7
	4	118.7	181.4	134.7	144.9
	5	113.6	175.0	129.5	139.4
	6	122.7	126.7	142.2	130.5
	Mean	101.7	145.5	122.0	123.1
	7	157.1	112.0	157.0	142.0
Period 2	8	154.4	93.5	144.2	130.7
	9	167.4	104.3	124.4	132.0
	Mean	159.6	103.3	141.9	134.9
	Overall Mean	121.0	131.4	128.6	

Table 5.14

Experiment 5 : Summary of Analysis of Variance for Prolactin Data.

Source of Variation	Contrast No.	D.F.	Variance Ratio
MAIN EFFECTS			
A. LIGHTING REGIMES		2	
Normal vs Reversed	1	1	2.84
Even vs Normal and Reversed	2	1	0.29
B. TIME PERIODS		8	
Period 1 – Linear	3	1	27.91***
" " – Quadratic	4	1	13.84***
Periods 1 vs 2	5	1	4.98*
Non significant contrasts		3	0.73
INTERACTION (AxB)		16	
Contrast 1 x Contrast 5	6	1	59.67***
Contrast 2 x Contrast 4	7	1	5.10*
Non significant contrasts		10	0.86
Residual Mean Square		81	670.58
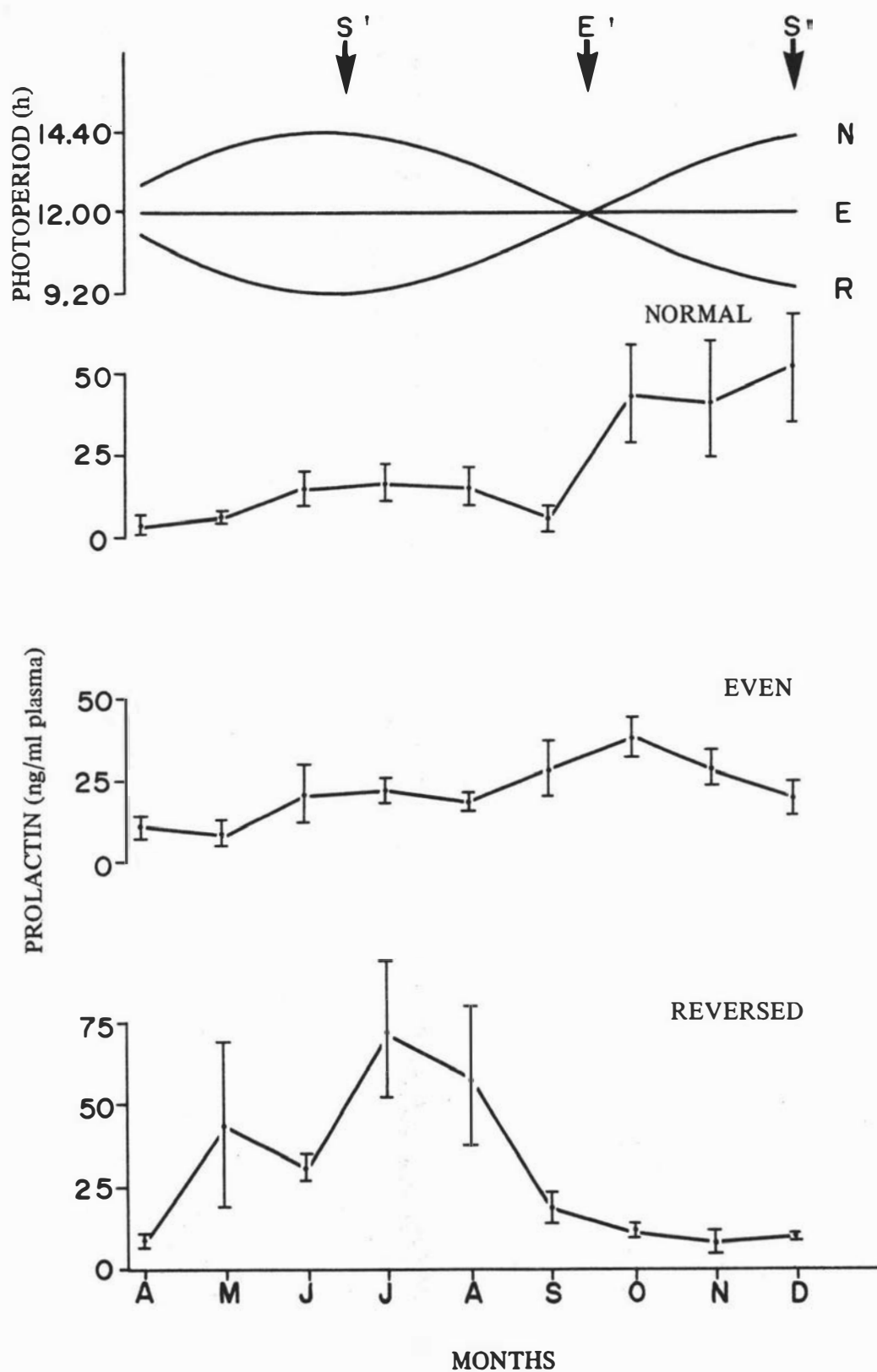
Figure 5.5 : Monthly variations in plasma prolactin concentrations (mean±S.E.) recorded from rams subjected to Normal (N), Even (E) or Reversed (R) lighting regimes. Daily photoperiod changes are ill-ustrated and the timing of the equinox (E') and solstices (S') indicated.

increasing photoperiod, was recorded from the rams on the Normal lighting regime during the October-December period. The above two results, in which plasma prolactin concentration increased with lengthening photoperiod, entirely accounted for the very highly significant component (Contrast 6) of the Lighting Regimes x Periods interaction.

Compared to the range of fluctuations in plasma prolactin concentrations recorded from the rams on the Normal and Reversed lighting regimes, only relatively minor changes were recorded from those on the Even lighting regime.

4. DISCUSSION

The results obtained in this experiment indicated that, in the absence of changes in other environmental factors, alterations in lighting influenced neuroendocrine mechanisms in N.Z. Romney rams. This finding supported the hypothesis that changes in daily photoperiod were the major stimulus for seasonal changes in semen characteristics and plasma hormone levels in rams at pasture.

Plasma LH concentrations recorded in this experiment were so low that detection of significant differences between treatment groups was unlikely. This failure to show any effect of photoperiod on plasma LH levels, similar to those reported by Pelletier and Ortavant (1975a), largely can be accounted for by the differences in concentrations of this hormone obtained in the two laboratories. While the mean levels for plasma LH from rams in the French study ranged from 1.5 to 4.0 ng/ml, those in the present experiment rarely exceeded 0.5 ng/ml. Bioassay comparisons have indicated that this difference probably could not be attributed to the different standards used in each assay : the LH-M1 standard used by Pelletier and Ortavant had 1.5 times the biological activity of NIH-LH-S1, whereas the NIH-LH-S18 used in this

laboratory was equivalent to 1.03 times the NIH-LH-S1 standard. Nevertheless, it is possible that a difference in immunoreactivity of the assay standards, which was not reflected as a difference in biological activity, or some other difference between the assays, accounted for the different levels of LH recorded in this and the French work. Alternatively, the low LH levels of the N.Z. Romney rams could have reflected either a breed difference, or there may have been a diurnal pattern of secretion, so that low levels of LH always were recorded from blood samples collected at 10.00 h (however, see Chapter VII).

Although there was little evidence for any seasonal change in plasma LH levels, the rise in plasma testosterone concentration recorded during the final four months of the experiment from the rams on the Reversed lighting regime, demonstrated that photoperiodic influences could alter gonadal function. Since changes in testosterone production would be expected to reflect altered LH secretion, the difference in pattern of results for these two hormones in Experiment 5 was surprising. This disparity may have reflected deficiencies in the LH assay, or it was possible that the frequency of pulses of LH output had altered, without necessarily changing mean plasma levels at the particular time of the day when samples were collected (Katongole, Naftolin and Short, 1974).

The photoperiodic influence on gonadal testosterone production was confirmed by the simultaneous increase in seminal fructose levels and in ejaculate volumes recorded from the Reversed lighting group. Only one previous report has indicated that seasonal changes in seminal fructose levels could be simulated by manipulating the photo-period (Jackson and Williams, 1973). In an Australian study no seasonal pattern of seminal fructose levels was observed from rams on normal or reversed lighting regimes (Moule, Braden and Mattner, 1966).

This latter result caused the authors to conclude that seasonal changes in seminal fructose levels in grazing rams were attributable to nutritional factors; in the present work, the diet was of constant composition throughout the experiment. However, under some extensive Australian grazing conditions, nutrition may have an influence on seminal fructose levels.

For rams on Reversed lighting the delay between the longest daily photoperiod and the increase in plasma testosterone levels was longer than that recorded from rams at pasture (Experiment 3). Jackson and Williams (1973) indicated that a transient phase occurs during adaptation of rams to an imposed light rhythm, so that gonadal responses do not become "in phase" with the new rhythm until after a few photoperiodic cycles. Presumably such an adaptation period is required for establishment of new "in phase" rhythms of LH secretion, and results from refractoriness at pituitary, hypothalamic, or other levels in the central nervous system.

Other studies on ram semen have shown that photoperiod reversal can reverse the seasonal changes in parameters related to spermatogenesis (Ortavant, 1956; Ortavant and Thibault, 1956; Fowler, 1961; Colas et al., 1972; Jackson and Williams, 1973); no such response for these parameters was seen in the present study nor in semen from grazing N.Z. Romney rams (Experiment 3). This apparent conflict of results could have resulted from breed differences. Alternatively, it may indicate that spermatogenic responses to photoperiodic rhythms are significant only when using rhythms with greater (8 h) amplitude, and a shorter (6 months) cycle length, than that used in the current experiment.

Results of experiments on the effects of different lighting regimes on epididymal spermatozoal reserves (Ortavant, Mauleon and Thibault, 1964) led to the authors making two important conclusions :

firstly, the effects of lighting reversal were more pronounced with a six-monthly cycle than with the natural lighting changes, and secondly, the effects were more pronounced in the second cycle than in the first. In Experiment 5 the natural changes in photoperiod which occur at this locality were simulated, thus the results of this experiment were more comparable to those obtained from rams at pasture in Experiments 3 and 4, than to findings from many of the studies reported in the literature.

One point of departure from natural daylight changes was the absence of allowance for twilight in the present experiment. At Palmerston North twilight provided grazing animals with a slightly variable period of additional lighting throughout the year (about 30 minutes per day), but the amplitude of the annual photoperiodic cycle was not significantly different from that used in this experiment (civil twilight = 32 minutes midsummer and 26 minutes midwinter, N.Z. Aeronautical Information Publication, Ministry of Transport). Thus, at this latitude twilight would not account for differences between results recorded from grazing rams and from those housed indoors. However at higher latitudes twilight becomes progressively longer and has a greater seasonal variation so that it could represent a significant factor in the annual photoperiodic changes.

The second point raised by Ortavant, Mauleon and Thibault (1964) exposes the major limitation of the present experiment; this was the fact that the experiment did not continue for more than one cycle of lighting changes. At the design stages of this preliminary experiment, it was not envisaged that light rhythm reversal would take so long to produce its effects. Consequently the experiment was concluded at the December solstice. It is now obvious that extension of the experiment for a few months may have shown whether the elevated

plasma testosterone and seminal fructose levels in the Reversed lighting group did in fact decline, while those for the Normal lighting group continued to rise. Furthermore, if the experiment had been continued for a year or two, it is possible that data for other semen characteristics and LH may have shown seasonal changes in phase with the photoperiodic cycle.

No significant changes in plasma testosterone or seminal fructose concentrations were recorded from the rams on Even lighting indicating that this group resembled the Normal lighting group in its seasonal pattern. This result was consistent with the conclusion of Ortavant (1961), who claimed that although ten to twelve hours of daylight represented the optimum for reproduction in rams, this only applied when daily photoperiod was being progressively decreased.

Rams on Normal lighting did not display major changes in semen production, or plasma LH or testosterone levels during the course of this study. As similar results were recorded from rams on Even lighting, no conclusion could be reached regarding the existence of residual reproductive rhythms in the latter group. Residual rhythms of semen production by rams were reported by Jackson and Williams (1973), but more than one photoperiodic cycle was required to demonstrate their existence.

Even with the short time-course of this experiment it was possible to reach definite conclusions about prolactin secretion in rams : all groups of rams showed changes in plasma prolactin levels which were in phase with the lighting regimes. Thus this experiment confirmed the finding of Pelletier (1973) that prolactin secretion in rams was directly related to the length of the photoperiod. Also, the relatively constant plasma prolactin levels of rams on Even lighting suggested that there was no inherent seasonal rhythm of prolactin release.

In some species, it has been shown that the pineal gland has an important role in the regulation of hormone release by lighting changes (Reiter, 1974a). It remains to be established whether the seasonal prolactin responses to changing photoperiods are regulated by the pineal gland in the ovine species, and in turn, whether the changes in prolactin secretion have any significant direct or indirect role in regulating ovine reproductive seasonality.

CHAPTER VI

EFFECTS OF PINEALECTOMY ON SEMEN PRODUCTION AND PLASMA HORMONE LEVELS

IN RAMS SUBJECTED TO CONTRASTING LIGHTING REGIMES

1. INTRODUCTION

Experiment 6 was undertaken to study the effects of pineal-
ectomy on semen production and plasma hormone levels in rams.

In Experiment 4, cranial cervical ganglionectomy produced
changes in a number of parameters associated with reproductive activity,
and also altered pineal gland function. This result indicated that
the pineal gland may have an important role in inducing seasonality of
reproduction in rams. Thus it was necessary to remove the pineal
gland to allow direct investigation of this possibility.

Reiter (1973b) noted that pinealectomy was a notoriously
unreliable means of demonstrating pineal gland influences on the
reproductive system. He suggested that often this was due to the use
of inappropriate lighting regimes, which by virtue of their excessive
photoperiod lengths, effectively "pinealectomized" experimental
animals whether they had been surgically pinealectomized or not. The
present experiment was designed to overcome this shortcoming : both
pinealectomized and control rams were submitted to normal or reversed
lighting regimes, in an attempt to evaluate the possible role of the
pineal gland in mediating the reproductive responses to photoperiod
reversal, which were recorded in Chapter V.

2. MATERIALS AND METHODS

(1) Animals and Experimental Procedure

Sixteen adult N.Z. Romney rams were allocated at random to the

pinealectomy (PX) or sham-operation (Controls) surgical treatments described in Chapter II. Surgery was carried out between six and one weeks immediately prior to the commencement of the experiment, by which time all rams appeared to have recovered from surgery and were in good health.

Post-mortem macroscopic examination of formalin-fixed brains verified the completeness of the pinealectomy operations, but histological examination of paramedian brain sections revealed minute remnants of pineal stalk in a few pinealectomized rams. These remnants were not considered to be of sufficient importance to warrant exclusion of the data obtained from any of the rams concerned. Brains from sham-operated rams were examined in a like manner and appeared normal in every respect. See Figures 6.1 and 6.2.

Commencing at the September equinox 1974, one light-controlled room (containing eight rams : four pinealectomized and four sham-operated) was submitted to the normal seasonal rhythm of photoperiodic change (Normal), whilst another room (also containing four pinealect-omized and four sham-operated rams) was subjected to a reversed pattern of photoperiodic changes (Reversed). The amplitude of the photoperiodic cycle was identical to that used in Experiment 5, with a minimum photoperiod of 9 hours and 20 minutes and a maximum of 14 hours and 40 minutes. These lighting treatments were continued until the end of the experiment in May 1975.

(2) Statistical Analyses

(a) Semen and Hormone Data. For each semen parameter, data were pooled to provide a monthly mean estimate for each ram. The eight complete months of the experiment were divided into two periods, demarcated by the December solstice, as shown in Figure 6.3. Orthogonal coefficients used to partition time period effects for semen data are

Figure 6.1 : Midsagittal section of brain from sham-operated
ram showing intact pineal gland (p).
Solochrome cyanin and cresyl fast violet stain.

Figure 6.2 : Midsagittal section of brain from pinealectomized
ram showing remnant of pineal stalk (r).
Solochrome cyanin and cresyl fast violet stain.

(Note : In Figures 6.1 and 6.2 the rostral end of the brain
is to the left of each figure.)
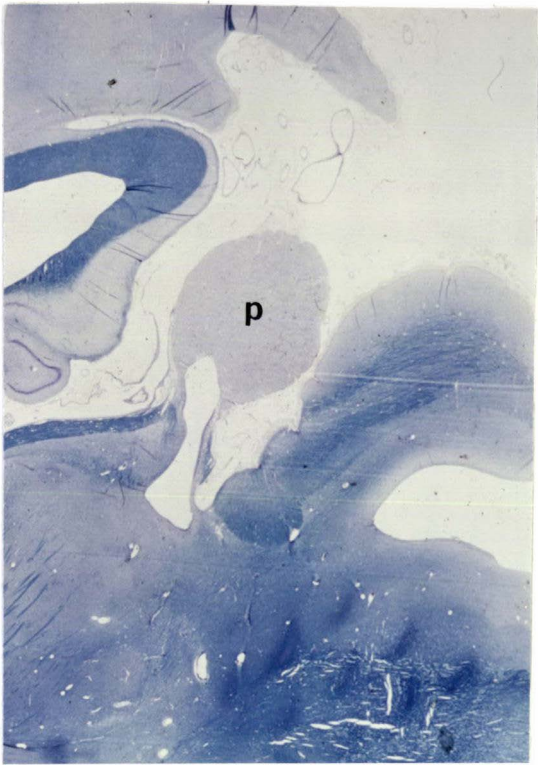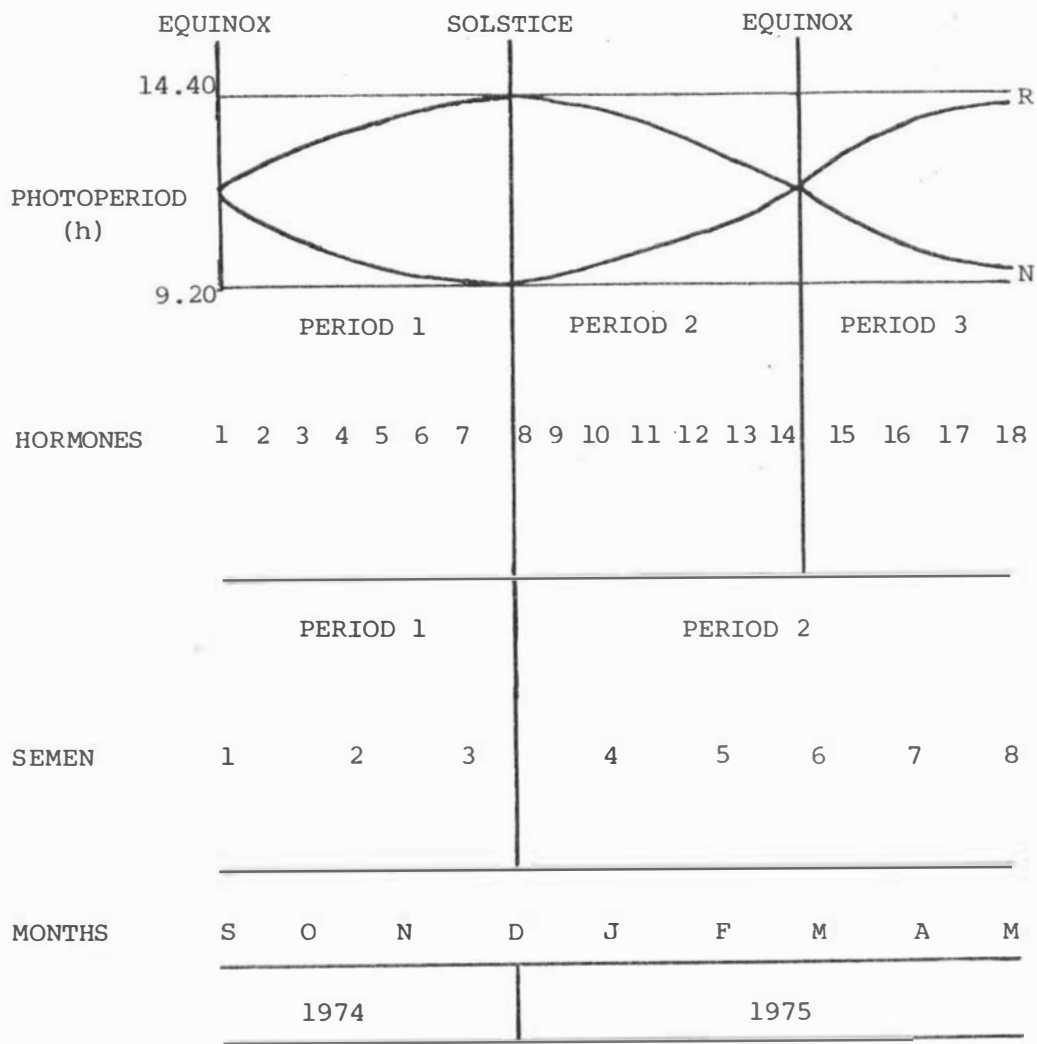
KEY: R – Reversed

       N – Normal

Figure 6.3 : Diagram showing the cyclic changes in daily photoperiod for the lighting regimes used in Experiment 6, and the subdivision of the time-course of the experiment for data analyses.

Table 6.1

Orthogonal coefficients used in partitioning time period effects for semen data in Experiment 6.

		Period 1				Period 2			
Sampling time –		1	2	3		4	5	6	7	8
Contrast										
Period 1 – Linear		−1	0	+1		0	0	0	0	0
" " – Quadratic		+1	−2	+1		0	0	0	0	0
Period 2 – Linear		0	0	0		−2	−1	0	+1	+2
" " – Quadratic		0	0	0		+2	−1	−2	−1	+1
" " – Cubic		0	0	0		−1	+2	0	−2	+1
Period 1 vs Period 2		+5	+5	+5		−3	−3	−3	−3	−3

Table 6.2

Orthogonal coefficients used in partitioning time period effects for plasma hormone data in Experiment 6.

	Period 1							Period 2							Period 3			
Sampling time —	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Contrast																		
Period 1 – Linear	−3	−2	−1	0	+1	+2	+3	0	0	0	0	0	0	0	0	0	0	0
" " – Quadratic	+5	0	−3	−4	−3	0	+5	0	0	0	0	0	0	0	0	0	0	0
" " – Cubic	−1	+1	+1	0	−1	−1	+1	0	0	0	0	0	0	0	0	0	0	0
Period 2 – Linear	0	0	0	0	0	0	0	−3	−2	−1	0	+1	+2	+3	0	0	0	0
" " – Quadratic	0	0	0	0	0	0	0	+5	0	−3	−4	−3	0	+5	0	0	0	0
" " – Cubic	0	0	0	0	0	0	0	−1	+1	+1	0	−1	−1	+1	0	0	0	0
Period 3 – Linear	0	0	0	0	0	0	0	0	0	0	0	0	0	0	−3	−1	+1	+3
" " – Quadratic	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+1	−1	−1	+1
" " – Cubic	0	0	0	0	0	0	0	0	0	0	0	0	0	0	−1	+3	−3	+1
Period 1 vs Period 2	+1	+1	+1	+1	+1	+1	+1	−1	−1	−1	−1	−1	−1	−1	0	0	0	0
Period 3 vs Periods 1 & 2	−2	−2	−2	−2	−2	−2	−2	−2	−2	−2	−2	−2	−2	−2	+7	+7	+7	+7

shown in Table 6.1.

For each hormone the 18 two-weekly estimates of plasma hormone concentrations were grouped into three periods, demarcated by the December solstice and March equinox, as shown in Figure 6.3. Orthogonal coefficients used to partition time period effects for hormone data are shown in Table 6.2. Results from Experiment 5 indicated that these orthogonal contrasts should have provided the most meaningful evaluation of photoperiodic effects.

(b) Treatment Contrasts. Treatment effects of lighting regimes, operations, and their interaction were examined using the orthogonal coefficients shown below.

Contrast	Normal Lighting		Reversed Lighting	
	Controls	Pinealectomized	Controls	Pinealectomized
Main Effects				
Lighting Regimes	+1	+1	−1	−1
Operations	+1	−1	+1	−1
Interaction				
Lighting Regimes x Operations	+1	−1	−1	+1

3. RESULTS

(1) Semen Data

See Tables 6.3 to 6.9 and Figures 6.4 to 6.7.

Semen from sham-operated rams had higher values for spermatozoal motility and percentage of motile spermatozoa than semen from pineal-ectomized rams. Also pinealectomized rams on Reversed lighting produced ejaculates with low motility indices and low percentages of motile spermatozoa during the final five months of the experiment (Contrasts 1, 2, 3, 4 and 6). In the period from the December solstice till the end of the experiment, mean ejaculate volumes tended to increase (Contrast 3) and were higher than in the previous three months;

Table 6.3

Mean motility indices (scale 0-4) and mean percentages of motile spermatozoa recorded from ejaculates collected in Experiment 6.

Motility index

	Sampling time	Normal Lighting		Reversed Lighting		Mean
		Controls	PX	Controls	PX	
Period 1	1	2.9	2.8	3.0	2.9	2.9
	2	3.1	3.0	3.1	3.2	3.1
	3	3.1	2.8	3.3	2.7	2.9
	Mean	3.0	2.9	3.1	2.9	3.0
Period 2	4	3.0	3.0	2.9	2.4	2.8
	5	2.9	3.2	2.5	2.3	2.7
	6	3.6	2.8	3.1	2.7	3.0
	7	3.0	2.9	3.0	2.0	2.7
	8	2.6	2.9	2.7	2.1	2.5
	Mean	3.0	3.0	2.8	2.3	2.7
Overall Mean		3.0	2.9	3.0	2.5	

% Motile Spermatozoa

	Sampling time	Normal Lighting		Reversed Lighting		Mean
		Controls	PX	Controls	PX	
Period 1	1	58.3	55.6	58.3	57.5	57.4
	2	63.8	60.6	63.8	59.4	61.9
	3	60.6	57.5	65.0	51.9	58.7
	Mean	60.9	57.9	62.4	56.3	59.3
Period 2	4	59.4	58.1	60.0	50.6	57.0
	5	53.8	59.4	52.6	45.1	52.7
	6	67.5	54.4	58.1	44.4	56.1
	7	61.3	54.4	56.3	41.9	53.4
	8	50.0	52.5	46.9	38.8	47.0
	Mean	58.3	55.8	54.8	44.2	53.2
Overall Mean		59.3	56.6	57.6	48.7	

(Key: Controls = Sham-operated;   PX = Pinealectomized)

Table 6.4

Mean ejaculate volumes and mean total fructose contents of ejaculates
collected in Experiment 6.

Ejaculate volume (ml)

		Normal Lighting		Reversed Lighting		
	Sampling time	Controls	PX	Controls	PX	Mean
Period 1	1	1.45	1.24	1.41	1.42	1.38
	2	1.11	1.04	0.95	1.11	1.05
	3	1.08	1.16	1.43	1.33	1.25
	Mean	1.21	1.14	1.26	1.28	1.22
Period 2	4	1.14	1.73	0.95	1.46	1.32
	5	1.19	1.66	1.30	2.06	1.55
	6	1.03	2.03	1.96	1.88	1.72
	7	1.09	2.60	1.70	1.89	1.82
	8	1.50	2.20	1.81	1.73	1.81
	Mean	1.19	2.04	1.54	1.80	1.64
Overall Mean		1.19	1.70	1.43	1.61	

Total ejaculate fructose content (mg)

		Normal Lighting		Reversed Lighting		
	Sampling time	Controls	PX	Controls	PX	Mean
Period 1	1	0.67	1.14	0.47	1.69	0.99
	2	0.64	0.75	0.47	1.32	0.80
	3	0.93	0.95	3.19	0.85	1.48
	Mean	0.75	0.94	1.38	1.29	1.09
Period 2	4	1.87	1.83	3.41	1.14	2.06
	5	2.34	3.02	5.76	5.23	4.08
	6	2.41	5.73	6.50	9.00	5.91
	7	3.64	7.39	3.08	8.57	5.67
	8	8.91	10.77	2.14	6.07	6.97
	Mean	3.84	5.75	4.18	6.00	4.94
Overall Mean		2.67	3.94	3.12	4.23	

(Key: Controls = Sham-operated;   PX = Pinealectomized)

Table 6.5

Mean concentrations of fructose in semen and in seminal plasma
of ejaculates collected in Experiment 6.

Seminal fructose concentration (mg/ml)

	Sampling time	Normal Lighting Controls	PX	Reversed Lighting Controls	PX	Mean
Period 1	1	0.48	1.05	0.37	1.18	0.77
	2	0.47	0.77	0.48	1.01	0.68
	3	0.87	0.75	1.39	0.76	0.94
Mean		0.61	0.86	0.75	0.98	0.80
Period 2	4	1.12	0.90	1.95	1.08	1.26
	5	1.38	2.04	3.59	2.72	2.43
	6	2.17	2.71	2.32	4.31	2.88
	7	2.84	2.64	2.16	4.31	2.99
	8	5.76	4.62	1.07	3.90	3.84
Mean		2.65	2.58	2.22	2.26	2.68
Overall Mean		1.88	1.93	1.66	2.40	

Seminal plasma fructose concentration (mg/ml)

	Sampling time	Normal Lighting Controls	PX	Reversed Lighting Controls	PX	Mean
Period 1	1	0.62	1.37	0.46	1.38	0.96
	2	0.62	0.95	0.51	1.23	0.83
	3	1.17	0.94	1.72	0.87	1.18
Mean		0.81	1.09	0.90	1.16	0.99
Period 2	4	1.42	1.15	2.27	1.23	1.52
	5	1.85	2.64	4.35	3.28	3.03
	6	2.63	3.32	3.37	4.90	3.56
	7	3.15	3.48	2.53	5.01	3.54
	8	6.71	5.49	1.40	4.27	4.47
Mean		3.15	3.22	2.78	3.74	3.22
Overall Mean		2.27	2.41	2.07	2.77	

(Key: Controls = Sham-operated; PX = Pinealectomized)

Table 6.6

Mean concentrations of spermatozoa/ml and mean numbers of
spermatozoa/ejaculate in semen collected in Experiment 6.

Spermatozoa/ml $(x10^9)$

		Normal Lighting		Reversed Lighting		
	Sampling time	Controls	PX	Controls	PX	Mean
Period 1	1	3.80	2.95	5.04	3.28	3.77
	2	3.25	2.60	3.22	3.11	3.04
	3	3.48	3.20	4.10	2.71	3.37
	Mean	3.51	2.92	4.12	3.03	3.39
Period 2	4	3.42	3.36	3.27	3.32	3.34
	5	3.10	3.69	3.06	2.91	3.19
	6	2.85	2.84	4.48	2.33	3.13
	7	2.47	3.41	3.88	2.39	3.04
	8	2.31	2.55	4.69	2.01	2.89
	Mean	2.83	3.17	3.88	2.59	3.12
Overall Mean		3.08	3.07	3.96	2.75	

Spermatozoa/ejaculate $(x10^9)$

		Normal Lighting		Reversed Lighting		
	Sampling time	Controls	PX	Controls	PX	Mean
Period 1	1	6.16	4.31	7.01	4.96	5.61
	2	3.93	2.57	3.05	3.69	3.31
	3	4.54	3.73	5.95	4.03	4.56
	Mean	4.88	3.53	5.34	4.22	4.49
Period 2	4	3.95	5.95	3.26	5.25	4.60
	5	4.20	6.22	4.56	5.92	5.22
	6	3.61	6.60	10.05	4.61	6.22
	7	3.49	9.66	6.91	5.05	6.28
	8	3.63	6.00	8.66	3.75	5.51
	Mean	3.77	6.88	6.69	4.92	5.57
Overall Mean		4.18	5.62	6.18	4.65	

(Key: Controls = Sham-operated; PX = Pinealectomized)

Table 6.7

Mean percentages of unstained and morphologically normal
spermatozoa in semen collected in Experiment 6.

% Unstained spermatozoa

		Normal Lighting		Reversed Lighting		
	Sampling time	Controls	PX	Controls	PX	Mean
Period 1	1	62.6	58.8	53.0	56.8	57.8
	2	65.9	64.0	60.4	58.5	62.2
	3	67.9	72.3	67.5	59.6	66.8
Mean		65.5	65.0	60.3	58.3	62.3
Period 2	4	64.3	75.5	62.5	64.5	66.7
	5	70.3	79.1	70.6	73.4	73.3
	6	78.9	79.6	65.1	71.3	73.7
	7	77.0	76.4	63.6	63.0	70.0
	8	71.0	72.6	62.5	66.9	68.2
Mean		72.3	76.6	64.9	67.8	70.4
Overall Mean		69.7	72.3	63.2	64.2	

% Morphologically normal spermatozoa

		Normal Lighting		Reversed Lighting		
	Sampling time	Controls	PX	Controls	PX	Mean
Period 1	1	82.0	68.9	70.7	71.5	73.3
	2	73.3	76.4	72.5	78.1	75.1
	3	77.3	74.5	69.3	73.4	73.6
Mean		77.5	73.3	70.8	74.3	74.0
Period 2	4	75.6	69.4	67.9	70.2	70.8
	5	80.1	75.8	68.3	60.3	71.1
	6	77.6	61.6	72.9	65.9	69.5
	7	77.0	65.5	72.9	67.6	70.7
	8	81.4	77.0	68.9	71.5	74.7
Mean		78.3	69.9	70.2	67.1	71.4
Overall Mean		78.0	71.1	70.4	69.9	

(Key: Controls = Sham-operated; PX = Pinealectomized)

Table 6.8

Experiment 6 : Summary of Analyses of Variance for Semen Data.

Source of Variation	Contrast No.	D.F.	Volume	Motility	% Motile	Sperm./ml	Sperm./ejac.
					Variance Ratios		
MAIN EFFECTS							
A. LIGHTING REGIMES	1	1	0.48	3.56	5.14*	1.70	0.84
B. OPERATIONS	2	1	10.84**	5.38*	7.66**	8.09**	0.01
C. TIME PERIODS		7					
Period 2 – Linear	3	1	7.33**	0.97	4.17*	1.21	1.33
Periods 1 <u>vs</u> 2	4	1	15.38***	3.13	7.80**	1.56	3.48
Non significant contrasts		4	0.89	0.60	0.87	0.71	1.52
INTERACTIONS							
A x B	5	1	2.72	2.07	2.13	7.76**	7.10**
A x C		7					
A x (Periods 1 <u>vs</u> 2)	6	1	0.02	4.67*	2.95	0.08	0.01
Non significant contrasts		5	0.68	0.05	0.14	0.55	0.26
B x C		7					
B x (Periods 1 <u>vs</u> 2)	7	1	7.41**	0.29	0.23	0.69	2.73
Non significant contrasts		5	0.24	0.65	0.42	0.70	0.56
A x B x C		7					
A x B x Period 2 – Linear	8	1	2.72	0.43	0.00	4.07*	4.85*
A x B x (Periods 1 <u>vs</u> 2)	9	1	2.61	0.69	0.31	1.63	4.95*
Non significant contrasts		4	0.66	0.98	0.19	0.31	0.48
Residual Mean Square		96	0.34	0.42	142.80	1.48	9.91

(Key, Semen Parameters : Volume = ejaculate volume; Motility = motility index; % Motile = percentage of motile spermatozoa; Sperm./ml = concentration of spermatozoa/ml; Sperm./ejac. = number of spermatozoa/ejaculate)

Table 6.9

Experiment 6 : Summary of Analyses of Variance for Semen Data.

Source of Variation	Contrast No.	D.F.	Variance Ratios				
			Fr. Conc.	Fr. Cont.	S.P. Fr. Conc.	% Unstained	% Normal
MAIN EFFECTS							
A. LIGHTING REGIMES	1	1	0.13	0.22	0.03	12.89***	4.70*
B. OPERATIONS	2	1	1.22	2.34	0.98	0.69	3.29
C. TIME PERIODS		7					
Period 1 – Linear	3	1	0.06	0.10	0.07	5.18*	0.00
Period 2 – Linear	4	1	12.70***	10.85**	11.44**	0.02	0.66
" " – Quadratic	5	1	0.26	0.73	0.58	4.57*	0.86
Periods 1 vs 2	6	1	25.86***	23.09***	26.05***	15.23***	1.52
Non significant contrasts		2	0.71	0.90	0.70	0.57	1.25
INTERACTIONS							
A x B	7	1	0.94	0.01	0.42	0.08	2.34
A x C		7					
A x Period 2 – Linear	8	1	5.31*	4.54*	5.01*	0.73	0.01
" " " – Quadratic	9	1	4.58*	6.63*	4.89*	0.46	0.20
Non significant contrasts		4	0.05	0.04	0.05	0.35	0.97
B x C		7					
Non significant contrasts		6	0.46	1.06	0.59	0.36	0.92
A x B x C		7					
A x B x Period 2 – Linear	10	1	4.21*	0.67	3.27	0.46	0.03
Non significant contrasts		5	0.21	0.88	0.18	0.34	0.19
Residual Mean Square		96	4.10	19.24	5.75	125.62	136.08

(Key, Semen Parameters : Fr. Conc. = fructose concentration of semen; Fr. Cont. = total ejaculate fructose content; S.P. Fr. Conc. = fructose concentration of seminal plasma; % Unstained = percentage of unstained spermatozoa; % Normal = percentage of morphologically normal spermatozoa)
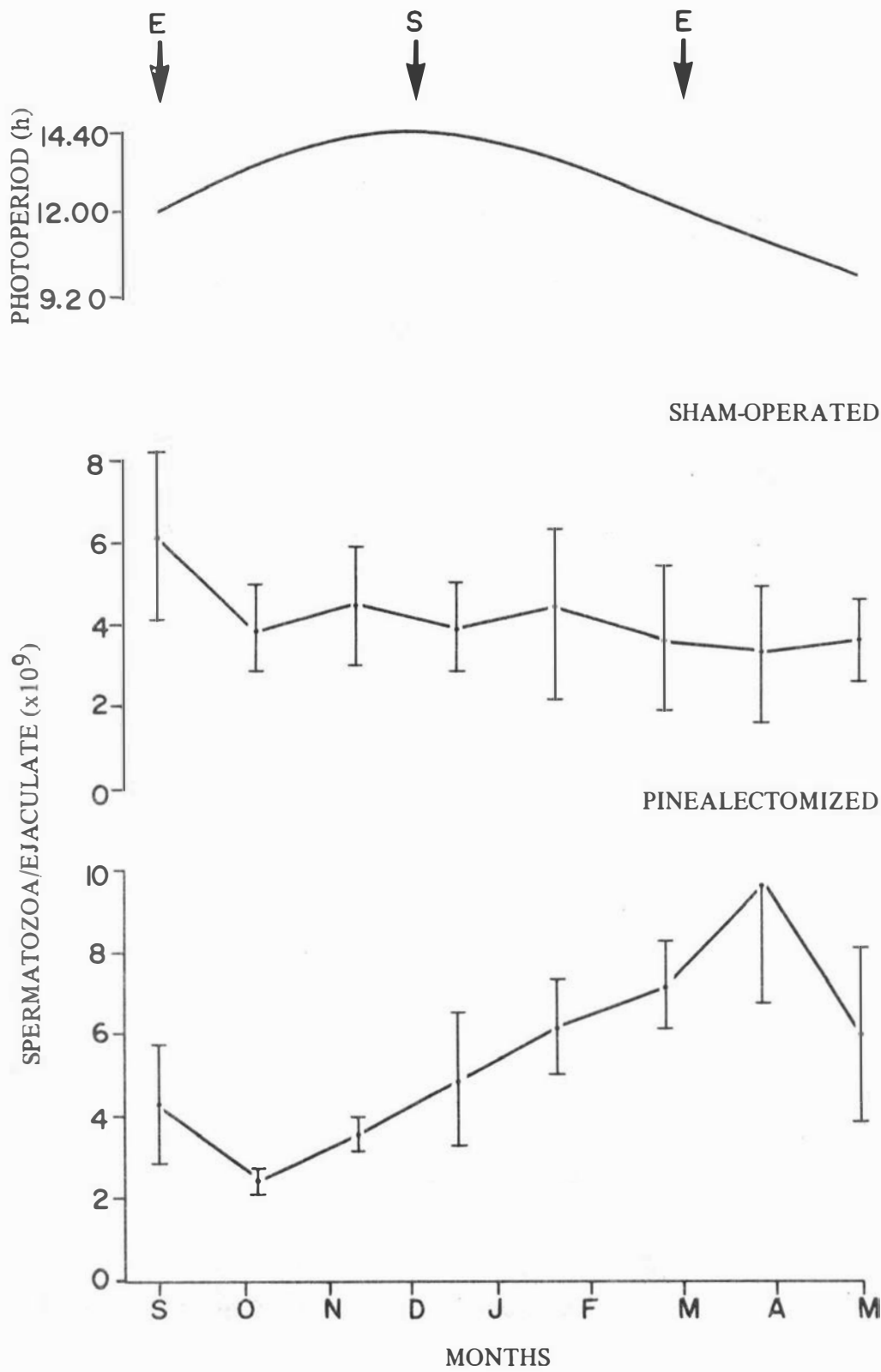
Figure 6.4: Monthly variations in numbers of spermatoza/ejaculate (mean+S.E.) in semen collected from sham-operated and pinealectomized rams subjected to Normal lighting regime. Daily photoperiod changes are illustrated and the timing of the equinoxes (E) and solstice (S) indicated.
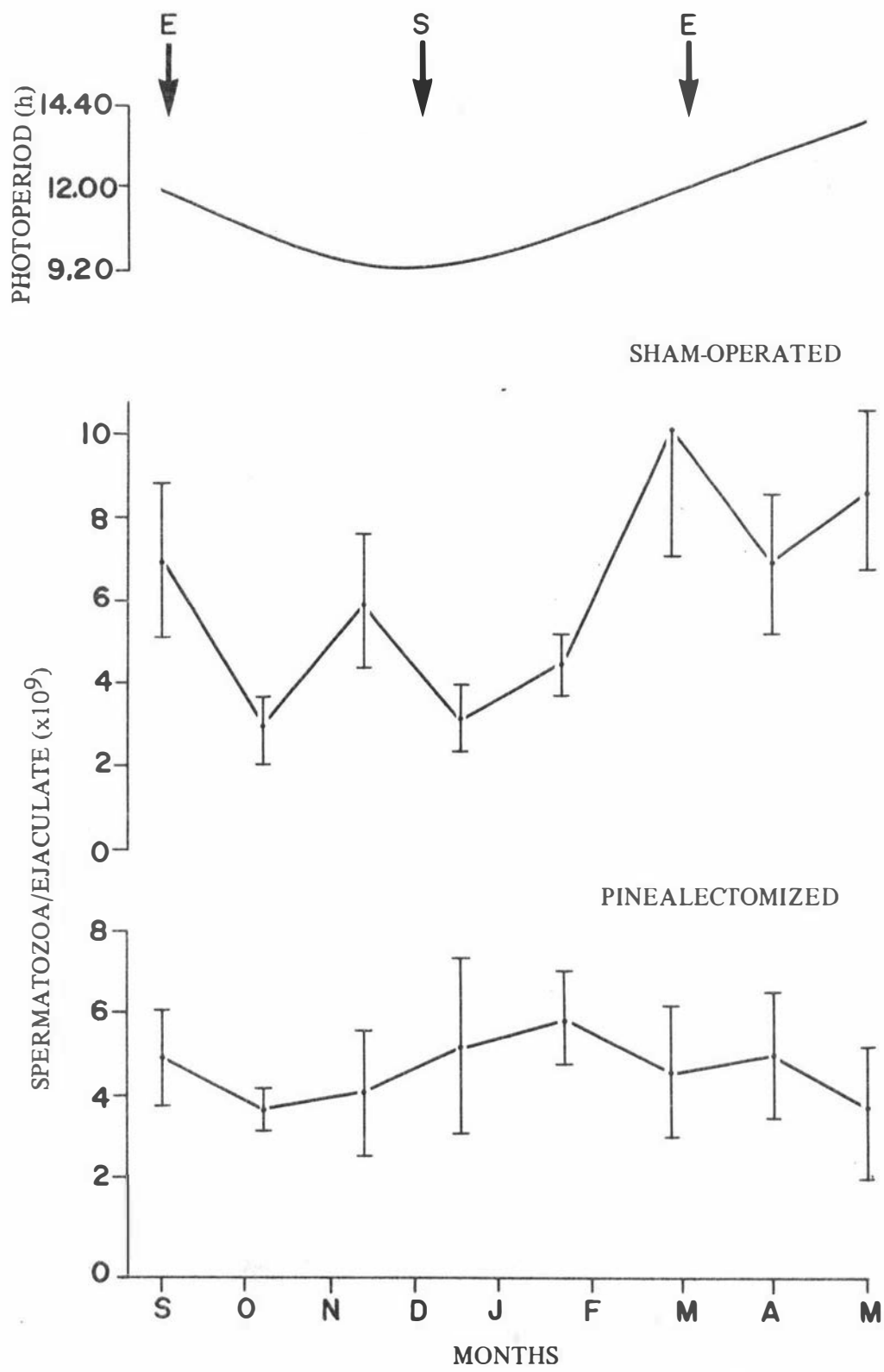
Figure 6.5 : Monthly variations in numbers of spermatoza/ejaculate (mean±S.E.) in semen collected from sham-operated and pinealectomized rams subjected to the Reversed lighting regime. Daily photoperiod changes are illustrated and the timing of the equinoxes (E) and solstice (S) indicated.

Figure 6.6 : Monthly variations in seminal plasma fructose concentrations (mean±S.E.) in semen collected from sham-operated and pinealectomized rams subjected to the Normal lighting regime. Daily photoperiod changes are illustrated and the timing of the equinoxes (E) and solstice (S) indicated.

Figure 6.7 : Monthly variations in seminal plasma fructose concen-
trations (mean+S.E.) in semen collected from sham-operated and
pinealectomized rams subjected to the Reversed lighting regime.
Daily photoperiod changes are illustrated and the timing of the
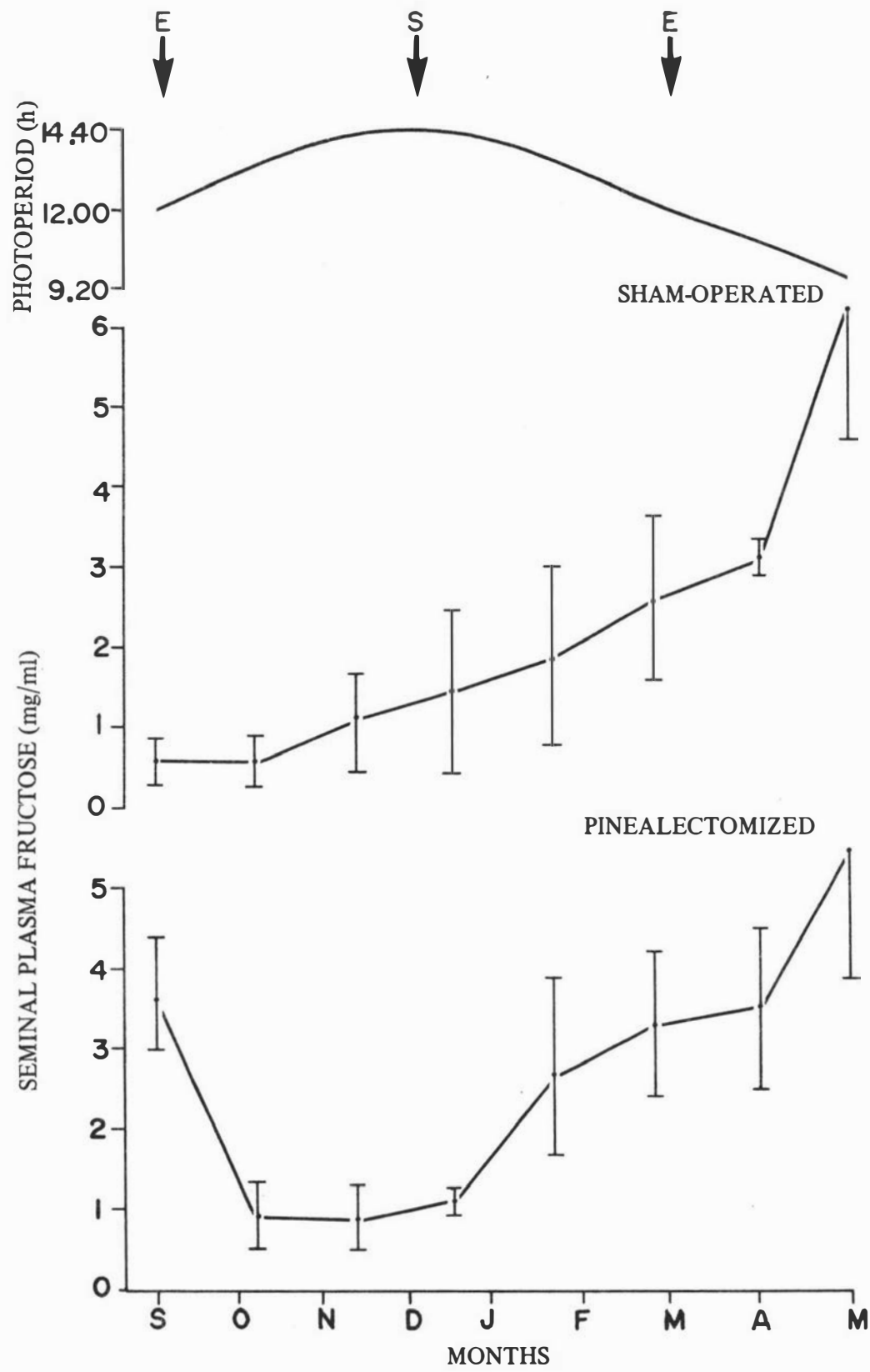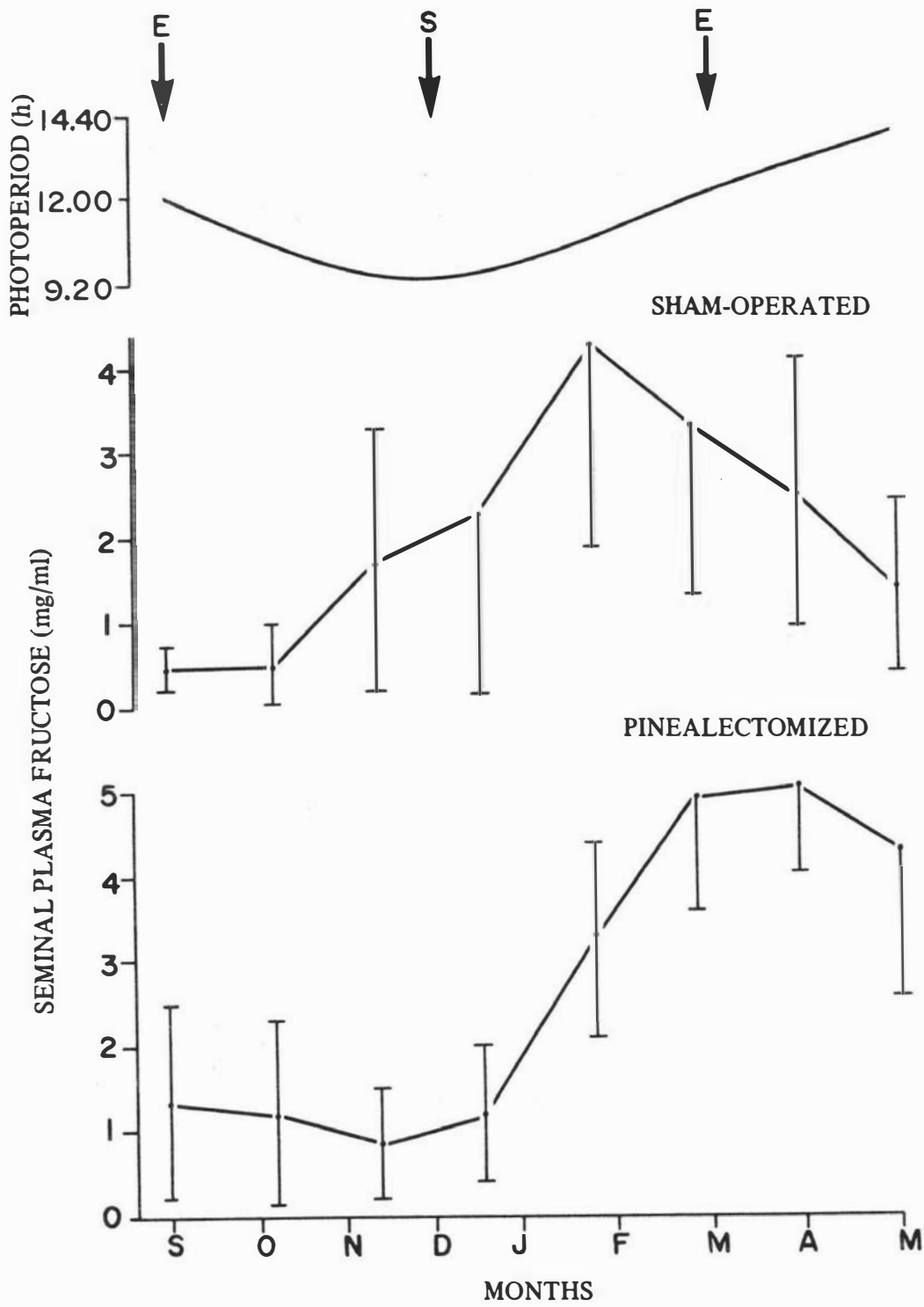equinoxes (E) and solstice (S) indicated.

this trend was most marked for pinealectomized rams (Contrasts 4 and 7). Overall, sham-operated rams had lower ejaculate volumes than pineal-ectomized rams (Contrast 2).

All three measures of seminal fructose levels displayed higher values during the last five months of the study. Although ejaculates from the rams on Reversed lighting showed peak fructose values during this period and declining levels towards the end of the experiment, the fructose content of semen from rams on the Normal lighting regime continued to increase until the end of the experiment (Contrasts 4, 6 and 8). Pinealectomy had no overall effect on seminal fructose levels, however the decline in seminal fructose concentrations in ejaculates from rams under Reversed lighting was significantly less marked in the PX rams than in the Controls (Contrasts 8, 9 and 10).

Ejaculates from sham-operated rams had higher mean concentrations of spermatozoa than those from pinealectomized rams (Contrast 2). This difference was largely attributable to results from rams on Reversed lighting; the opposite trend was seen in results from rams on Normal lighting during the final five months of the experiment, both for spermatozoal concentration and for total numbers of spermatozoa per ejaculate (Contrasts 5, 8 and 9).

Rams under Normal lighting produced semen with higher overall percentages of unstained and morphologically normal spermatozoa than did rams under Reversed lighting. The mean percentage of unstained spermatozoa, calculated from all four groups of rams, rose steadily from September 1974, until February 1975, then showed a slight decline (Contrasts 1, 3, 5 and 6).

(2) Plasma Hormone Data

(a) LH. See Tables 6.10, 6.13 and Figures 6.8 and 6.9.

All four groups of rams displayed peaks of LH secretion during

Table 6.10

Mean plasma LH concentrations recorded from rams in Experiment 6.
(Values presented are 100 $\log_{10}(x + 1.1)$, where x is hormone concentration in ng/ml.)

	Sampling time	Normal Lighting		Reversed Lighting		Mean
		Controls	PX	Controls	PX	
Period 1	1	16.8	14.3	12.2	13.0	14.1
	2	18.4	18.6	11.1	16.9	16.2
	3	16.8	17.1	13.6	16.9	16.1
	4	25.1	26.3	16.6	23.8	22.9
	5	14.9	16.3	12.7	14.6	14.6
	6	15.9	15.2	20.6	20.6	18.1
	7	12.0	13.7	11.6	11.7	12.2
	Mean	17.1	17.4	14.1	16.8	16.3
Period 2	8	17.9	16.2	18.5	10.8	15.8
	9	8.9	12.2	12.3	13.0	11.6
	10	9.4	9.5	8.8	12.9	10.1
	11	9.3	10.9	9.6	15.6	11.3
	12	17.9	9.1	8.9	11.1	11.7
	13	15.0	9.1	8.3	17.2	12.4
	14	21.1	7.3	5.8	9.7	11.0
	Mean	14.2	10.6	10.3	12.9	12.0
Period 3	15	14.5	10.2	6.1	10.8	10.4
	16	10.9	9.8	7.3	7.3	8.8
	17	12.5	8.4	5.8	14.5	10.3
	18	11.4	8.9	7.8	7.0	8.8
	Mean	12.3	9.3	6.7	9.9	9.6
Overall Mean		14.9	13.0	11.0	13.7	

(Key: Controls = Sham-operated rams; PX = Pinealectomized rams)

Figure 6.8 : Fortnightly variations in plasma LH concentrations
(mean±S.E.) recorded from sham-operated and pinealectomized rams
subjected to the Normal lighting regime.   Daily photoperiod changes
are illustrated and the timing of the equinoxes (E) and solstice
(S) indicated.

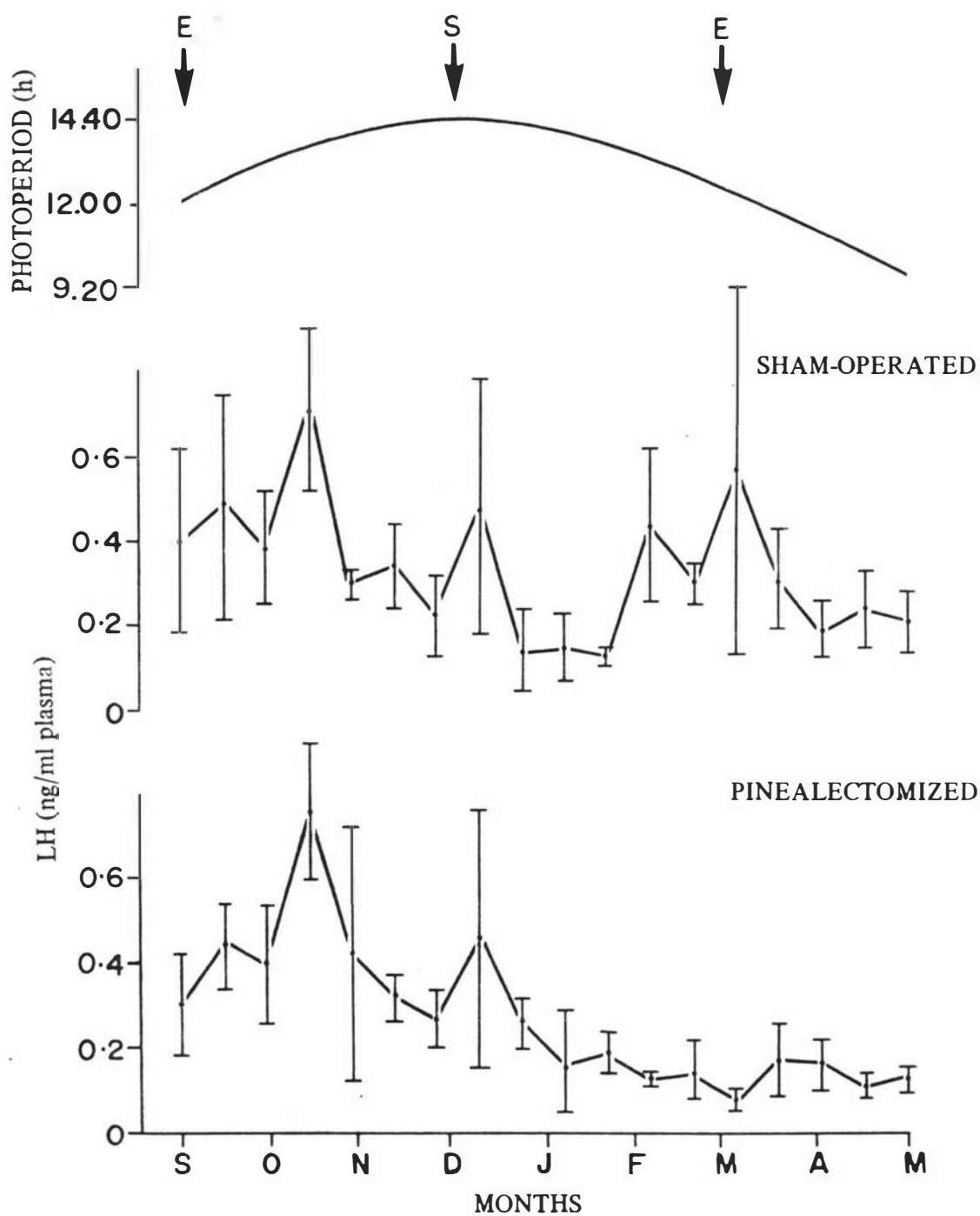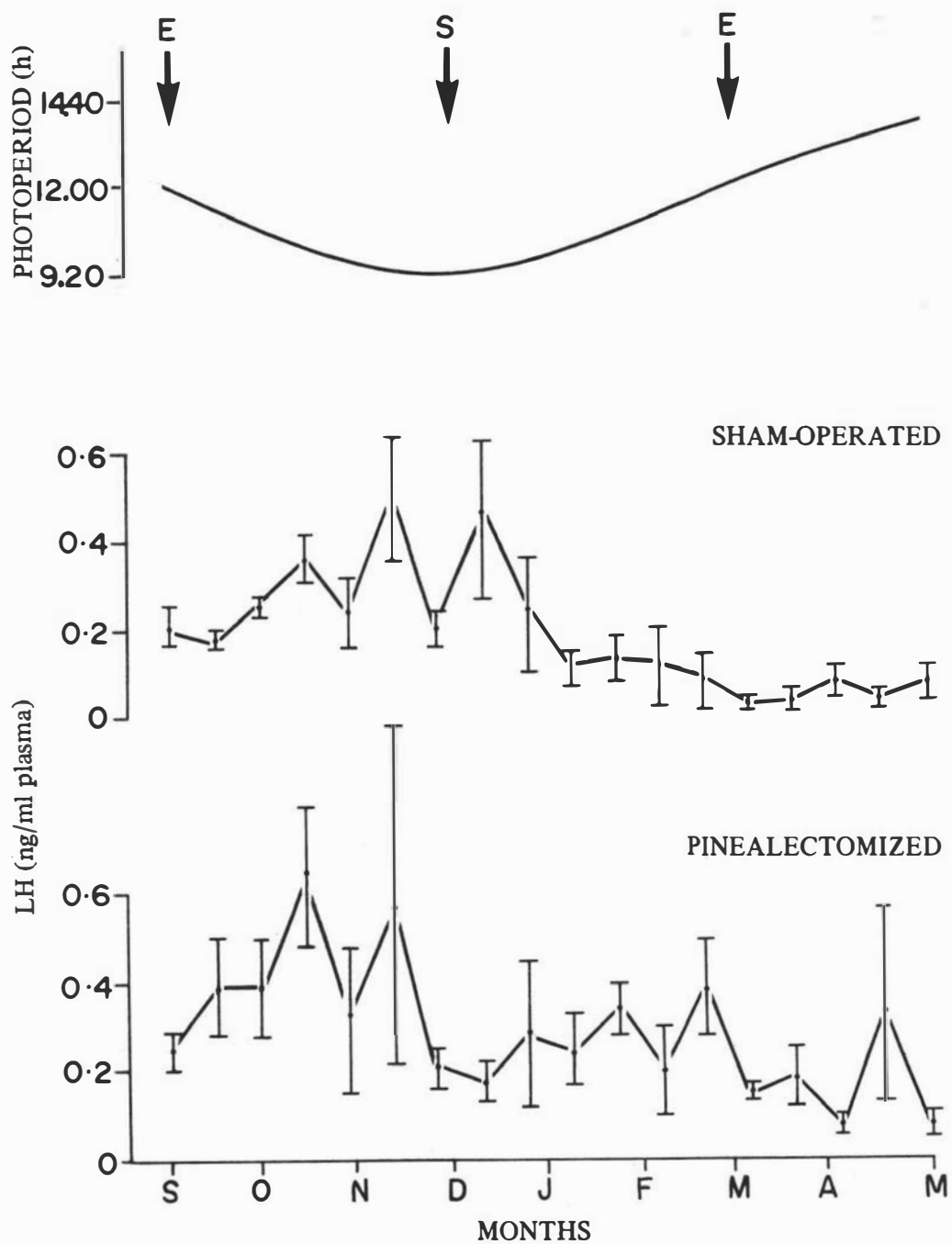Figure 6.9 : Fortnightly variations in plasma LH concentrations
(mean+S.E.) recorded from sham-operated and pinealectomized rams
subjected to the Reversed lighting regime. Daily photoperiod
changes are illustrated and the timing of the equinoxes (E) and
solstice (S) indicated.

the period from September to December (Period 1), followed by lower mean levels during the final five months (Contrasts 6 and 7). Rams under the Normal lighting regime had higher mean plasma LH levels than those subjected to the Reversed lighting regime. This result, and the significant Lighting Regimes x Operations interaction, arose from the lower mean plasma LH levels recorded from the sham-operated rams subjected to the Reversed lighting regime (Contrasts 1 and 8).

From December 1974 to March 1975 (Period 2), plasma LH levels of sham-operated rams under Normal lighting displayed rising values whilst those of sham-operated rams under Reversed lighting declined, as also did those of pinealectomized rams under Normal lighting (Contrast 20).

(b) <u>Testosterone</u>. See Tables 6.11, 6.13 and Figures 6.10 and 6.11.

Regardless of lighting regime, sham-operated rams showed a well-defined peak of plasma testosterone levels at around the time of the shortest daily photoperiod. On the other hand, plasma from pineal-ectomized rams showed no consistent pattern of change in testosterone levels.

The above statements accounted for all the significant interaction contrasts (Contrasts 8, 10, 14 and 20), while the fact that sham-operated rams on both lighting regimes did not have elevated plasma testosterone levels during the first four months gave rise to the highly significant between-periods contrast (Contrast 6).

(c) <u>Prolactin</u>. See Tables 6.12, 6.13 and Figures 6.12 and 6.13.

Substantial fluctuations in plasma prolactin levels were recorded from all groups of rams in this experiment. Generally these changes were such that mean prolactin levels were related directly to the photoperiods to which the animals were exposed. However, the fluctuations in plasma prolactin concentration were not as marked in pinealectomized rams as they were in sham-operated rams. Also the PX

Table 6.11

Mean plasma testosterone concentrations recorded from rams in
Experiment 6. (Values presented are $100 \log_{10}(x + 1.1)$, where
x is hormone concentration in ng/ml.)

	Sampling time	Normal Lighting		Reversed Lighting		
		Controls	PX	Controls	PX	Mean
	1	20.9	33.6	22.7	33.8	27.7
	2	21.6	20.1	16.4	28.7	21.7
	3	34.8	32.8	15.3	29.8	28.2
Period 1	4	40.8	52.0	12.8	52.5	39.5
	5	56.9	33.2	29.5	25.2	36.2
	6	22.0	26.6	57.0	42.9	37.1
	7	34.9	19.5	34.6	30.5	29.9
	Mean	33.1	31.1	26.9	34.8	31.5
	8	47.8	36.8	88.6	43.8	54.2
	9	33.1	48.1	78.3	54.3	53.4
	10	37.2	40.4	50.5	32.5	40.1
Period 2	11	31.1	46.5	48.6	55.5	45.4
	12	64.4	41.8	55.3	54.8	54.1
	13	76.4	56.8	33.5	68.1	58.7
	14	82.4	23.4	19.0	50.7	43.9
	Mean	53.2	42.0	53.4	51.4	50.0
	15	82.7	35.9	28.0	40.3	46.7
Period 3	16	57.0	40.8	24.8	31.2	38.4
	17	69.6	31.8	20.3	29.2	37.7
	18	32.7	27.3	32.4	27.7	30.0
	Mean	60.5	33.9	26.4	32.1	38.2
Overall Mean		47.0	36.0	37.1	40.6	

(Key: Controls = Sham-operated rams; PX = Pinealectomized rams)

Figure 6.10 : Fortnightly variations in plasma testosterone con-
centrations (mean±S.E.) recorded from sham-operated and pineal-
ectomized rams subjected to the Normal lighting regime. Daily
photoperiod changes are illustrated and the timing of the equin-
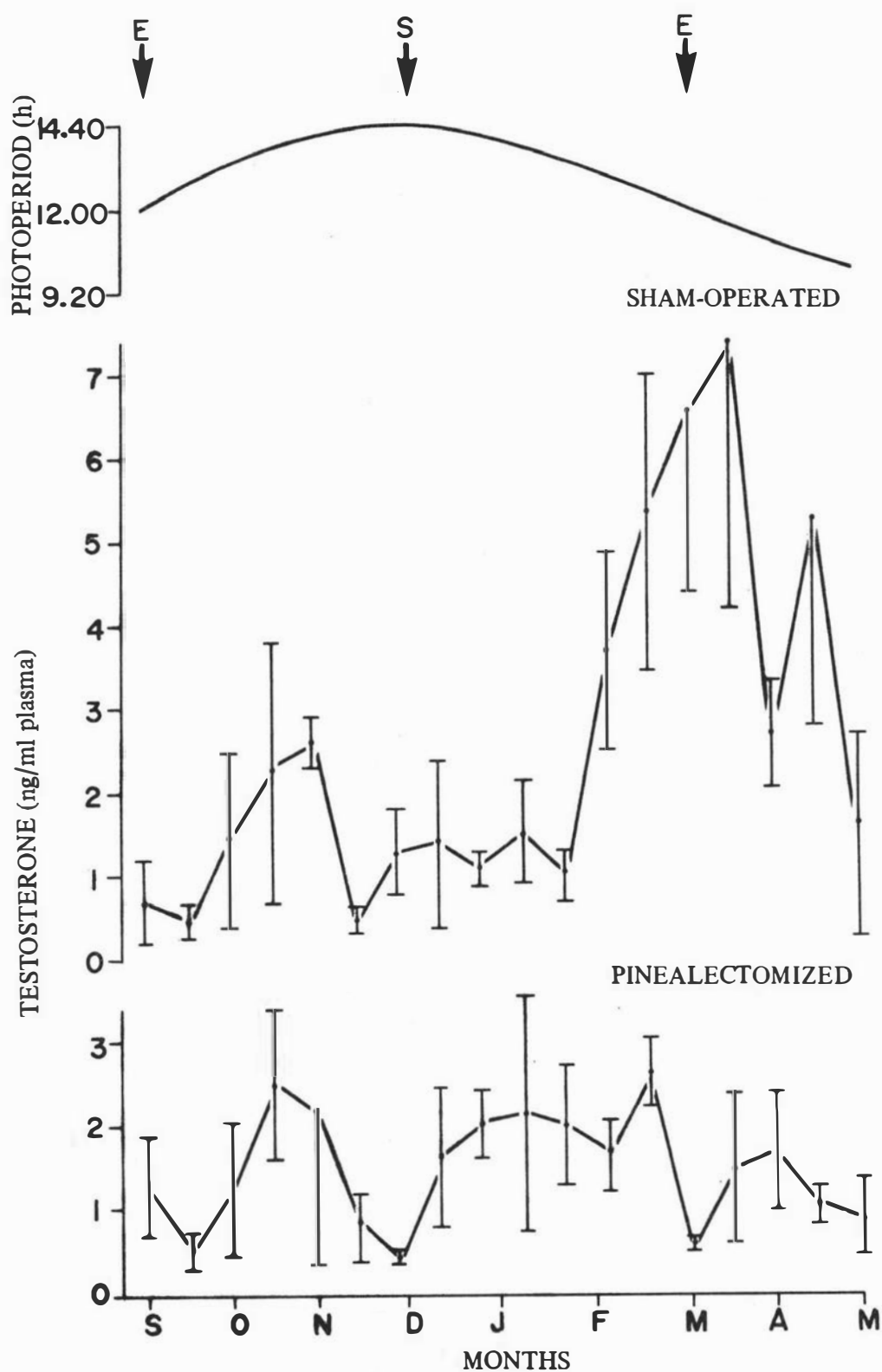oxes (E) and solstice (S) indicated.

Figure 6.11 : Fortnightly variations in plasma testosterone con-
centrations (mean±S.E.) recorded from sham-operated and pineal-
ectomized rams subjected to the Reversed lighting regime.
Daily photoperiod changes are illustrated and the timing of the
equinoxes (E) and solstice (S) indicated.

Table 6.12

Mean plasma prolactin concentrations recorded from rams in Experiment 6. (Values presented are $100 \log_{10}(x + 1.1)$, where x is hormone concentration in ng/ml.)

| | Sampling time | Normal Lighting | | Reversed Lighting | | Mean |
		Controls	PX	Controls	PX	
	1	173.2	188.0	165.4	133.1	164.9
	2	165.0	157.8	152.6	157.5	158.2
	3	173.8	155.1	92.5	145.4	141.7
Period 1	4	181.2	162.0	90.4	154.7	147.1
	5	187.5	174.5	106.1	151.6	154.9
	6	189.9	173.7	99.7	125.5	147.2
	7	190.6	149.8	102.1	138.7	145.3
Mean		180.2	165.8	115.5	143.8	151.3
	8	175.2	155.5	53.6	128.6	128.2
	9	157.1	145.2	96.7	161.8	140.2
	10	172.6	156.9	101.0	148.5	144.7
Period 2	11	137.0	163.3	110.6	157.6	142.1
	12	177.4	189.4	195.2	201.1	190.8
	13	114.4	167.2	138.6	177.1	149.3
	14	104.7	178.1	169.0	209.7	165.4
Mean		148.3	165.1	123.5	169.2	151.5
	15	60.1	161.4	166.2	195.5	145.8
	16	48.0	166.1	192.8	182.0	147.2
Period 3	17	128.3	183.5	178.6	168.6	164.7
	18	115.1	163.1	188.9	176.5	160.9
Mean		87.9	168.5	181.6	180.6	154.6
Overall Mean		147.3	166.1	133.3	161.9	

(Key: Controls = Sham-operated rams; PX = Pinealectomized rams)

Figure 6.12 : Fortnightly variations in plasma prolactin concentrations (mean±S.E.) recorded from sham-operated and pinealectomized rams subjected to the Normal lighting regime. Daily photoperiod changes are illustrated and the timing of the equinoxes (E) and solstice (S) indicated.
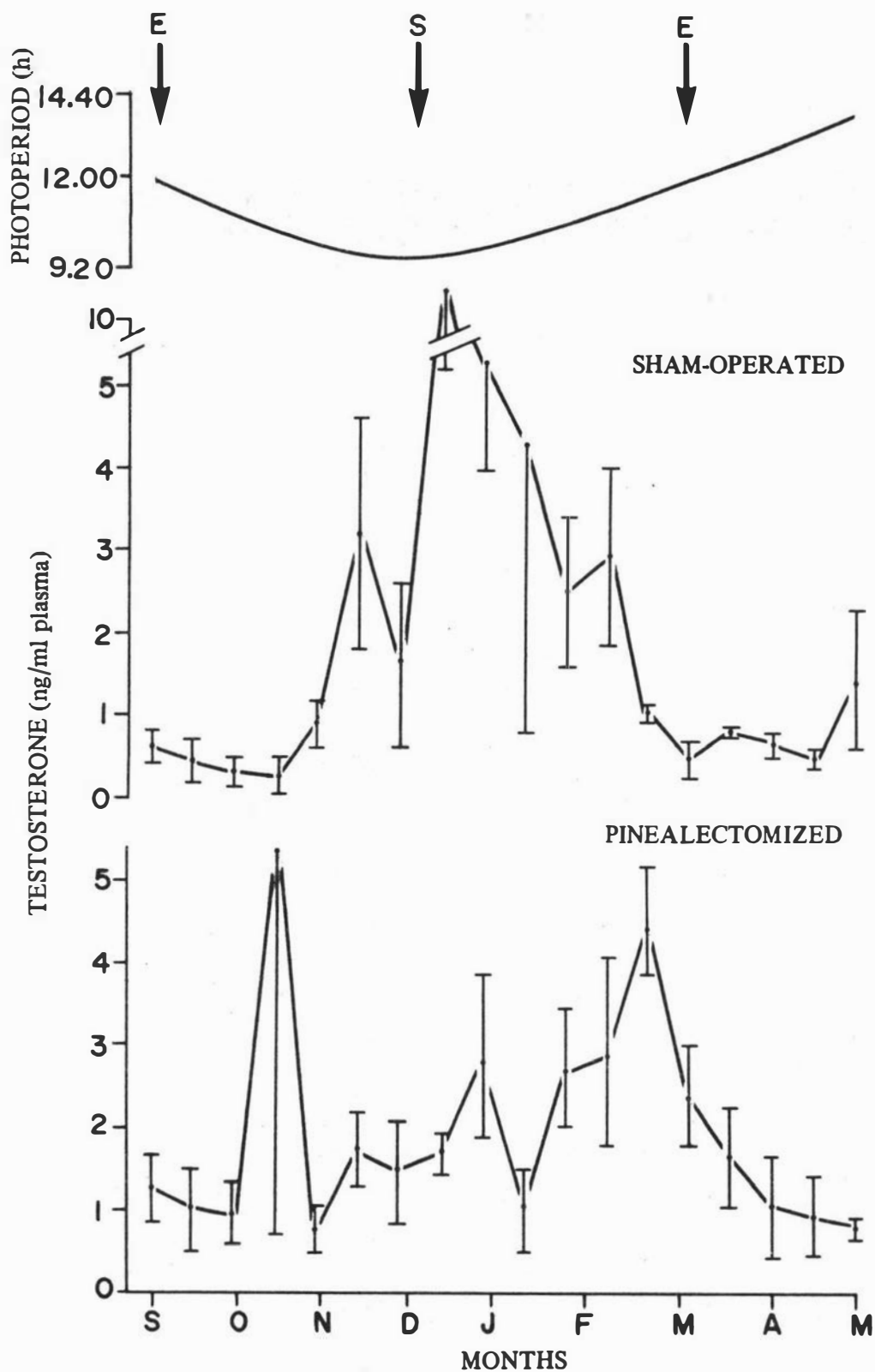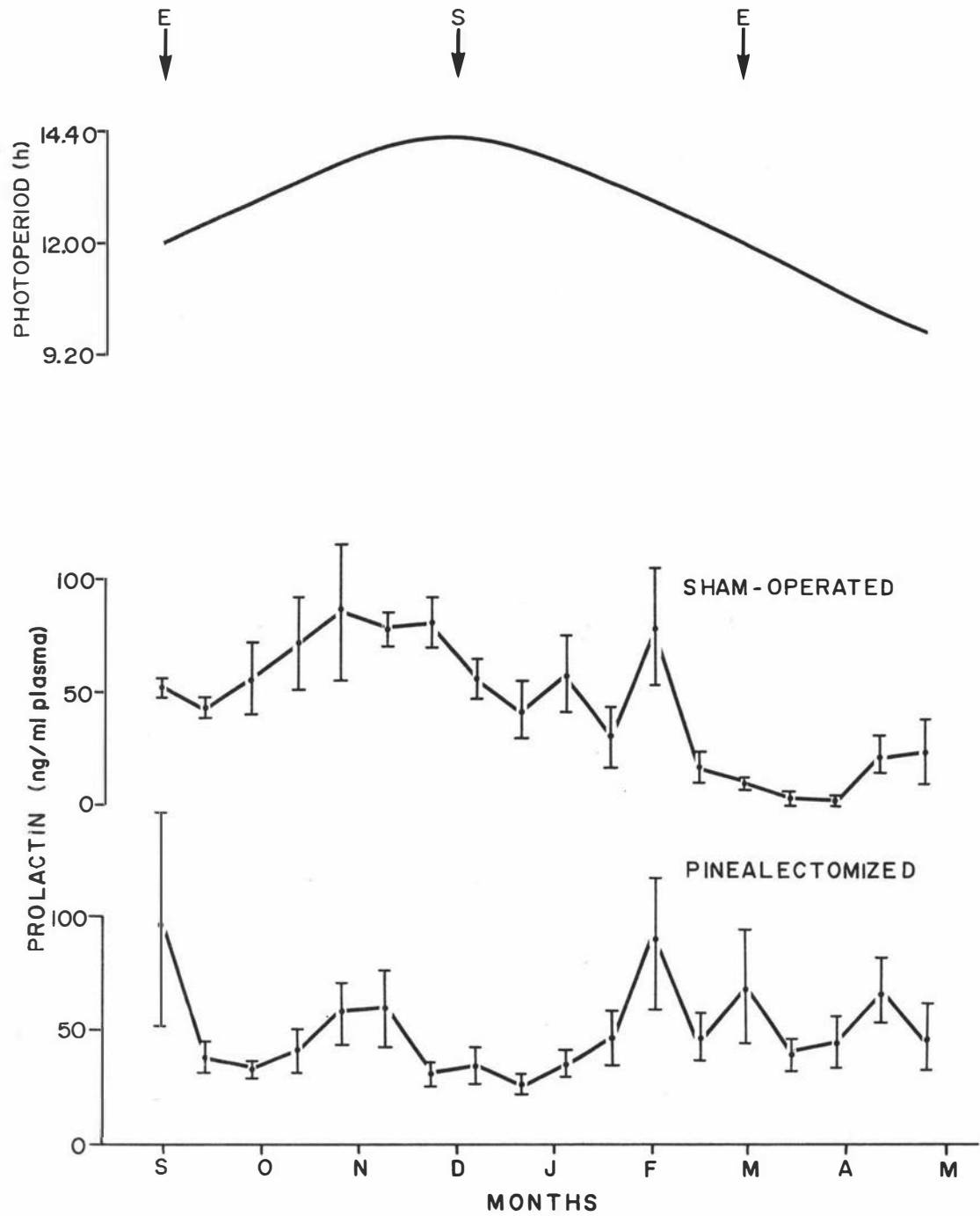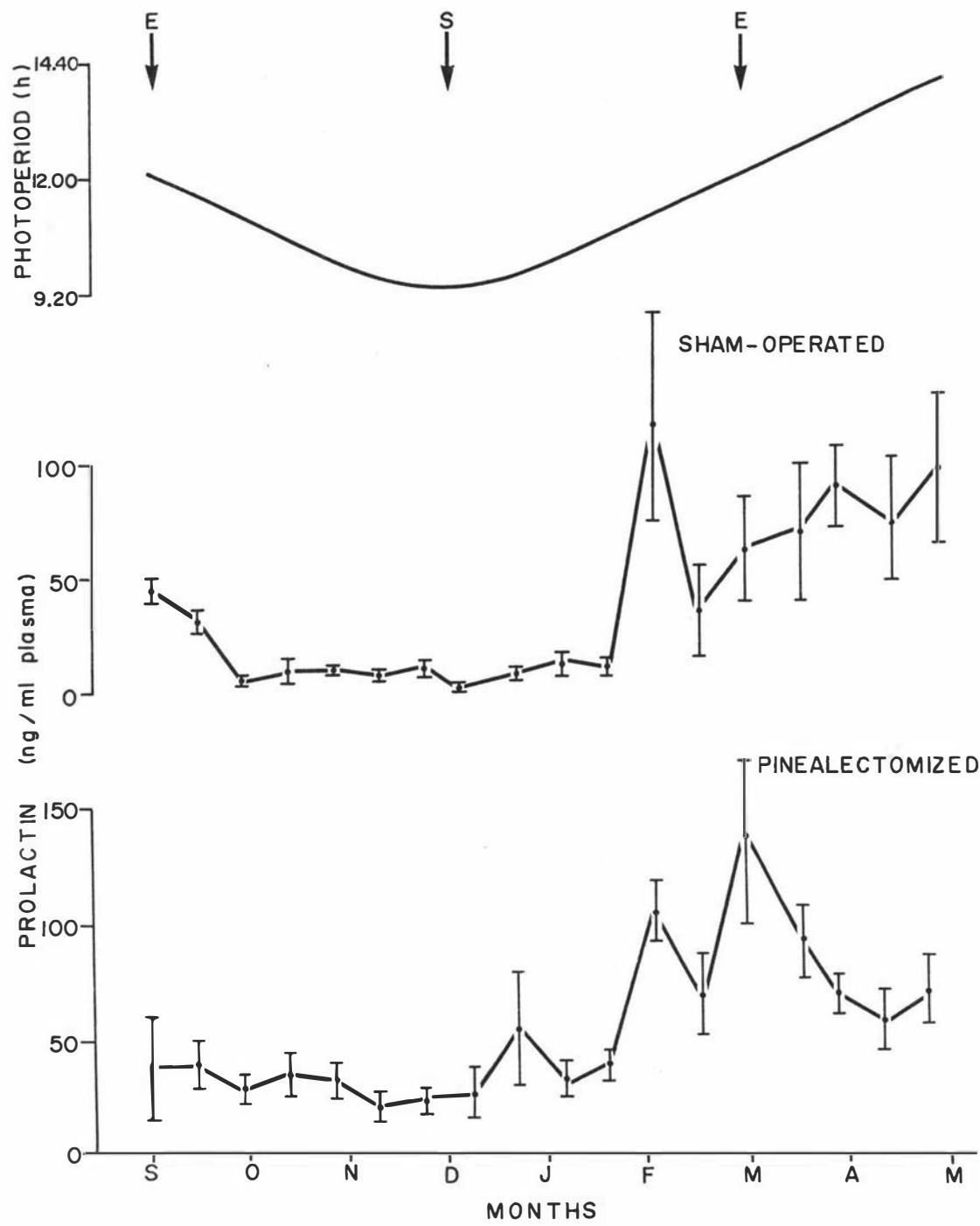
Figure 6.13 : Fortnightly variations in plasma prolactin concentrations (mean±S.E.) recorded from sham-operated and pinealectomized rams subjected to the Reversed lighting regime. Daily photoperiod changes are illustrated and the timing of the equinoxes (E) and solstice (S) indicated.

Table 6.13

Experiment 6 : Summary of Analyses of Variance for LH, Testosterone and Prolactin Data.

Source of Variation	Contrast No.	D.F.	LH	Testost- erone	Prolactin
MAIN EFFECTS					
A. LIGHTING REGIMES	1	1	3.99*	0.89	7.19**
B. OPERATIONS	2	1	0.20	1.82	48.11***
C. PERIODS		17			
Period 1 – Quadratic	3	1	10.71***	1.29	1.26
Period 2 – Linear	4	1	1.62	0.04	21.01***
" " – Cubic	5	1	3.03	4.10*	1.02
Period 1 vs Period 2	6	1	22.93***	33.72***	0.00
Period 3 vs Periods 1 & 2	7	1	22.58***	0.56	0.61
Non significant contrasts		6	0.59	1.39	2.10
INTERACTIONS					
A x B	8	1	8.95**	6.83**	2.04
A x C		17			
A x Period 1 – Linear	9	1	2.08	0.80	5.90*
A x Period 2 – Linear	10	1	1.24	11.08**	42.45***
·A x Period 3 – Linear	11	1	0.17	1.76	4.83*
" " " – Cubic	12	1	0.22	0.58	6.23*
A x (Period 1 vs Period 2)	13	1	0.36	0.94	18.26***
A x (Period 3 vs Periods 1 & 2)	14	1	0.35	8.54**	94.51***
Non significant contrasts		5	0.70	0.72	0.68
B x C		17			
B x Period 3 – Linear	15	1	0.03	0.26	7.17**
B x (Period 1 vs Period 2)	16	1	1.13	2.22	9.89**
B x (Period 3 vs Periods 1 & 2)	17	1	0.04	1.61	6.41*
Non significant contrasts		8	0.79	1.01	1.15
A x B x C		17			
A x B x Period 1 – Linear	18	1	0.70	0.04	7.40**
" " " " – Quadratic	19	1	0.11	0.60	4.57*
A x B x Period 2 – Linear	20	1	9.88**	22.90***	17.09***
A x B x (Period 3 vs Periods1 & 2)	21	1	0.20	2.82	51.21***
Non significant contrasts		7	0.44	0.89	1.01
Residual Mean Square		216	46.21	566.44	838.60

group had higher mean plasma prolactin levels than the Controls, while the overall mean estimates were higher in rams under Normal lighting than in those under Reversed lighting (Contrasts 1 and 2).

Sham-operated rams under the Normal lighting regime had maximal plasma prolactin concentrations at about the time of the longest photo-period; these subsequently waned with the approach of the shortest daily photoperiod at the end of the experiment. No such changes were shown by the pinealectomized rams in the same room, in fact this group displayed a series of only relatively minor deviations from its mean prolactin level. In contrast, the sham-operated rams under Reversed lighting had very low plasma prolactin concentrations during the first five months of this experiment, corresponding to the short daily photoperiods, then steadily rising levels as the photoperiod increased towards the end of the experiment. Again pinealectomized rams in the same room displayed less-marked changes : a rise in plasma prolactin levels during February and March 1975 was followed by a fall, even though photoperiods were still increasing (Contrasts 9-21).

(3) Autopsy Data

See Tables 6.14 and 6.15.

Compared to the rams on the Reversed lighting regime those under Normal lighting had higher overall mean values for : testicular weights, epididymal weights, epididymal spermatozoal reserves, and seminal vesicular weights, fructose contents and fructose concentrations. In fact the highest and lowest mean values for these parameters were obtained from the sham-operated rams under Normal and Reversed lighting, respectively; the pinealectomized rams had mean values intermediate between these two extremes. This trend was indicated by the significant Lighting Regimes x Operations interactions recorded for all the parameters mentioned above, except testicular weights and seminal

Table 6.14

Data (means* ± S.E.) collected following autopsy of rams utilized in Experiment 6.

	Body weights (Kg)	Testicular weights (g)	Seminiferous tubule diameters (μm)	Epididymal weights (g)	Epididymal spermatozoal reserves ($\times 10^9$)
NORMAL LIGHTING REGIME					
Sham-operated	75.3±1.8	429.6±20.1	207.1±4.3	84.7±5.9	101.44±6.24
Pinealectomized	80.1±3.9	417.5±50.3	204.2±7.7	70.2±5.7	74.82±5.64
REVERSED LIGHTING REGIME					
Sham-operated	75.9±2.1	238.7±35.1	188.4±7.0	45.6±2.6	31.52± 6.00
Pinealectomized	81.4±2.5	337.2÷31.6	204.6±2.3	59.1±3.3	50.44±13.65

	Ampullar weights (g)	Seminal vesicular weights (g)	Seminal vesicular fructose — Total content (mg)	Seminal vesicular fructose — Concentration (mg/g)	Thyroid weights (g)	Pituitary weights (mg)
NORMAL LIGHTING REGIME						
Sham-operated	5.48±0.48	15.63±2.16	81.8±15.4	510.8± 35.2	9.15±0.44	1049±137
Pinealectomized	4.54±0.63	8.10±1.38	35.4± 8.7	545.7±151.8	10.90±1.02	1426±165
REVERSED LIGHTING REGIME						
Sham-operated	3.96÷0.12	6.49±0.76	9.0± 1.7	174.9± 28.9	10.24±1.13	1197± 91
Pinealectomized	4.26±0.32	9.58±0.43	32.3± 7.3	334.4± 67.0	8.75±1.18	1238±206

*Where data have been obtained from paired organs, means for each group were based on totals per ram.

Table 6.15

Variance ratios for contrasts in the analyses of variance of data presented in Table 6.14. (D.F. = 1,12)

Contrast	Body weights	Testicular weights	Seminiferous tubule diameters	Epididymal weights	Epididymal spermatozoal reserves
Lighting Regimes	0.12	14.24**	2.54	29.71***	30.33***
Operations	3.65	1.44	1.33	0.01	0.20
Lighting Regimes x Operations	0.01	2.37	2.76	9.27**	7.09*
Error Mean Square	29.42	5165.33	131.75	84.54	73.29

Contrast	Ampullar weights	Seminal vesicular weights	Seminal vesicular fructose Total content	Seminal vesicular fructose Concentration	Thyroid weights	Pituitary weights
Lighting Regimes	4.34	7.98*	15.72**	10.10**	0.29	0.02
Operations	0.56	2.67	1.46	1.27	0.02	1.80
Lighting Regimes x Operations	2.04	15.29**	13.26**	0.52	2.68	1.16
Error Mean Square	0.75	7.37	366.75	29612.00	3.93	0.10

vesicular fructose concentrations. A similar trend was shown by the mean values for ampullar weights and seminiferous tubule diameters, but was not statistically significant.

4. DISCUSSION

Experiment 6 showed that reproductive functions of rams could be modified significantly by pinealectomy, with the most dramatic influences being effects on plasma levels of reproductive hormones. Most of these effects appeared as modifications to the influences of changes in photoperiod length, which were recorded from intact rams in Experiment 5.

(1) Semen Production

Elevated values for ejaculate volumes, spermatozoal motility, and percentages of motile and unstained spermatozoa, in semen collected from rams under Normal lighting were recorded during the period of decreasing daily photoperiod. Similar changes were not seen in semen from rams on Reversed lighting (with the exception of ejaculate volumes), thus indicating that the seasonal pattern of change in these parameters was altered by reversal of the lighting cycle. However, as the abovenamed parameters did not display seasonal changes in the earlier experiments in this thesis, and the rise in ejaculate volumes occurred in all groups of rams regardless of lighting regime, conclusions about the photoperiodicity of these particular semen characteristics must be viewed with caution.

Results for percentages of unstained spermatozoa contrasted sharply with those of Fowler (1961), who reported reduced percentages of live spermatozoa from December to March, irrespective of lighting treatment. Temperature was not controlled in Fowler's study and probably contributed to his result.

Ejaculate volumes generally increased during the latter five months of the experiment and this increase may have been attributable to a residual seasonal pattern in all groups; however, higher values were recorded from the pinealectomized rams indicating an effect of this operation.

Most rams in this experiment had rising seminal fructose levels over the final months of the study, however the sham-operated rams on the Reversed lighting regime showed an early rise followed by declining levels over the last three months. This latter result indicated that lighting regime reversal advanced the period of elevated seminal fructose levels, as seen in Experiment 5. Moreover, since this effect was confined to the sham-operated rams, it could be attributed to pineal gland function in these intact rams.

Significant Lighting Regimes x Operations interactions for spermatozoal concentrations and total numbers of spermatozoa per ejaculate resulted from the higher mean levels in semen from sham-operated rams under Reversed lighting throughout the whole study period, and from pinealectomized rams under the Normal lighting regime during the final five months. The latter finding supported a similar result obtained from cranial cervical ganglionectomized rams in Experiment 4.

Surprisingly, there was no change in mean numbers of spermatozoa per ejaculate in semen from the sham-operated rams under Normal lighting, as some evidence for seasonal changes in this parameter had been found in Experiments 3 and 4. This disparity may well have arisen from the difference in animal management procedures used in this and the earlier two experiments.

(2) Plasma Hormone Levels

(a) LH. Elevated plasma LH levels were recorded from all groups during October and November and probably represented a residual

seasonal rhythm similar to that displayed by grazing rams (Experiment 3); however, the period of elevated LH secretion was of much shorter duration in Experiment 6.

There was little concurrence between the results obtained from pinealectomized rams in this experiment, and from the cranial cervical ganglionectomized rams in Experiment 4 which displayed irregular changes in plasma LH levels. Also, as in Experiment 5, there was little evidence for the pronounced photoperiodicity of plasma LH levels in rams reported by Pelletier and Ortavant (1975a) and Lincoln (1976a).

Lack of significant effects of treatments on plasma LH levels may have resulted from the very low plasma concentrations which were much lower than those recorded in the comparable studies of Pelletier (1971), Pelletier and Ortavant (1975a) and Lincoln (1976a). Differences between the highest and lowest LH concentrations were small, making the detection of statistically significant changes in concentration unlikely. Since castrated rams have elevated plasma LH levels (Pelletier, 1968), they would be useful experimental animals for this type of research, since pro- or antigonadotrophic effects of pinealectomy, or lighting treatments, could be studied without interference from negative feedback by gonadal steroids.

An antigonadotrophic role for the pineal gland in rams was suggested by the plasma LH results, as the significant Lighting Regimes x Operations interaction indicated that the pineal gland of sham-operated rams inhibited LH release during increasing daily photoperiods, although not during decreasing photoperiods. In fact under decreasing photoperiod conditions plasma LH data from rams under Normal lighting suggested a progonadotrophic role for the pineal gland. This possibility has also been raised by research with rats, in which pinealectomy was followed by a reduction in serum levels of both LH and FSH (van Bronswijk et al., 1975). Early work in France indicated

that pinealectomy did not alter the pituitary LH content of rams subjected to either increasing or decreasing daily photoperiods (J. Pelletier, personal communication), however much further research will be required to evaluate the relationship between research based on pituitary hormone content and experiments such as the present one in which peripheral plasma hormone concentrations were measured.

(b) <u>Testosterone</u>. Plasma testosterone levels of sham-operated rams displayed marked seasonal patterns which were similar to those obtained in Experiment 5. Control rams under Reversed lighting had maximal plasma testosterone levels from November to February, corresponding with the Reversed lighting group in Experiment 5 which had elevated plasma testosterone levels in December when that experiment was terminated. Also both the sham-operated rams under the Normal lighting regime in this experiment and the rams under Normal lighting in Experiment 5, did not have elevated plasma testosterone levels during this same period. In the present experiment, the sham-operated rams under Normal lighting had peak plasma testosterone levels during February, March and April whereas intact rams on pasture had similar peaks from January to March (Experiment 3). The slightly later timing of peak plasma testosterone secretion in the present study could reflect differences in experimental conditions, such as the absence of temperature changes in Experiment 6. A similar finding has been reported from an experiment in which red deer stags were placed in controlled-lighting conditions (six-monthly photoperiodic cycle) : peak plasma testosterone levels occurred six weeks later than was normal (Pollock, 1975).

Pinealectomized rams under both lighting regimes did not display any definite seasonal peak of testosterone secretion, which suggested that the pineal gland is in fact a vital organ in the mediation of the marked seasonal fluctuations in androgen production normally recorded

from rams.

(c) <u>Prolactin.</u> Plasma prolactin data recorded from control animals in this experiment exhibited a direct relationship with photoperiod. Similar results were recorded with intact rams in Chapter V, with castrated and intact rams by Pelletier (1973), and with bull calves by Bourne and Tucker (1975). On the other hand pinealectomized rams showed a diminished prolactin response to photoperiodic changes. A similar finding, based on results from castrated male lambs subjected to sixteen or eight hours light per day, has been reported by Forbes (1975). These findings demonstrated that the pineal gland has an important role in mediating the photoperiod change-induced seasonal fluctuations in prolactin secretion of rams.

Plasma prolactin data from Experiment 6 indicated that the pineal gland of rams was stimulatory to prolactin release during long daily photoperiods and inhibitory during short photoperiods. This conclusion concurs with that reached in Chapter IV (based on plasma prolactin data from cranial cervical ganglionectomized rams in that experiment, and from cranial cervical ganglionectomized goats (H.L. Buttle, personal communication)), that a major role of the pineal gland was inhibition of prolactin secretion during the winter months.

The mechanism by which the pineal gland influenced prolactin release is not clear. It almost certainly involves a complex interaction with the effect of photoperiod which might be clarified by carrying out an experiment in which blinded as well as pinealectomized rams are studied.

(3) <u>Autopsy Data</u>

All rams were killed in May, when those under Normal lighting should have been experiencing their autumnal peak of reproductive activity and associated maximal reproductive organ weights (Lemay and

Corrivault, 1973). This conclusion was confirmed by the fact that, for most characteristics which may have relevance to reproductive function, the highest values were recorded from sham-operated rams on Normal lighting and lowest values from sham-operated rams on Reversed lighting. Data from the pinealectomized rams mostly were intermediate in value between the extreme values from the sham-operated rams and were little influenced by lighting regimes. This significant interaction suggested that the pineal gland could be stimulatory or inhibitory to reproductive functions according to the length of the photoperiod; Hoffman and Reiter (1966) reached an identical conclusion about similar findings in female hamsters. Thus the pineal gland apparently fulfils the requirements for an organ which can synchronize the reproductive system of rams with seasonal changes in daylength.

(4) General discussion

Autopsy data from sham-operated rams under Normal lighting reflected the peak values for plasma testosterone levels and semen quality recorded in the few weeks prior to killing. This pattern of changes indicated that high levels of circulating testosterone were accompanied by increased gonadal activity and an associated improvement in semen quality. However, this group of rams did not have higher values than the other groups for all variables at the termination of the experiment, notable exceptions being : ejaculate volumes, spermatozoal numbers and concentrations, and plasma prolactin levels.

The relationship between plasma LH and testosterone levels was not as direct as might have been expected from earlier experiments in this thesis, or from previous reports in the literature (Galloway et al., 1974; Sanford et al., 1974b; Bremner et al., 1976; Lee et al., 1976). This poor correlation between the secretory patterns for these hormones may reflect deficiencies in the plasma sampling

regime. LH is secreted in a highly pulsatile manner (see Chapter VII), and information gained from single samples collected every two weeks probably gives a poor indication of the pattern of secretion of this hormone.

The apparent lack of association between plasma prolactin levels and reproductive parameters did not aid clarification of the reproductive function(s) of this hormone in males. This result might indicate that prolactin has no important role in the control of reproduction in rams, or that its effects (on the gonads) are not fully manifested until several months after their exposure to high circulating levels of this hormone. This latter possibility may also apply to LH, plasma levels of which appear to have been suppressed by the increased negative feedback of testosterone by the time that reproductive parameters reach their maximal levels. The effects of testosterone on prolactin secretion have not been fully investigated.

Results from the present experiment have shown that the pineal gland is not simply progonadotrophic. It was readily apparent that exposure to reducing photoperiods stimulated gonadal activity more in the control rams than in the pinealectomized rams. The possibility that the pineal gland may be progonadotrophic is not novel (Reiter, 1974a) since pinealectomy depressed gonadal activity of female hamsters in certain circumstances (Hoffman and Reiter, 1966), and reduced serum gonadotrophin levels in male rats (van Bronswijk et al. 1975). Also French workers have shown that melatonin injections raised pituitary gonadotrophin levels in rats (Thieblot, Berthelay and Blaise, 1966), a result which the authors claimed represented a progonadotrophic role for this pineal hormone. The pineal gland has been reported to contain large stores of GnRH (White et al., 1974) but it is not known whether this hormone was synthesized de novo by the gland, or merely accumulated from other sources. In either case,

such stores could still represent an important source of GnRH activity.

In contrast, the results obtained during exposure of sham-operated and pinealectomized rams to long photoperiods, especially in the Reversed lighting group near the end of this project, have clearly demonstrated that the pineal gland is antigonadotrophic in rams under such circumstances. Although the antigonadotrophic properties of the pineal gland in various rodent species have been widely accepted (Reiter, 1973b), this is the first report of a similar function of the gland in the ovine species.

Very little research has been undertaken to investigate the direct actions of pineal principles on the gonads, probably because of the difficulties involved in designing experiments to examine this possibility. However, Liu and Kinson (1973) have reported direct inhibitory effects of melatonin and serotonin on rat testicles. Thus, effects of pineal gland secretions at the level of the gonads can not be ruled out in Experiment 6, especially in view of the fact that no major changes in plasma LH levels were seen in both groups of sham-operated rams, even despite the wide variations in gonadal activity recorded from these animals

It is generally accepted that the pineal gland exerts its major influence on the reproductive system at the level of the hypothalamus or the anterior pituitary (Reiter, 1973b). In vitro experiments using tissue culture systems which contained bovine anterior pituitary and/or hypothalamic tissue indicated that the antigonadotrophic activity of the pineal gland was exerted principally at the level of the hypothalamus (Hayes, Knight and Symington, 1974). In the current experiment, definitive conclusions about the level of action of the pineal principles could not be made. This was partly due to the relatively short duration of the experiment. A longer study of the effects of pinealectomy, involving a number of breeding seasons, may

have overcome the problem of distinguishing between residual rhythms in plasma LH levels and some of the semen data, and changes directly attributable to the lighting regimes. Also a modification of the plasma sampling routine to provide a number of observations on any one day might have overcome a possible deficiency in Experiment 6. Furthermore, in view of the low plasma LH levels recorded, a modification of the radioimmunoassay method may be necessary.

In spite of these shortcomings, this experiment has shown that rams require an intact pineal gland to display their normal responses to the annual rhythmic fluctuations in daily photoperiod.

CHAPTER VII

PERIPHERAL PLASMA HORMONE LEVELS RECORDED FROM PINEALECTOMIZED OR

SHAM-OPERATED RAMS SUBJECTED TO CONTRASTING LIGHTING REGIMES

1. INTRODUCTION

Considerable evidence published in recent years has indicated

that in many species some hormones are released in a pulsatile manner,

consequently peripheral plasma concentrations of these hormones

undergo frequent and rapid fluctuations.

Anterior pituitary secretion of LH in rams has been shown to

be highly variable (Katongole, Naftolin and Short, 1974; Sanford

et al., 1974b; Falvo et al., 1975). Likewise, oscillations in

peripheral plasma concentrations of testosterone in rams have been

reported (Attal, 1970; Katongole, Naftolin and Short, 1974; Purvis,

Illius and Haynes, 1974; Sanford et al., 1974b; Falvo et al., 1975),

and it has been assumed that these changes in testosterone

concentration were the direct result of alterations in circulating LH

levels. Such a relationship between LH and testosterone secretion

has been established by studies which showed that testosterone peaks

always were preceded by an LH peak (Katongole, Naftolin and Short,

1974; Sanford et al., 1974b). None of the authors referred to above

have shown any evidence to suggest a circadian pattern of release for

either hormone.

Using plasma samples collected at four hourly intervals, Forbes

et al., (1975) reported that prolactin levels of ram lambs showed a

significant nocturnal rise. In contrast, Chamley et al. (1974)

found no evidence for a circadian pattern of secretion of this hormone

by adult rams. The latter authors based their finding on two-hourly

"pooled" blood samples. Circadian variations in plasma cortisol levels in rams have been described by Holley, Beckman and Evans (1975) and in ewes by McNatty, Cashmore and Young (1972).

Thus it is likely that information obtained from single blood samples, taken at one time during the day, has restricted value as a measure of the endocrine status of rams. With this point in mind, three intensive studies were carried out to determine the patterns of secretion of plasma hormones in the rams utilized in Experiment 6.

At the time that these experiments were planned, Experiment 6 had not been completed so samples collected in that experiment had not been assayed. Hence the effects of pinealectomy, or lighting regimes, on the secretion of hormones in rams, had not been revealed. It was possible that such treatments might have altered the patterns of hormone secretion, without changing the mean plasma concentration at the morning sampling time used in Experiment 6.

The only evidence that pinealectomy might alter diurnal hormonal secretion patterns in sheep was that of Thurley, Gibb and Russell (1975) who presented data which indicated that the diurnal cortisol rhythm tended to be abolished in pinealectomized ewes. No reports have been published on the effects of different lighting regimes on the plasma hormone profiles of rams. Thus Experiments 7.1, 7.2, and 7.3 were designed to examine whether it was possible to detect any effects of pinealectomy or daily photoperiod on the patterns of reproductive hormone secretion in rams, in a series of experiments in which plasma samples were collected at frequent intervals for extended periods of time.

2. MATERIALS AND METHODS

(1) Animals and Experimental Procedure

In Experiment 7.1 the experimental animals used were the rams on

the Normal lighting regime of Experiment 6. Four rams were sampled on November 20, 1974, and the remaining four rams were sampled on December 4, 1974. On each occasion two pinealectomized and two sham-operated rams were sampled and blood was collected via indwelling venous cannulae (see Chapter II) every twenty minutes for twenty-six hours, commencing at 06.00 h. Occasional blockages in the cannulae resulted in a few samples not being collected. In Experiment 7.2 all sixteen rams in Experiment 6 were sampled on March 3, 1975, blood being collected by venepuncture at 30 minute intervals for four hours commencing at 13.00 h. A similar procedure was adopted for Experiment 7.3, which was performed on May 20, 1975. However, in this study venepuncture blood samples were collected at 30 minute intervals for six hours commencing at 10.30 h.

As disturbance to the rams was minimal, and no changes were made to their lighting and feeding routine, it was considered that these acute studies did not interfere with the results of Experiment 6.

(2) Hormone Assay Procedure

The particular interest in circadian hormone secretion profiles, which has appeared in the field of reproductive endocrinology was a stimulus to measure a number of different hormones in the plasma samples obtained from Experiment 7.1 LH, prolactin, testosterone and cortisol were assayed as described in Chapter II. LH and prolactin only were measured in samples from Experiments 7.2 and 7.3.

(3) Statistical Analyses

Estimated values for the few missing samples were calculated as the mean of the preceding and subsequent samples.

Hormone concentrations were transformed to their logarithm using the relationship : logarithm of $x = 10 \log_{10}(x + 1.1)$

where, x is hormone concentration in ng/ml plasma.

For each ram, transformed values were progressively summed in numerical order of sampling, to form a series of cumulative totals. The gradient of this cumulative distribution against time, which was tested for linearity by analysis of variance, was used as a variable in between-treatment contrasts. It was considered that this gradient provided a parameter which reflected hormone output more comprehensively than the arithmetic mean of the hormone concentrations. Between-group comparisons of the gradients of the cumulative hormone distributions in the first study were performed by Student's 't' test. In Experiments 7.2 and 7.3 treatment effects of lighting regimes, operations, and their interaction were examined using the orthogonal coefficients shown below :

Contrast	Normal Lighting		Reversed Lighting	
	Sham-Operated	Pineal-ectomized	Sham-Operated	Pineal-ectomized
Lighting Regimes	+1	+1	−1	−1
Operations	+1	−1	+1	−1
Lighting Regimes x Operations	+1	−1	−1	+1

Studies on goats have shown that venepuncture initially elevates prolactin levels which, in subsequent samples, return to normal as the animals become accustomed to this stress (Hart, 1973). On this basis, the prolactin data for the first samples collected by venepuncture in Experiments 7.2 and 7.3 were excluded from the statistical analyses.

3. RESULTS

Experiment 7.1 was designed as a study of the normal patterns of secretion of LH, testosterone, prolactin and cortisol, and also to examine the effects of pinealectomy on these secretory patterns.

All eight rams exhibited a highly irregular pattern of plasma LH secretion. (See Figures 7.1 and 7.2). These pulses occasionally

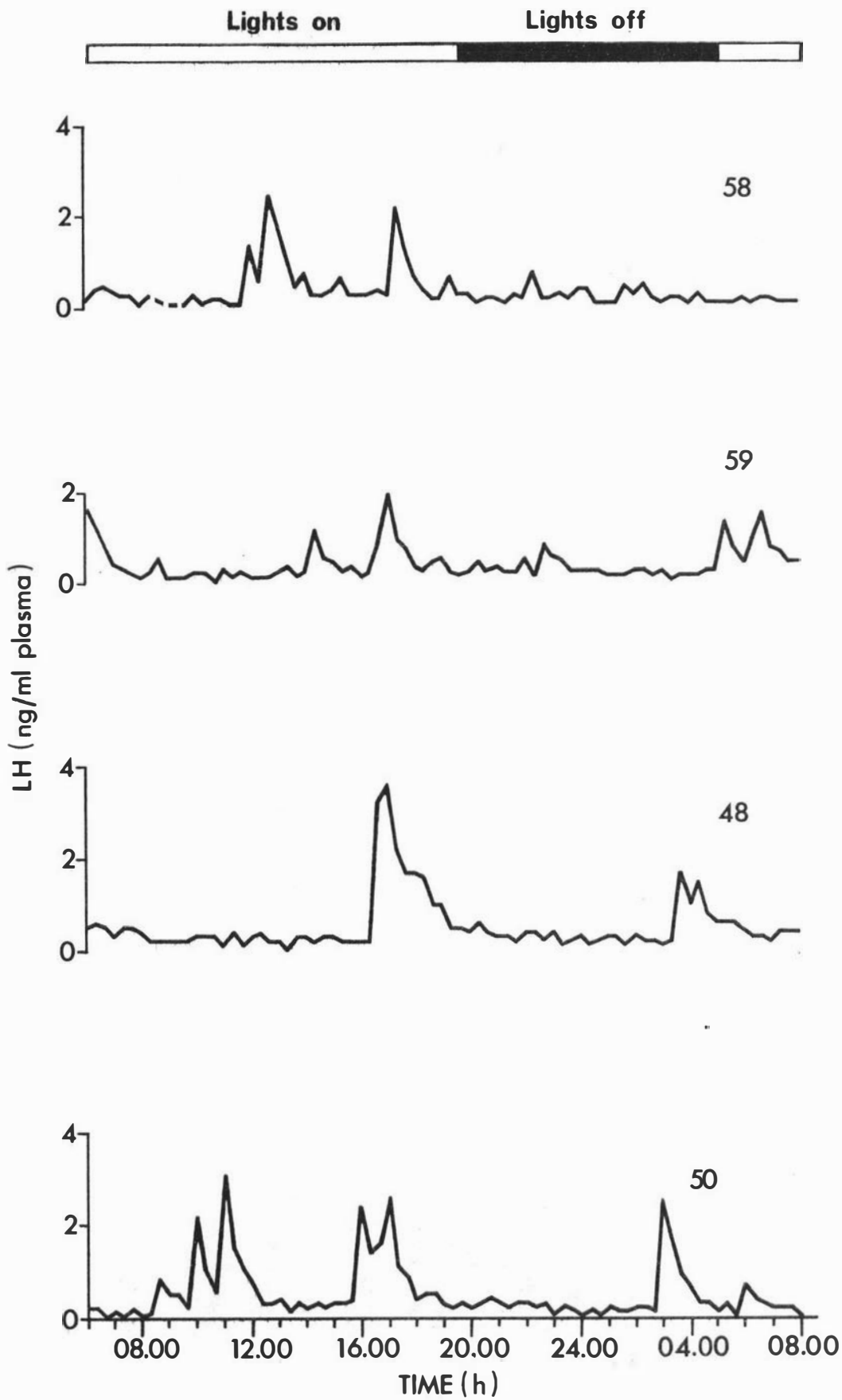Figure 7.1 : LH secretion patterns recorded from plasma samples collected from four sham-operated rams, sampled at 20 minute intervals for 26 hours.
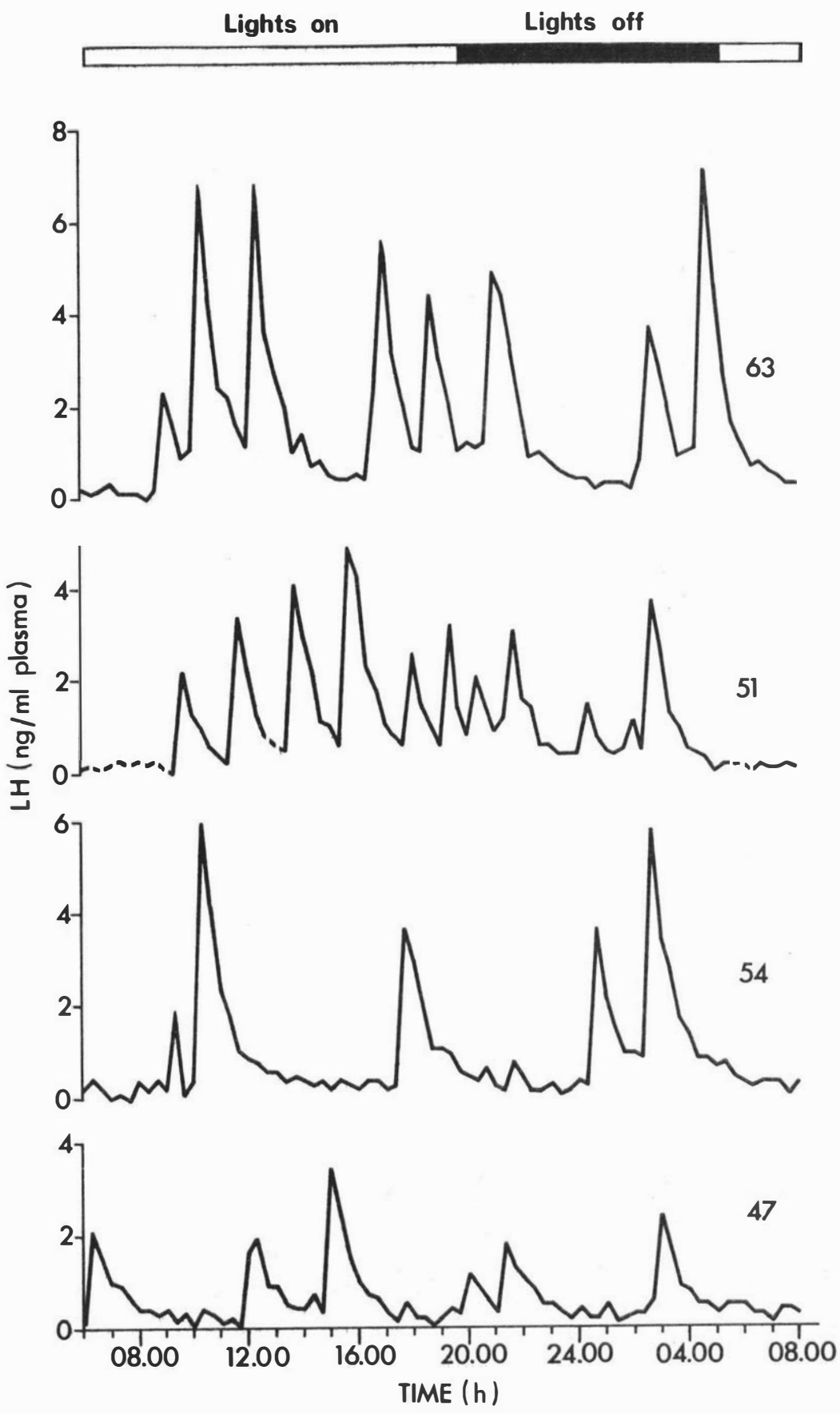
Figure 7.2 : LH secretion patterns recorded from plasma samples collected from four pinealectomized rams, sampled at 20 minute intervals for 26 hours.
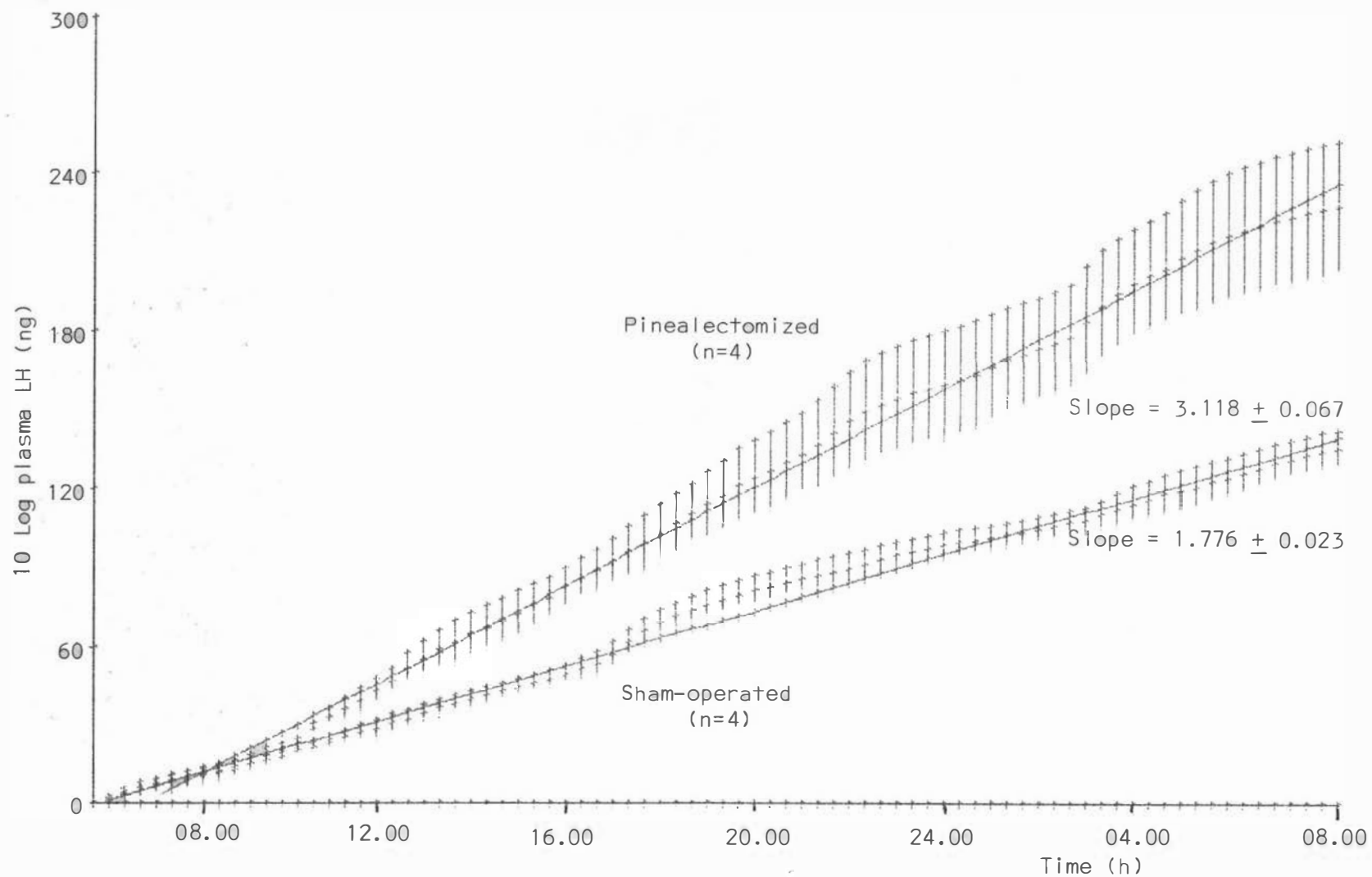
Figure 7.3 : 26-Hour cumulative LH levels (Means ± S.E.) showing pooled cumulative regression lines.

raised plasma LH concentrations from barely detectable levels to more than six ng/ml in the next twenty-minute sample. There was no circadian pattern of distribution of the pulses. Pinealectomized rams appeared to produce more LH peaks, with higher peak hormone levels, than did the sham-operated rams. Student's 't' test confirmed that the slopes of the cumulative LH distributions for the pinealectomized rams were greater than those for the sham-operated animals (3.118 vs 1.776, p < 0.05). (See Figure 7.3 and Table 7.1).

Plasma testosterone levels (Figures 7.4 and 7.5) displayed a pulsatile pattern of secretion very similar to that of LH. Major peaks of testosterone concentration corresponded with pulses of LH release and each elevation in testosterone levels usually occurred twenty to forty minutes following an LH pulse. Testosterone peaks usually exceeded 5 ng/ml. Again, as with LH, there was no indication of a circadian pattern of testosterone secretion. Pinealectomized rams had an output of testosterone which was higher than that of sham-operated rams, however comparison of the slopes of the cumulative distributions indicated that this difference just failed to reach statistical significance (5.141 vs 4.004, p = 0.052). (See Table 7.1).

In all rams plasma prolactin levels fluctuated markedly throughout the twenty-six hour sampling period (Figures 7.6 and 7.7). Although the slopes of the cumulative prolactin distributions were not significantly different for the two groups (Table 7.1), a distinct circadian pattern was displayed by the sham-operated rams, but not by the pinealectomized rams. The circadian pattern recorded from control animals took the form of an elevation of plasma prolactin concentrations, commencing in the early afternoon and reaching maximum values (in excess of 100 ng/ml) approximately between 20.00 h and 22.00 h; subsequently prolactin levels fell relatively rapidly to reach low levels at about 24.00 h.

Figure 7.4 : Testosterone secretion patterns recorded from plasma samples collected from four sham-operated rams, sampled at 20 minute intervals for 26 hours.

Figure 7.5 : Testosterone secretion patterns recorded from plasma samples collected from four pinealectomized rams, sampled at 20 minute intervals for 26 hours.
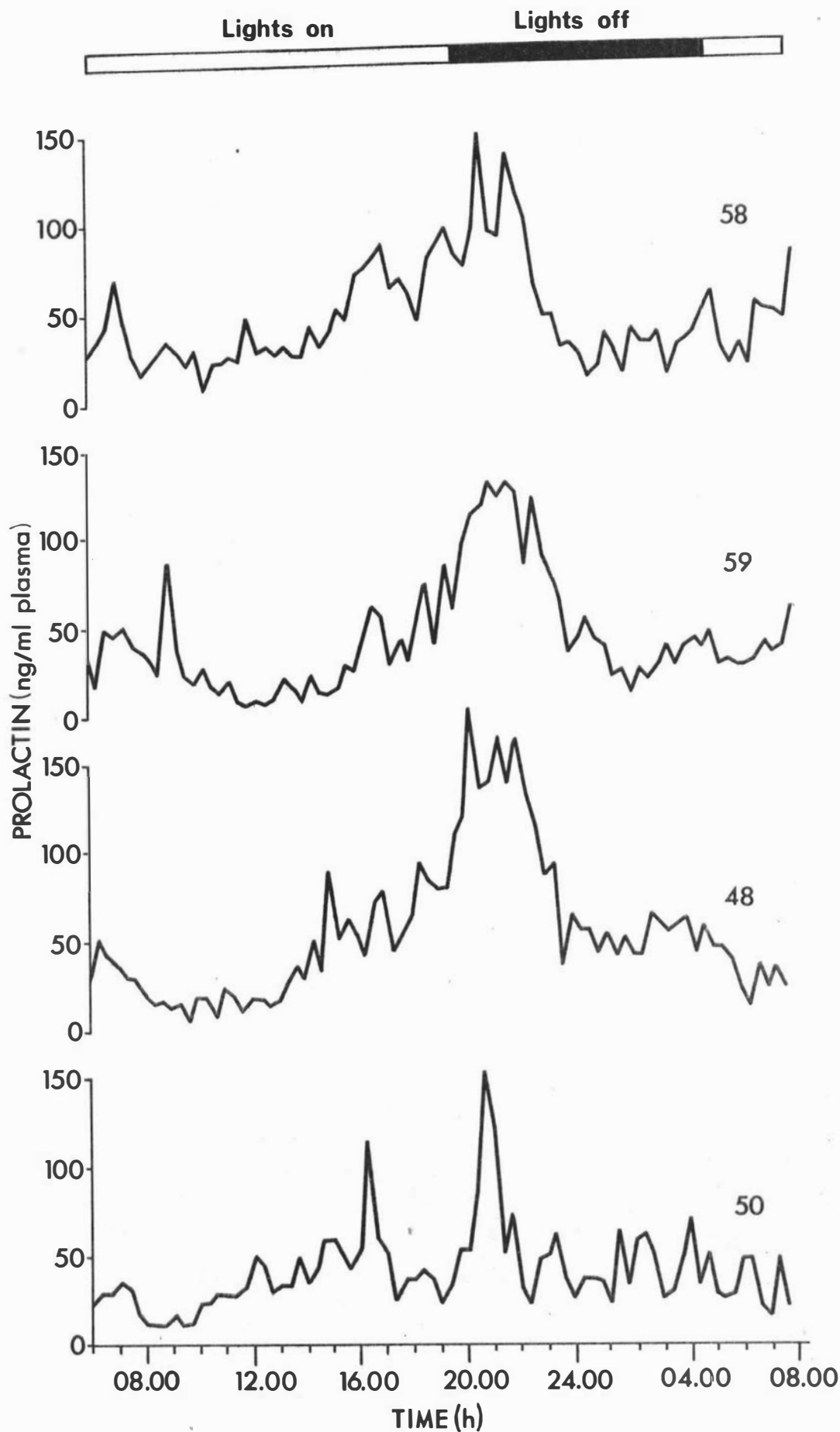
Figure 7.6 : Prolactin secretion patterns recorded from plasma samples collected from four sham-operated rams, sampled at 20. minute intervals for 26 hours.
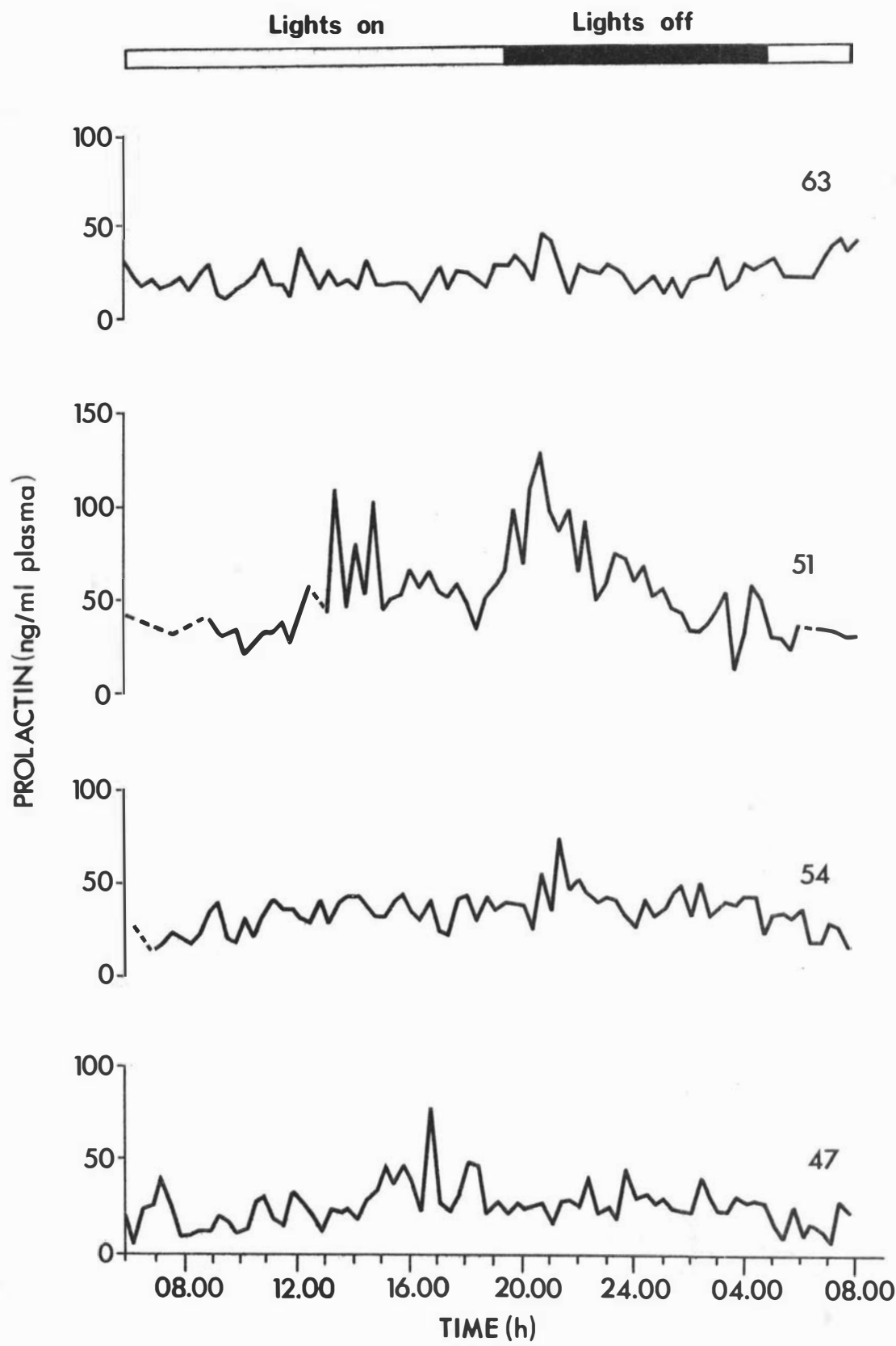
Figure 7.7 : Prolactin secretion patterns recorded from plasma samples collected from four pinealectomized rams, sampled at 20 minute intervals for 26 hours.
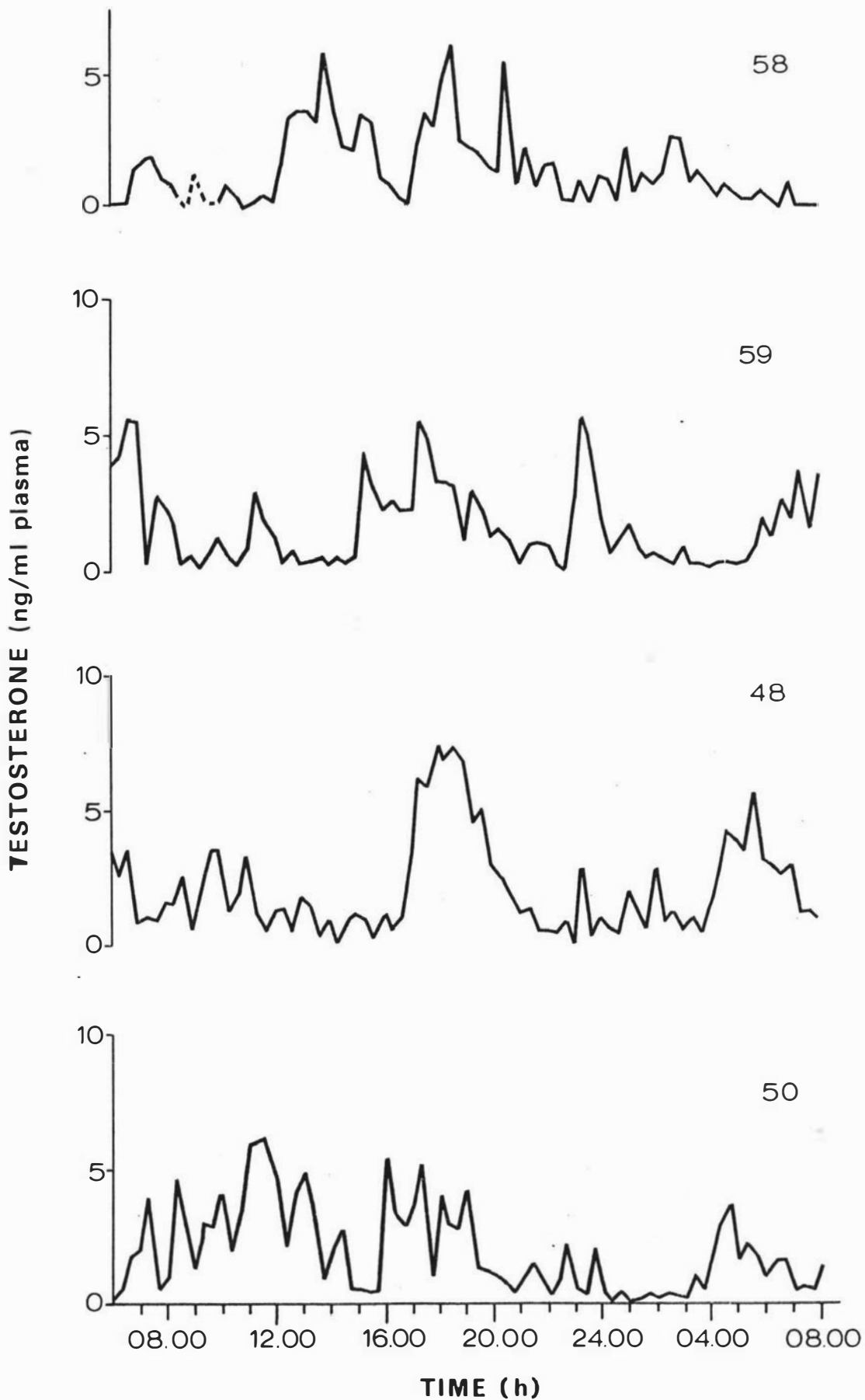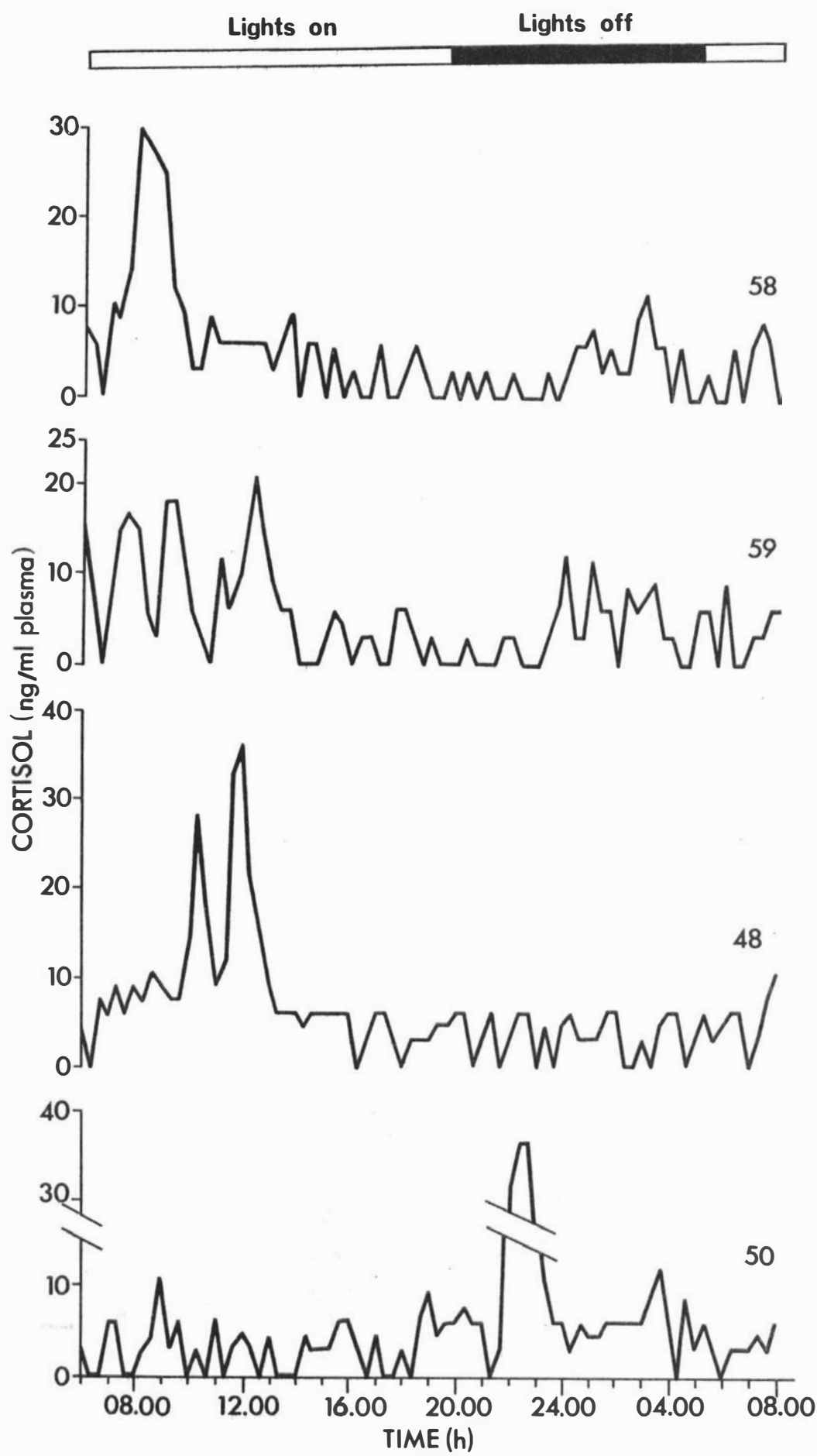
Figure 7.8 : Cortisol secretion patterns recorded from plasma samples collected from four sham-operated rams, sampled at 20 minute intervals for 26 hours.
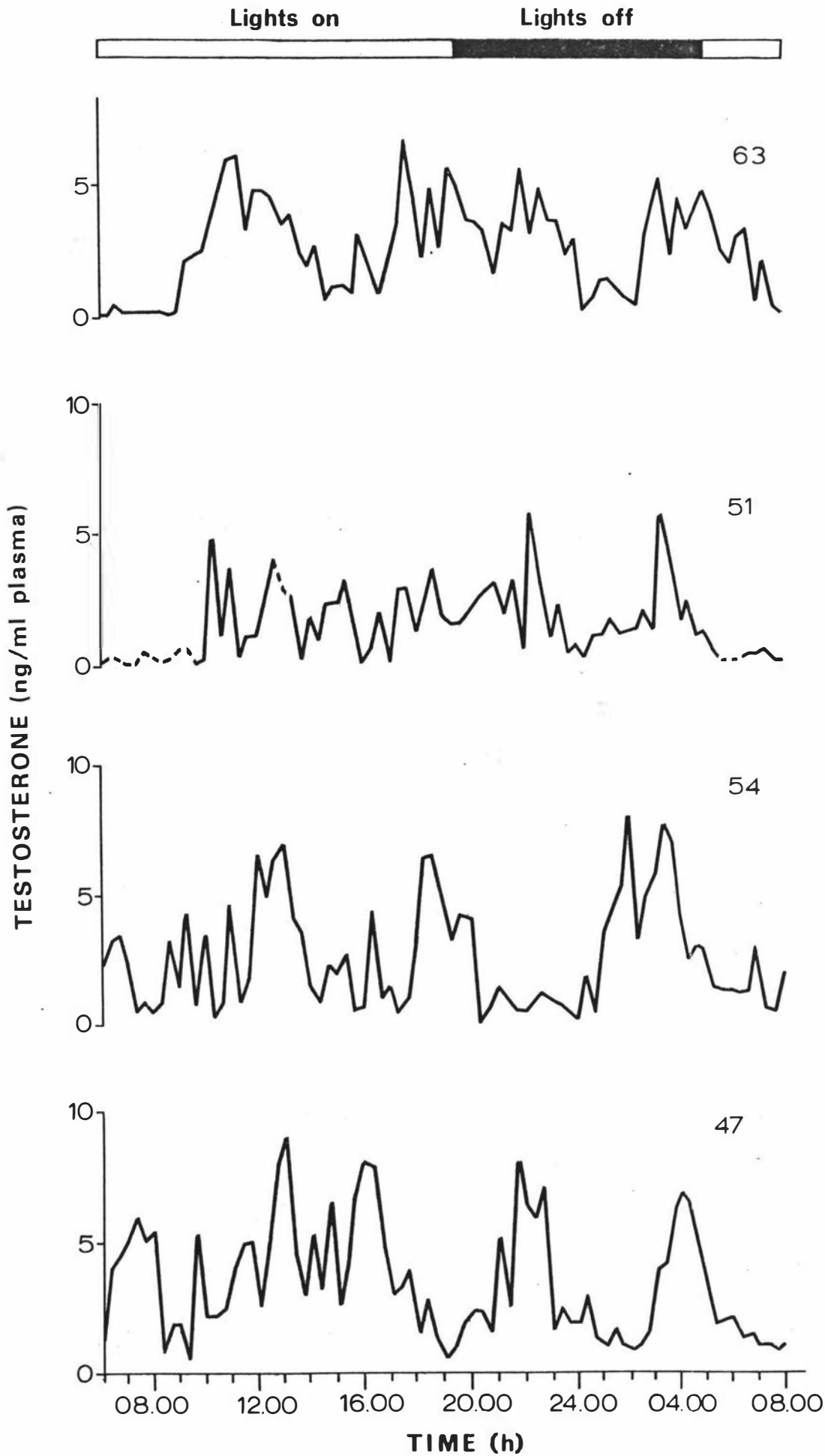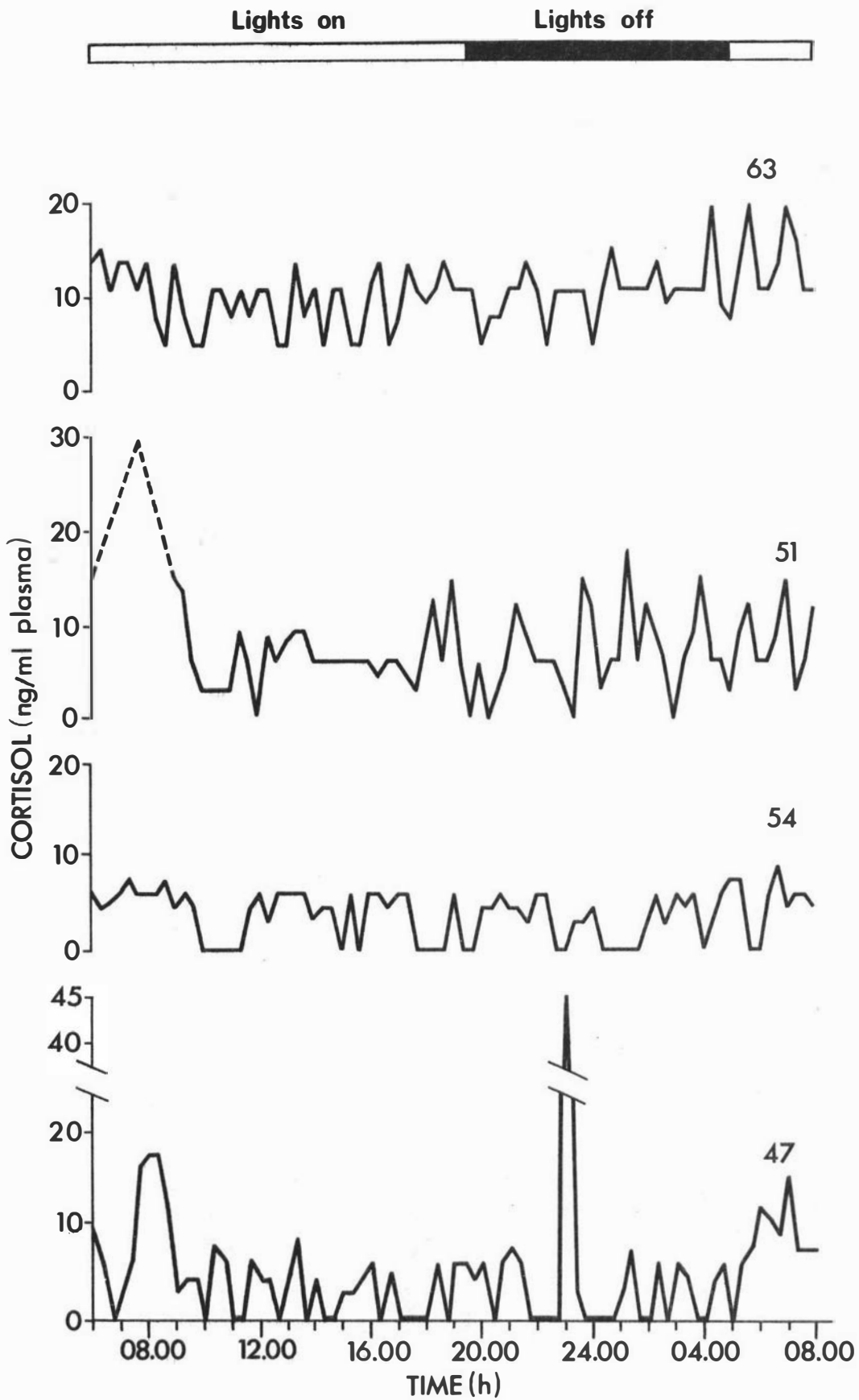
Figure 7.9 : Cortisol secretion patterns recorded from plasma samples collected from four pinealectomized rams, sampled at 20 minute intervals for 26 hours.

Table 7.1

Gradients of Cumulative LH, Testosterone, Prolactin and Cortisol Distributions Recorded from Plasma Samples Collected over 26 hours from Individual Sham-operated or Pinealectomized Rams.

	Log LH	Log Testosterone	Log Prolactin	Log Cortisol
Pinealectomized	4.045	5.439	13.66	27.32
	3.388	4.003	17.29	36.35
	2.808	5.009	15.26	19.47
	2.230	6.114	14.25	22.40
Mean+S.E.	3.118+0.067	5.141+0.442	15.12+0.15	26.39+1.10
Sham-operated	1.689	3.935	17.67	22.27
	1.481	3.609	15.91	23.97
	1.989	4.312	17.18	31.11
	1.944	4.158	16.26	30.92
Mean+S.E.	1.776+0.023	4.004+0.152	16.76+0.10	27.07+0.71
$\underline{t}_6$	3.298*	2.432[+]	−0.126	−0.156

($^{+}$ p = 0.052)

All rams exhibited a cortisol secretion pattern which appeared as a sequence of peaks, most of which had a duration of approximately one hour, and maximum values of about 7 ng/ml. (See Figures 7.8 and 7.9). However, much larger peaks (some higher than 30 ng/ml) were recorded, particularly between 07.00 h and 14.00 h. Peaks of this type were evident in three of the four sham-operated rams (Nos. 48, 58 and 59), and, to a lesser extent, in two of the pinealectomized rams (Nos. 47 and 51). There were, however, no significant differences between the slopes of the cumulative cortisol distributions for the two groups of rams (Table 7.1). Two of the rams (Nos. 47 and 50) displayed a large cortisol peak at about 22.30 h. This might have been a stress effect, since these two rams were in adjacent crates.

Experiment 7.2 was carried out with several aims in mind : firstly to determine whether the effects of pinealectomy on LH secretion, which were revealed in Experiment 7.1, could be detected in a shorter term experiment; secondly to see whether these effects of pinealectomy were apparent later in the year; and thirdly to investigate possible effects of lighting regimes on LH and prolactin secretion.

Very few peaks of LH secretion were recorded from any of the rams and, in the case of pinealectomized rams under Normal lighting, there were no peaks at all. (See Figure 7.10). In the analysis of variance of LH data, none of the treatment contrasts were significant. (Tables 7.2 and 7.3).

Individual rams showed quite considerable variations in plasma prolactin levels, and nine out of the sixteen had elevated levels at the initial sampling. presumably as a result of the venepuncture sampling procedure. Pinealectomized rams under both lighting regimes had a higher output of prolactin than did sham-operated rams ($p < 0.05$). (See Figure 7.11 and Tables 7.2 and 7.3). Lighting regimes had no

Figure 7.10 : (Experiment 7.2) LH levels (mean+S.E.) in sham-operated
and pinealectomized rams recorded from plasma samples collected at 30
minute intervals for a four hour period on March 5, 1975.

Figure 7.11 : (Experiment 7.2) Prolactin levels (mean±S.E.) in sham-operated and pinealectomized rams recorded from plasma samples collected at 30 minute intervals for a four hour period on March 5, 1975.

Table 7.2

Gradients of Cumulative LH and Prolactin Distributions Recorded from
Plasma Samples Collected over 4 hours from Individual Sham-operated or
Pinealectomized Rams on Contrasting Lighting Regimes.

		Log LH	Log Prolactin
Normal Lighting Regime			
Sham-operated		2.102	14.27
		2.294	14.70
		1.452	11.40
		1.115	15.17
	Mean+S.E.	1.741±0.276	13.89±0.85
Pinealectomized		1.041	19.41
		1.090	17.00
		1.313	20.35
		1.769	19.06
	Mean+S.E.	1.303±0.166	18.96±0.71
Reversed Lighting Regime			
Sham-operated		0.895	16.48
		1.979	14.25
		0.884	12.70
		2.722	18.00
	Mean+S.E.	1.620±0.448	15.36±1.17
Pinealectomized		2.180	17.42
		0.900	17.75
		0.834	20.50
		3.504	17.91
	Mean+S.E.	1.854±0.631	18.39±0.71

Table 7.3

Summary of Analyses of Variance for LH and Prolactin Data Presented in Table 7.2.

Source of Variation	D.F.	Variance Ratios	
		LH	Prolactin
Lighting Regimes	1	0.26	0.27
Operations	1	0.06	21.20***
Lighting Regimes x Operations	1	0.64	1.33
Residual Mean Square	12	0.70	3.10

significant effect on mean plasma prolactin levels.

As the LH data for plasma samples collected over a four hour period in Experiment 7.2 was inconclusive, a further short-term study (Experiment 7.3) was undertaken to examine the effects of pinealectomy and lighting regimes on LH and prolactin production rates. In this experiment, however, the sampling period was extended to six hours.

Plasma LH concentrations from individual rams again indicated a pulsatile release of this hormone with most animals displaying one or more peaks of LH secretion. However, the sham-operated rams on the Normal lighting regime were an exception in that their plasma LH levels changed little during the study (Figure 7.12). Analysis of variance of the gradients of the cumulative LH distributions revealed that the rams on Reversed lighting had a higher mean level of LH output than those on normal lighting ($p < 0.05$). (See Tables 7.4 and 7.5).

Pinealectomized rams tended to have higher mean levels of LH production that did the sham-operated animals, but in the analysis of variance this difference failed to reach significance ($p = 0.052$).

In Experiment 7.3 (Figure 7.13) individual rams again had markedly variable plasma levels of prolactin, and thirteen of the sixteen rams had elevated levels at the first sampling. Analysis of variance of the gradients of the cumulative prolactin distributions (Tables 7.4 and 7.5) indicated that the output of prolactin was higher in pinealectomized rams than in sham-operated rams, whilst both groups subjected to Reversed lighting apparently had higher prolactin production rates than those under Normal lighting. However, inspection of Figure 7.13 and the cumulative distribution data (Table 7.4) indicated that both of these significant results could be misleading as they occurred virtually entirely as a result of the low rate of prolactin output from the sham-operated rams on the Normal
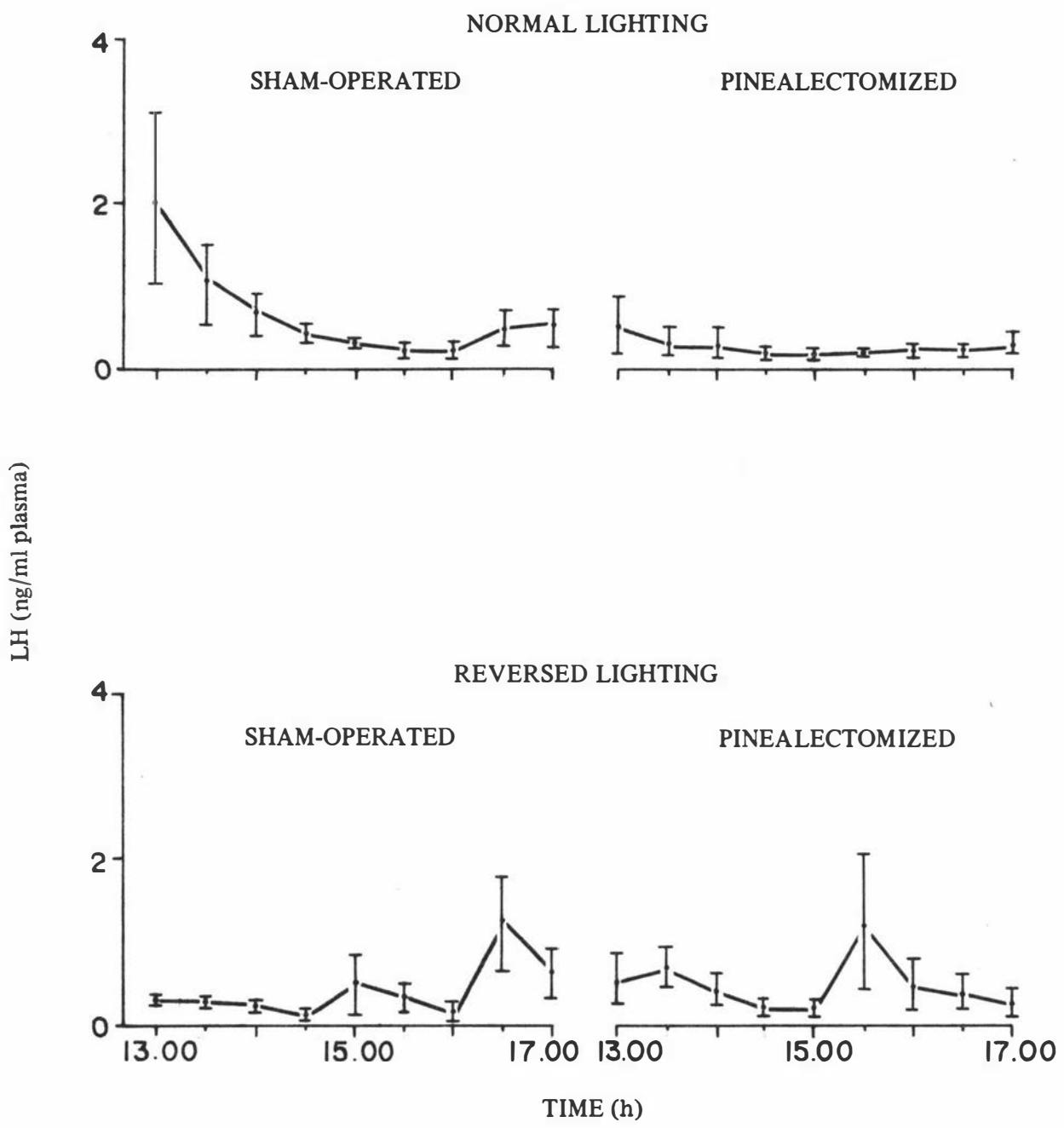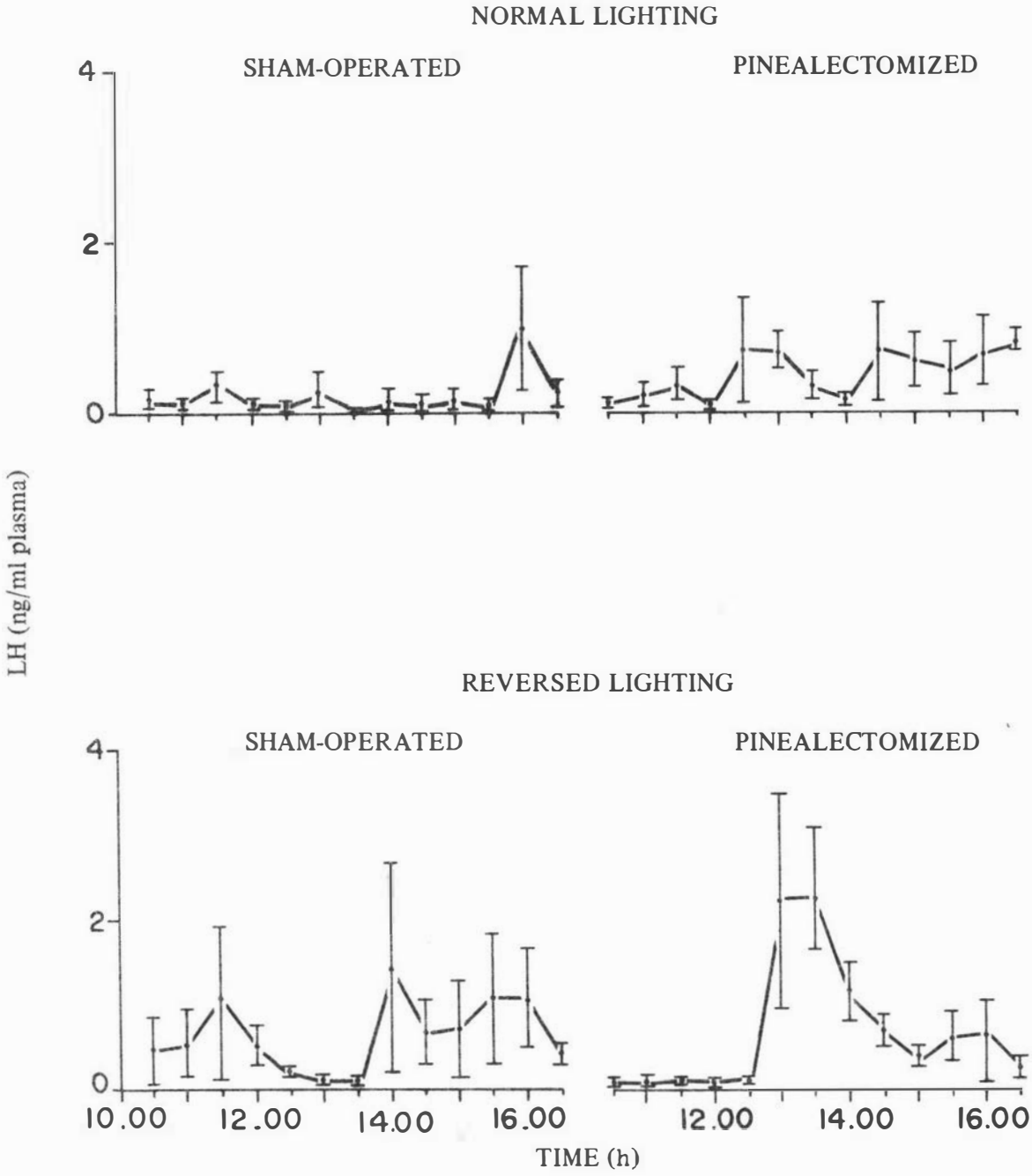
Figure 7.l2 : (Experiment 7.3) LH levels (mean±S.E.) in sham-operated and pinealectomized rams recorded from plasma samples collected at 30 minute intervals for a six hour period on May 20, 1975.
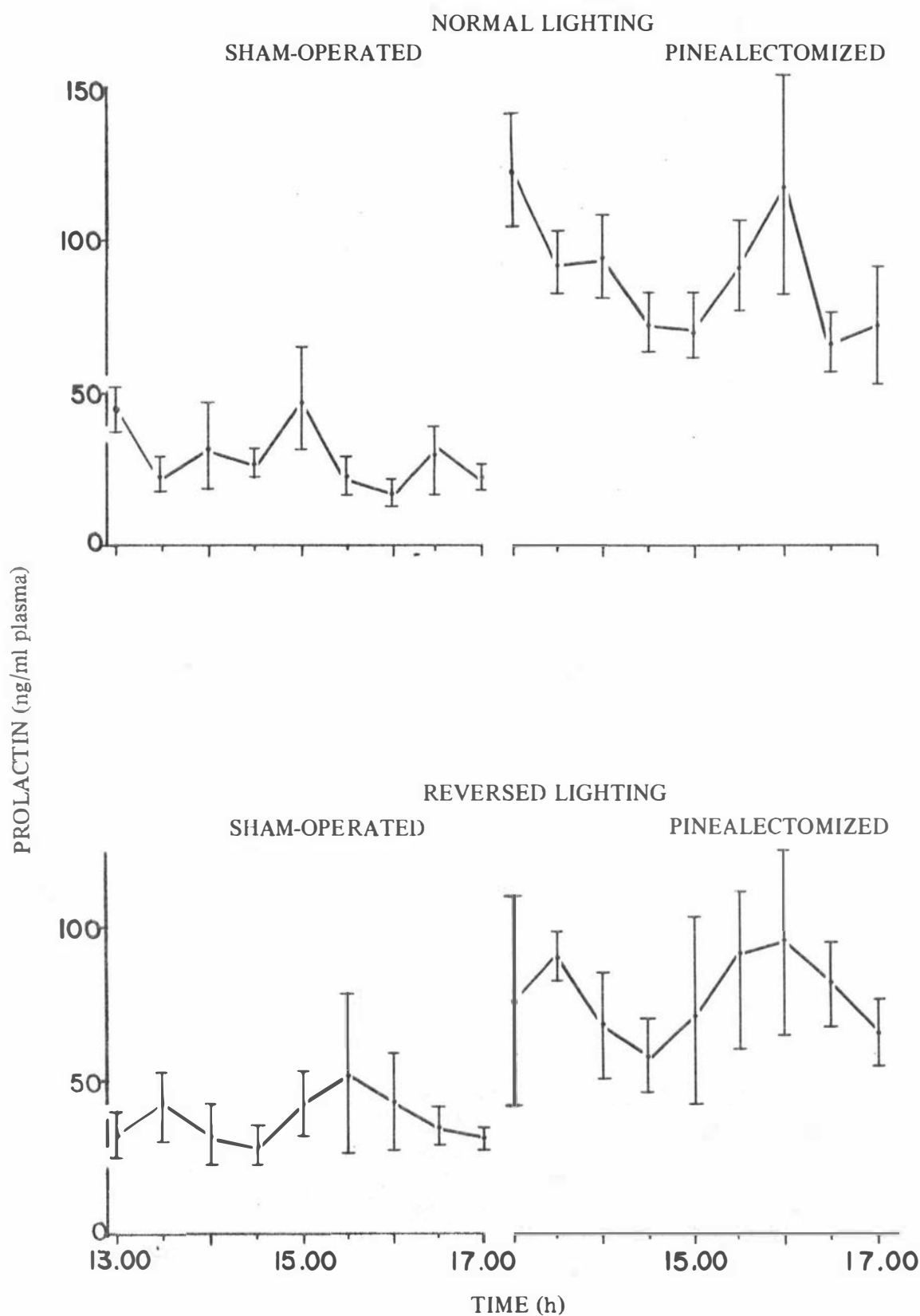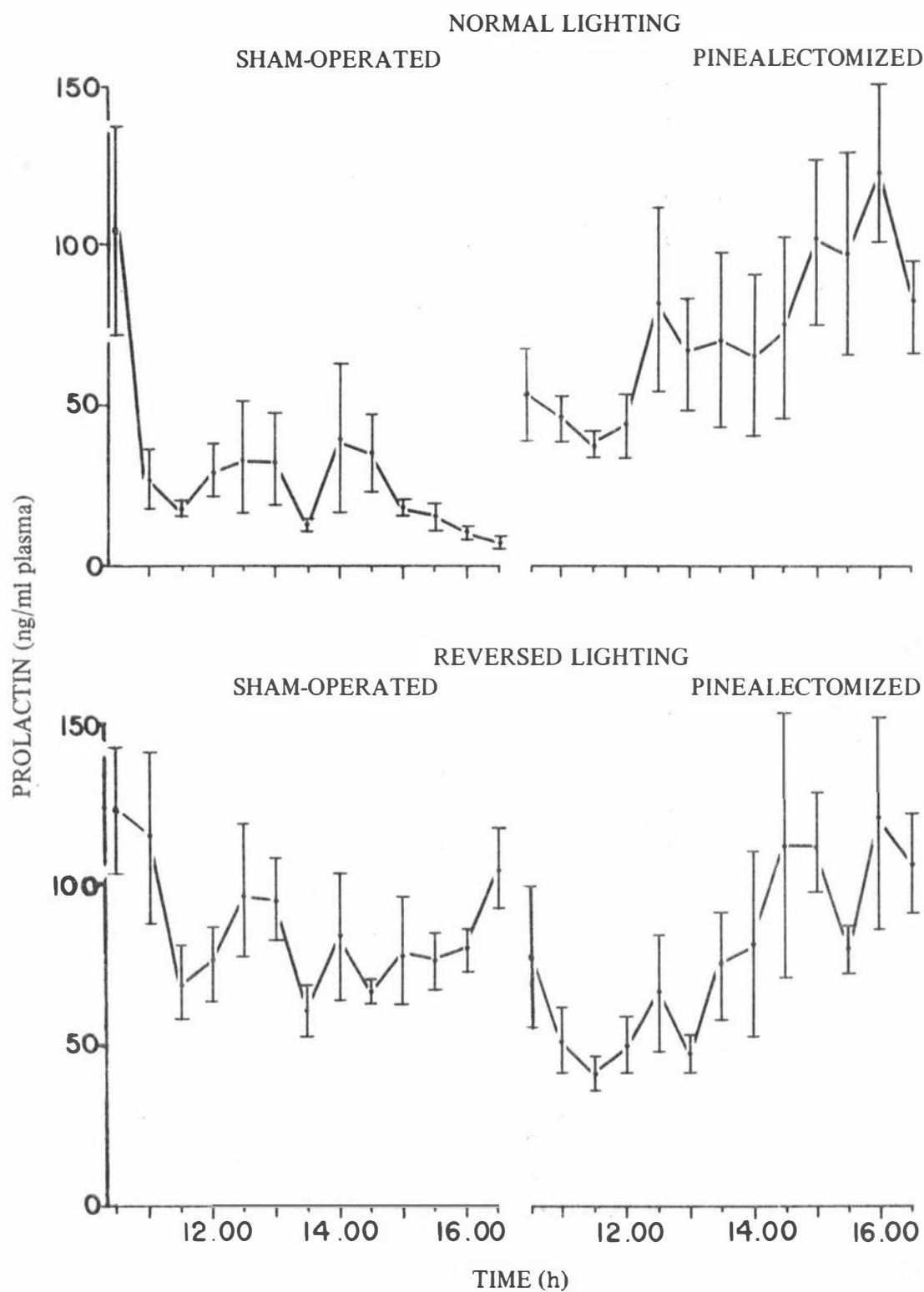
Figure 7.13 : (Experiment 7.3) Prolactin levels (mean±S.E.) in sham-
operated and pinealectomized rams recorded from plasma samples coll-
ected at 30 minute intervals for a six hour period on May 20, 1975.

Table 7.4

Gradients of Cumulative LH and Prolactin Distributions Recorded from Plasma Samples Collected over 6 hours from Individual Sham-operated or Pinealectomized Rams on Contrasting Lighting Regimes.

		Log LH	Log Prolactin
Normal Lighting Regime			
Sham-operated		1.179	16.55
		0.622	18.95
		1.225	17.38
		0.736	15.85
	Mean+S.E.	0.940+0.153	17.18+0.67
Pinealectomized		2.815	24.12
		0.971	21.04
		1.851	26.35
		1.644	21.66
	Mean+S.E.	1.820+0.381	23.29+1.22
Reversed Lighting Regime			
Sham-operated		1.564	25.37
		2.763	25.01
		1.956	25.44
		1.235	23.83
	Mean+S.E.	1.880+0.329	24.91+0.37
Pinealectomized		1.707	21.69
		1.838	25.54
		2.797	25.69
		3.227	22.75
	Mean+S.E.	2.392+0.369	23.92+1.00

Table 7.5

Summary of Analyses of Variance for LH and Prolactin Data Presented in Table 7.4.

Source of Variation	D.F.	Variance Ratios	
		LH	Prolactin
Lighting Regimes	1	5.53*	22.74***
Operations	1	4.69+	8.52*
Lighting Regimes x Operations	1	0.30	.16.44**
Residual Mean Square	12	0.41	3.07

($^+$ p = 0.052)

lighting regime. This conclusion was confirmed by the highly significant Lighting Regimes x Operations interaction.

4. DISCUSSION

(1) LH

Highly pulsatile patterns of LH release in rams have been described in several earlier reports (Bolt, 1971; Katongole, Naftolin Short. 1974; Sanford et al., 1974b; Falvo et al., 1975) but the physiological significance of this type of secretory pattern is not clear. It is possible that LH responsive tissues require a digital rather than an analogue control, such that they respond to hormone pulse frequency rather than to absolute concentrations of the gonadotrophin. On the other hand, the rapid increase in peripheral concentrations of LH which follows such pulses, could represent a means for momentarily saturating binding sites on receptor tissues, thereby obviating the need for constant production of large amounts of the hormone.

Inspection of the records of LH output for individual rams (Figures 7.1 and 7.2) revealed that most of the secretory pulses ·caused concentrations to rise very steeply but to decline in an exponential fashion. A similar pattern was seen in the data presented by Sanford et al. (1974b) who took blood samples at twenty minute intervals also. This finding suggested that the anterior pituitary releases LH in pulses and that between each pulse, little or no further secretion takes place.

It was estimated that many of the higher peaks in the present study fell to fifty per cent of their peak value in approximately thirty minutes. Similar or slightly lower figures could be estimated by inspection of the data of Sanford et al. (1974b). This half-life of approximately thirty minutes is similar to that reported by

Butler et al. (1972) and by Geschwind (1972) for ovine LH in ewes. The fact that this figure was fairly constant for secretory peaks with a wide range of heights gave further support to Geschwind's (1972) concept that a basal level of LH secretion does not occur between secretory phases. A half-life of this duration indicates that the sampling interval of twenty minutes used in the present study would enable detection of every LH pulse during the observation period, even if not allowing measurement of absolute peak values.

As noted in the previous reports mentioned above, there was no evidence for a circadian pattern of LH release in the present study. It is interesting to note that no circadian rhythm occurred in the output of a hormone which shows annual secretory fluctuations (Chapter III and Hochereau-de Reviers, Loir and Pelletier (1976)) which presumably are caused by seasonal changes in daily photoperiod.

Steeper slopes for the cumulative LH distributions in pineal-ectomized rams indicated that these rams had a greater output of LH than the sham-operated animals. This result was in accord with the widely accepted belief that the pineal gland has an antigonadotrophic role (Kappers, 1969; Reiter, 1973b). Previous workers have demonstrated antigonadotrophic properties of sheep pineal glands in in vitro systems (Citharel et al., 1972), but this is the first report which has presented direct evidence that the sheep pineal gland probably has a true antigonadotrophic function.

A similar tendency for the pinealectomized rams to have elevated LH levels was recorded in Experiment 7.3. However, in the analysis of variance this result just failed to reach significance, probably because the sham-operated rams on the Reversed lighting regime were subjected to a long daily photoperiod, which in turn caused a stimulation of LH secretion such as recorded in Experiment 3.

Little importance is attached to the fact that in Experiment 7.2 mean LH levels were not elevated in the pinealectomized rams. This is a result which may be interpreted as contradictory to those obtained in the other experiments in this chapter. More likely, however, it was a spurious result which arose due to the blood sampling period being too short and coinciding with a time in which the pinealectomized rams experienced virtually none of the randomly occurring spikes of LH secretion. Also it was possible that seasonal changes modified between-operation differences to the extent that the effects of pinealectomy were apparent only at particular times of the year.

The significant Lighting Regimes x Operations interaction seen in Experiment 7.3 indicated that pinealectomized rams continued to display seasonal changes in LH secretion, even in the absence of a pineal gland. This finding may have resulted from the presence of residual seasonal rhythms similar to those which Herbert (1972) claimed persisted in ferrets until the second year after pinealectomy.

The effects of the two different lighting regimes were not examined in Experiment 7.1, while in Experiment 7.2 lighting regimes had no effect on LH secretion. Again this latter result may merely have reflected the short period of blood sampling. More pertinent was the fact that the time of sampling was close to the equinox in both regimes, at which time little or no difference in LH output was expected. For example in an earlier study (Experiment 3) rams on pasture displayed a seasonal pattern of plasma LH levels which indicated that highest LH levels were associated with the longest daily photoperiods. This effect of photoperiod was confirmed in Experiment 7.3 in which the LH output of rams on Reversed lighting was higher than for those on Normal lighting. However these results contrast sharply with those of Pelletier and Ortavant (1975a) and Lincoln (1976a), in which highest plasma LH levels were associated

with decreasing daily photoperiods. This disparity in results undoubtedly was attributable mainly to the photoperiodic cycles used by Pelletier and Ortavant (6 months cycle length, 8 h amplitude) and Lincoln (abrupt change in daily photoperiod from 18 h to 8 h), which were vastly different from those used in Experiment 6.

In Experiments 5 and 6 lighting regimes had little effect on plasma LH levels; this result may reflect the inadequacy of the blood sampling regimes used in those particular experiments. Collectively, the results of the experiments described in this and earlier chapters can be interpreted as emphasizing the fact that experiments involving collection of single plasma samples or multiple samples over a short period of time (e.g. Experiments 7.2 and 7.3), will not reveal the full extent of the effects of pinealectomy or lighting regimes, on LH secretion patterns. These effects probably will only be elucidated by experiments which incorporate : plasma sampling regimes similar to that used in Experiment 7.1; with samplings of this type at monthly intervals throughout the year; and which utilize animals which have been exposed to their various lighting regimes for an extended period of time prior to commencing sample collection, in order that residual seasonal rhythms may be abolished.

(2) Testosterone

Fluctuations in plasma testosterone levels recorded throughout twenty-six hours from rams in Experiment 7.1 resembled similar patterns of secretion reported by earlier workers (Katongole, Naftolin and Short, 1974; Purvis, Illius and Haynes, 1974; Sanford et al., 1974b) Furthermore, elevations in plasma testosterone following LH pulses in these rams were consistent with the results of previous studies in which both LH and testosterone were measured (Katongole, Naftolin and Short, 1974; Sanford et al., 1974b). The twenty to forty minute interval between LH pulses and the subsequent peak in plasma

testosterone levels recorded in those two papers and in the present studies, confirmed similar results obtained by Bremner et al. (1976) and Lee et al. (1976) following GnRH administration to rams. On the other hand Galloway et al. (1974) recorded a simultaneous elevation in plasma levels of both hormones following GnRH injection, however this unusual result may have resulted from the large doses of releasing hormone used by these workers in comparison to the studies reported above.

None of the research mentioned above, including the present experiments, revealed any evidence for the presence of a circadian pattern of testosterone secretion, nor did other studies in which ram plasma was sampled at less frequent intervals (Attal, 1970; Falvo et al., 1975). In view of the absence of an LH rhythm of this type, this result was anticipated.

The testosterone output of pinealectomized rams was almost significantly higher than that of sham-operated rams, a result which undoubtedly was related directly to the difference in LH output between the two groups. A separate experiment would be required to determine whether these differences were due to an effect of pineal gland activity on LH release per se, or on the sensitivity of LH release to testosterone feedback. The fact that fortnightly plasma testosterone levels recorded during November and December (Experiment 6) did not reveal any differences between the two groups of rams (0.68 $\pm$ 0.23 vs 0.94 $\pm$ 0.33 ng/ml; t_{14} = 1.690, n.s.), again indicated the need to perform a number of studies similar to Experiment 7.1, at various times throughout the year.

(3) Prolactin

Sham-operated rams in Experiment 7.1 displayed consistent nocturnal increases in plasma prolactin concentrations. Similar

nocturnal elevations have been recorded by Forbes et al. (1975) from castrated male lambs, and by Davis and Borger (1974) from ovariect-omized ewes. In contrast, other workers have failed to detect circadian patterns of prolactin release in entire rams (Chamley et al., 1974) and vasectomized rams (Davis and Borger, 1973; S.L. Davis, personal communication). However Chamley et al. (1974), measured prolactin levels in "pooled" two-hourly samples, so they could have failed to detect short peaks of prolactin release; also presumably they had no control over possible degradation or loss of immunoreactivity of the hormone during each two-hour collection period. Also Davis and Borger (1973) may have conducted their study at a time of the year when circadian patterns of prolactin release did not occur; moreover blood sampling was performed only two weeks after placement of the animals in metabolism crates, so the rams may not have become accustomed to their surroundings when the experiments were carried out.

The nocturnal rise in plasma prolactin levels shown by sham-operated rams in Experiment 7.1 could not be attributed to feeding or stress. Although its onset was during the early afternoon, it appeared to increase sharply at about the time that the lights were switched off (19.30 h). The apparent relationship between the rise in prolactin levels and the change from light to darkness may have involved an alteration in pineal gland activity in response to the change in intensity of photic stimulation of the eyes. Although the possibility of pineal gland involvement in this mechanism is speculative, the fact that this nocturnal elevation of plasma prolactin levels appeared to be abolished in pinealectomized rams provided strong support for the idea.

It is possible that the prolactin secretion patterns of the two groups of rams might have been proven to be significantly

different if a more appropriate statistical test had been applied. The technique of fitting a linear regression to the cumulative output probably was inadequate for detection of the type of nocturnal prolactin peak displayed by the sham-operated rams. This short-coming may have been overcome by fitting polynomial expressions to the logarithms of the hormone concentrations, or by performing non-parametric analyses, although this latter technique usually requires larger numbers of different observations to show significant differences. As the data has been inspected, it would be inappropriate to apply further tests of significance in the analysis of these data.

In Experiment 6, the pineal gland was shown to be involved in the prolactin response to changing daily photoperiod, as the response was diminished in pinealectomized rams. The possible involvement of the pineal gland in the prolactin response to diurnal light-dark changes provided evidence that the pineal gland influences the effects of both short term (daily) and long term (seasonal) changes in photo-period on neuroendocrine mechanisms in rams.

There did not appear to be any association between the plasma LH and prolactin levels recorded in Experiment 7.1. Likewise, similar dissociations between LH and prolactin secretion patterns have been observed in ewes (Butler et al., 1972; Fell et al., 1972) and in men (McNeilly et al., 1974). Also, peaks of prolactin release in bulls did not coincide with serum testosterone peaks (Smith et al., 1973). Thus the reproductive significance, if any, of a nocturnal peak of prolactin secretion in rams is difficult to assess.

The effects of stress, including stress of venepuncture, on prolactin release in ruminants have been reported by a number of authors (Johke, 1969; Raud, Kiddy and Odell, 1971; Butler et al., 1972; Hart, 1973). In all cases, stress elevated plasma prolactin

levels. Temporary elevation of plasma prolactin levels was shown by many of the rams studied in Experiments 7.2 and 7.3 and indicated that caution must be exercised when comparing the results of experiments in which blood was collected by venepuncture with those in which indwelling cannulae were used. Also, it must be recognised that differences in plasma prolactin levels between groups of rams studied in this thesis (except Experiment 7.1) may reflect differences in response to the stress of venepuncture, rather than differences in the hormone levels in undisturbed animals. However, stress effects could not have accounted for differences in seasonal or daily patterns of prolactin secretion between the various groups of rams, as such patterns were dependent on relative changes in hormone levels rather than on differences in absolute concentrations. Nevertheless, it is probably better to study prolactin levels in sheep which have been cannulated on the day before sampling (Forbes et al., 1975).

In Experiment 7.2, higher prolactin output of pinealectomized rams under both lighting regimes reflected the general pattern of differences between the groups shown in early March in Experiment 6. In that experiment pinealectomized rams maintained relatively high and constant plasma prolactin levels throughout the whole nine months. On the other hand sham-operated rams under Normal lighting showed diminishing plasma prolactin concentrations and those on the Reversed lighting regime had increasing levels of this hormone. These previous observations account for the present results from sham-operated rams, including the low prolactin levels recorded from those on the Normal lighting treatment.

Results from Experiment 7.3 confirmed those of Pelletier (1973) and those of Experiment 6 which showed that plasma prolactin levels were reduced in rams exposed to shortened daily photoperiods. This conclusion was indicated by the lowered prolactin output recorded

from sham-operated rams on Normal lighting in May. No similar depression of prolactin output occurred in pinealectomized rams on Normal lighting; this result provided further evidence that the pineal gland influences the effects of seasonal changes in photoperiod on prolactin secretion by rams. A longer sampling period would have been required to determine whether the suggestion of an afternoon rise in plasma prolactin levels in pinealectomized rams resembled that recorded from sham-operated rams over a twenty-six hour period (Experiment 7.1). A circadian pattern of prolactin secretion by pinealectomized rams would be difficult to explain, as the evidence from Experiments 6, 7.1 and 7.2 indicated that seasonal, and probably circadian, patterns of prolactin secretion were abolished in pineal-ectomized rams, because the pineal gland diminished endocrine responses to changing light stimuli. A change in susceptibility of pineal-ectomized rams to stress of venepuncture could explain a rise in plasma prolactin levels, but there was no indication of a similar change in Experiment 7.2. Although it is probable that the afternoon prolactin elevation was a random event, a longer-duration study using indwelling cannulae for blood sampling would be necessary to clarify this point.

(4) <u>Cortisol</u>

It is assumed that the pulsatile pattern of cortisol secretion recorded in Experiment 7.1 reflected the pattern of ACTH secretion by the anterior pituitary. The indication of a diurnal pattern of cortisol secretion, with elevated levels during the morning daylight hours, confirmed similar findings in intact (McNatty, Cashmore and Young, 1972) and ovariectomized ewes (Butler <u>et al</u>., 1972). A slightly different diurnal pattern in rams was reported by Holley, Beckman and Evans (1975), who found one peak at 16.00 h and a second smaller peak at approximately 04.00 h. These authors suggested that

the 16.00 h peak may have resulted from the animals being fed at that time each day. Disturbance of the rams during feeding and cleaning operations may have contributed to the morning peak of cortisol levels seen in the present experiment. A similar explanation could not account for the results of McNatty, Cashmore and Young (1972), whose animals were fed ad libitum; however these authors did not state when cleaning operations were performed.

Two of the four pinealectomized rams (Nos. 54 and 63) showed cortisol secretion patterns which indicated that pinealectomy abolished the normal diurnal pattern of secretion. A similar, but also inconclusive trend was recorded from ewes by Thurley, Gibb and Russell (1975). Although it has been reported that the pineal gland may suppress adrenal cortical function in rats (Defronzo and Roth, 1972) and mice (Dickson and Hasty, 1972), there is insufficient evidence available to support any suggestion of a link between pineal gland and adrenal cortex function in sheep. Coghlan et al. (1960) found that pinealectomy did not appear to alter electrolyte balance in two salt-depleted ewes, which indicated that the pineal gland at least did not influence mineralocorticoid secretion by the adrenal cortex.

Further studies, similar to Experiment 7.1, would be required to investigate possible pineal-adrenal cortex links in detail, but such experiments would be of only peripheral interest to the study of reproductive endocrinology.

(5) General Discussion

Hormone secretion profiles studied in the experiments described in this chapter have demonstrated that the anterior pituitary of rams releases hormones in a highly pulsatile manner. It is difficult to say whether this reflects : a dynamic and highly variable pattern of control from the hypothalamus; a constant reverberation between hormone secretion and negative feedback; or the combined effects of

both types of control. The independent fluctuations in plasma levels of LH, prolactin, and cortisol argue strongly for independent mechanisms regulating the release of these hormones, and probably ACTH as well.

More information about the circadian patterns of secretion of these hormones and the possible influence of the pineal gland on such patterns, would undoubtedly have been obtained by performing twenty-six hour sampling studies similar to Experiment 7.1 at monthly intervals. Such a sampling procedure would have amplified the findings of Experiment 6, in which pinealectomized and sham-operated rams on contrasting lighting regimes were housed for a period of nine months. Many of the results which merely indicated possible patterns of hormone secretion, or showed non-significant trends, would have been firmly established by a more intensive study.

In spite of the shortcomings in experimental technique outlined above, results from Experiments 7.2 and 7.3 indicated that seasonal changes in the secretion patterns of LH and prolactin in rams did occur. A similar observation, based on plasma LH data from rams was made by Katongole, Naftolin and Short (1974) who suggested that it was the frequency of discharge, rather than the peak hormone level, that altered during the course of the year. This statement was confirmed recently by Lincoln (1976a) who described an increased frequency of plasma LH peaks recorded in Soay rams during the period of testicular activity.

In experiments designed to investigate seasonal changes in hormone production by measuring hormone levels in single weekly or monthly blood samples, the need to sample at the same time of the day is firmly established by the probable existence of regular circadian secretion patterns, particularly in the case of prolactin and cortisol. The present group of acute studies has also highlighted the fact that studies based on single blood samples each week or month do not provide the complete picture about seasonal effects of hormone production.

CHAPTER VIII

GENERAL DISCUSSION AND CONCLUSIONS

The experiments which have been described in this thesis were designed to establish basic information about the degree of seasonal variations in reproductive parameters, particularly in New Zealand Romney rams. In addition to providing data on seasonality of semen production, it was hoped to obtain comparable data on plasma levels of the hormones which may have influenced the reproductive tract. Finally, as a result of conducting experiments which involved surgical modification of the nervous system, and artificial manipulation of the environment, an attempt was made to elucidate the neuroendocrine and environmental mechanisms modulating the seasonality of reproduction in N.Z. Romney rams.

Experiments 3, 4 and 5 did not produce such definitive results as expected, largely due to small numbers of animals, insufficient frequency of sampling, and the relatively brief duration of the studies. These deficiencies arose largely because of the dearth of previous information on these characteristics for the N.Z. Romney ram, and also because the experiments were time-sequenced in such a manner that it was not possible to utilize data from the earlier two studies when designing Experiment 5. Nevertheless the initial three experiments did provide information which was valuable for the design of Experiments 6, 7.1, 7.2 and 7.3.

In the latter experiments important observations were made on the role of daily photoperiod as the principal stimulus for seasonal fluctuations in reproduction of rams, and indicated that the pineal gland was a vital mediator of these reproductive effects of photic stimuli.

1.   SEASONALITY OF REPRODUCTION IN GRAZING RAMS

Pelletier (1971) pointed out that seasonally rhythmic changes in plasma LH levels of rams were more pronounced when they were subjected to a six-monthly photoperiodic cycle rather than the normal twelve month cycle.   This observation by Pelletier may have accounted for the relatively small number of significant changes in plasma LH levels recorded in Experiments 5 and 6 in which the annual cycle of daily photoperiodic change was simulated.   Furthermore, the photoperiodic stimulation of LH secretion in rams reported by Lincoln (1976a) may have been partly the result of natural seasonal changes, on to which a shortened (32 week) photoperiodic cycle was superimposed.   However in Experiment 3, grazing rams displayed a seasonal pattern of changes in plasma LH levels which reached peak values during midsummer.   Although a summer peak of LH secretion might allow a period for conditioning gonadal tissue prior to the breeding season, this result was not expected since peaks of LH secretion during autumn were reported by Pelletier (1971), whilst other workers had not found any significant seasonal changes in plasma LH concentrations (Sanford, Palmer and Howland, 1974a).   On the other hand, Hochereau-de Reviers, Loir and Pelletier (1976) also have described elevated plasma LH levels in rams during midsummer months.   Several points mentioned in Chapter VII, about the need for an increased plasma sampling frequency, may partially have accounted for differences in results between the present and previous studies, particularly the lack of significant seasonal effects in the work of Sanford, Palmer and Howland (1974a).   The low LH values obtained throughout the present experiments raised some doubts concerning the accuracy of absolute hormone concentrations obtained with the present LH assay.   However, the appropriate assay validation tests were adequate to dispel such doubts, and furthermore, there was

no reason to suspect inaccuracies in the patterns of changing LH
secretion obtained when using this assay.

In contrast to the LH results, plasma testosterone and prolactin
levels followed clearly defined annual cycles in rams of all three
breeds. Regardless of any spermatogenic effects of testosterone, the
annual cycle of changes in secretion of this hormone undoubtedly is of
major importance to the seasonality of reproduction in rams through its
influence on libido. Difficulties in obtaining semen when using an
artificial vagina during the winter months were encountered in
Experiments 3 and 4, as well as by Smyth and Gordon (1967). It was
assumed that this observation represented a seasonal depression of
libido related to low plasma testosterone concentrations. Possible
implications of the seasonal pattern of prolactin secretion are
discussed later (Section 3).

In Experiment 3, N.Z. Romney rams displayed marked seasonal
fluctuations in seminal fructose levels as well as in plasma levels of
LH, testosterone and prolactin. Polled Dorset and Merino rams also
displayed annual changes in these parameters, which were similar to
those recorded from N.Z. Romneys, except that the seminal fructose
levels of Polled Dorset rams and plasma LH levels of Merino rams, did
not follow such clearly-defined patterns. Autumnal peaks of seminal
fructose production in all three breeds of rams confirmed similar
patterns reported from other breeds (Hiroe et al., 1960; Amir and
Volcani, 1965).

Distinct seasonal patterns of plasma hormone and seminal
fructose levels in Merino and Polled Dorset rams did not reflect the
less well-defined breeding seasons of ewes of these breeds reported by
Hafez (1952). Together, these results may have indicated considerable
sex differences in reproductive seasonality, or the fact that seasonal
changes in plasma hormone levels do not necessarily have to coincide

with changes in other reproductive characteristics.

Although fructose levels in semen showed annual rhythms in this study, other semen parameters tended to be more variable and did not display autumnal peaks. This difference from results of previous reports in the literature (see review by Lodge and Salisbury, 1970) may have resulted from the relatively mild seasonal changes in climatic conditions in New Zealand. The enforced change in semen collection technique during Experiments 3 and 4 also may have masked seasonal changes in some seminal parameters. A more likely explanation was that at Palmerston North (latitude $40^{\circ}21'$ S) the annual changes in daily photoperiod were less marked than at the higher latitudes where much of the other reported research has taken place.

Many seminal parameters studied in this experiment are not highly correlated with fertility (Wiggins, Terrill and Emik, 1953; Hulet and Ercanbrack, 1962; Hulet, Foote and Blackwell, 1964), so it was not possible to comment on seasonal changes in relative fertility of the semen. In view of this shortcoming, investigation of semen with respect to fertility may have been a worthwhile exercise, if not fraught with enormous practical difficulties. Stress tests such as viability after incubation at body temperature (Ludwick, Olds and Carpenter, 1948; Buckner, Willett and Bayley, 1954), or after freezing (Colas et al., 1972), provide more accurate indirect methods for assessing the fertilizing capacity of semen, and may have been useful additions to the present semen studies.

Surgical modification of olfactory and pineal gland functions in Experiment 4 did not alter the seasonal changes in plasma hormone levels or semen production of rams to any great extent. These treatments did, however, provide some insight into which neural structures might have mediated the normal responses to environmental changes. Removal of the olfactory bulbs altered the regular seasonal cycles of changes in

plasma LH and testosterone concentrations, so it was possible that olfactory stimuli provided some regulatory influence over hormonal secretion patterns. Olfactory cues could have been derived from pheromones produced by ewes, or other rams, during the breeding season. Evidence that pheromones produced by rams stimulated oestrous activity in ewes at the onset of the breeding season has been presented by Schinckel (1954) and Morgan, Arnold and Lindsay (1972). It has been suggested that in some male ungulates such olfactory functions involve the vomeronasal organ (Estes, 1972). In the present studies the function of this organ was not studied directly, but olfactory bulbectomy may have modified vomeronasal function (Alberts, 1974; Powers and Winans, 1975).

Significant effects of cranial cervical ganglionectomy on pineal cell volumes and HIOMT activity, indicated that this surgical procedure modified pineal gland activity. Consequently the disruption of regular seasonal changes in LH and testosterone secretion patterns in ganglionectomized rams in Experiment 4 was attributed to altered pineal function. A major difficulty arose from the finding that seminal fructose levels continued to display regular seasonal changes in all surgically treated groups of rams in Experiment 4 (Figure 4.2). Since seminal fructose levels were expected to reflect changes in plasma testosterone concentration, regular patterns of seminal fructose output from these rams were difficult to explain. However, inspection of the annual fluctuations in seminal fructose, plasma testosterone and plasma LH levels recorded in Experiment 4 (Figures 4.2, 4.3 and 4.4) revealed that normal seasonal rhythms were disrupted most of all in the case of LH, to a lesser extent for testosterone, and least for seminal fructose. A tentative hypothesis could have been that the pineal gland and/or olfactory system exerted direct influences on LH release, so that parameters

further removed from these direct influences were more likely to retain residual seasonal rhythms. Alternatively, it must be concluded that accessory sex gland function can be regulated by factors for which seasonality of activity was not abolished by either surgical treatment. The recent development of a radioimmunoassay for plasma melatonin levels in sheep (Rollag and Niswender, 1976) will allow investigation of the effects of ganglionectomy and olfactory bulbectomy on any seasonal pattern of secretion of this compound.

With respect to plasma prolactin data and much of the autopsy data, it was noted that the untreated and olfactory bulbectomized groups were similar to each other; also there were few major differences between the ganglionectomized and bulbectomized/ganglion-ectomized groups. As a consequence, it was concluded that the changes shown by the double-operated groups resulted principally from the ganglionectomy operation, and there was no synergism between olfactory and pineal influences on these parameters. Likewise, there was no reason to suspect that either surgical treatment reversed or nullified any effects of the other, a possibility that was suggested by work on blinded anosmic rats (Blask and Reiter, 1975).

Moule, Braden and Mattner (1966) ascribed seasonal changes in seminal fructose concentrations recorded from rams on pasture solely to changes in the quality and quantity of the forage available. However, that study was conducted in Australia where severe nutritional and temperature stresses can be expected; also seasonal changes in daily photoperiod would have been smaller because of the lower latitude at which their experiment was performed. In Experiments 3 and 4 rams were never underfed, while adverse effects of temperature on their reproductive systems could be ruled out because extreme temperatures did not occur (Figure 3.7). Nevertheless temperature may be an important environmental factor in other regions of the world (VanDemark

and Free, 1970).

A major hypothesis in this thesis was that the most important environmental factor contributing to the seasonal pattern of reproduction in rams was daily photoperiod. It has been shown in Experiment 4 that the pineal gland might have been involved in mediating the influence of changing daily photoperiod on reproduction in rams; this influence presumably was caused by optic stimuli which reached the pineal gland by way of the cranial cervical ganglia. Since the pineal gland of ganglionectomized rams could not perceive photoperiodic changes via this neural pathway, ganglionectomy should have produced results equivalent to pinealectomy. Furthermore blinding would be expected to generate results similar to those recorded following ganglionectomy, since blinding would abolish optic activity. As these assumptions have not been explored, future field experiments with blinded and/or hooded rams, as well as pinealectomized rams, would appear to be justified.

2. EFFECTS OF LIGHTING REGIMES ON REPRODUCTION IN RAMS

In their review of the photoperiodic control of gonadal and hypophyseal activity in domestic animals, Ortavant, Mauleon and Thibault (1964) deduced a set of four hypotheses :

1. An optimal photoperiod for gonadal stimulation existed, this being ten to eleven hours per day for most rams.

2. Continued exposure to the optimal photoperiod did not sustain gonadal activity indefinitely, which suggested the existence of a "refractory period" which varied according to the species or breed, and to the preceding photoperiodic treatment.

3. Light facilitated the release of gonadotrophins whereas darkness favoured their synthesis.

4. Sensitivity to photoperiodic stimulation varied between

breeds and between individual animals, as well as between species.

These deductions of Ortavant, Mauleon and Thibault (1964) were based on the literature available at that time as well as on their own studies with rams and ewes. Their studies had included observations of : spermatogenic activity in testicular tissue, testicular and epididymal weights, and pituitary gonadotrophin measurements.

Further to the above hypotheses is the question of whether seasonal patterns of reproductive activity are controlled by decreasing or increasing daily photoperiods. Yeates (1949) concluded that the autumnal peak of breeding activity in sheep was induced by the period of increasing daily photoperiods during the previous spring. In contrast, more recent evidence has shown that increased spermatogenic activity in rams was a direct response to decreasing daily photoperiods (Ortavant and Thibault, 1956; Ortavant, 1961).

The findings of Pelletier and Ortavant (1975a) showed that decreasing daily photoperiods were stimulatory to LH release in rams, whereas increasing photoperiods were inhibitory or less stimulatory; this result corresponded with that of a previous study by Pelletier (1971). Likewise, an abrupt decrease in daily photoperiod produced high plasma levels in Soay rams (Lincoln, 1976a). There was not always such a close relationship between decreasing daily photoperiods and plasma LH levels in the experiments described in this thesis. For instance, in rams on pasture (Experiment 3), maximal plasma LH levels were recorded during midsummer rather than in the autumn period, yet for rams on artificial lighting regimes (Experiments 5 and 6) plasma LH data did not provide conclusive evidence of any relationship with photoperiod. Part of the difficulty in reconciling the present results with those of other workers may have arisen from differences in breeds, latitudes, LH assays, or the photoperiodic

cycles to which the animals were subjected.

Peak levels of testosterone secretion were associated with the late summer-early autumn months in rams on pasture (Experiment 3), and with decreasing and short daily photoperiods in Experiments 5 and 6. These results for plasma testosterone levels gave substance to conclusions reached by Pelletier (1971) and Pelletier and Ortavant (1975a), which were based on plasma LH levels of rams subjected to six-monthly photoperiodic cycles. In addition, the effects of lighting regimes on testes and accessory sex glands recorded in Experiment 6 confirmed the findings of Ortavant and Thibault (1956). It is interesting to speculate about whether or not the French workers would have reached the same conclusions had they used twelve-monthly instead of six-monthly photoperiodic cycles. This group of authors apparently has never published plasma testosterone data from rams submitted to artificial lighting regimes, although Pelletier and Ortavant (1975b) have described the effects of testosterone propionate injections on pituitary and plasma LH levels in such rams. Their results showed that the negative feedback of testosterone on LH release was less effective during short daily photoperiods (8 h light) than during long daily photoperiods (16 h light). Also they pointed out that alteration in sensitivity to androgen feedback might be more important in natural (12-monthly) lighting cycles, since in the latter case light may be a less effective modifier of LH release because of its reduced rate of change compared to that in a six-monthly cycle.

A model for the control of gonadotrophin secretion by light and gonadal steroid negative feedback was proposed by Hoffmann (1973). The major component of this model was a hypothetical comparator which controlled releasing hormone secretion in response to changes in gonadal steroid feedback, plus stimuli from another source which in turn depended on photic information. Two possibilities for the

control of gonadotrophin release arose from the model proposed by Hoffmann. One such possibility required releasing hormone output to alter directly under the influence of light-derived stimuli and steriod levels. The other possible mechanisms involved an alteration in hypothalamic sensitivity to steroid feedback in response to changes in photic stimuli.

Although Pelletier and Ortavant (1968) found that hypothalamic LH-releasing activity was higher in rams on sixteen hours light per day than in those on eight hours per day, this result did not distinguish between impaired secretion of the releasing hormone (causing a build up in hypothalamic levels) or its increased synthesis and release, under long daily photoperiods. However alterations in releasing hormone secretion are consistent with the mechanisms proposed by Hoffmann.

Another hypothetical model to account for the influence of photoperiod on the control of gonadotrophin secretion in rams was postulated by Pelletier and Ortavant (1975b). This particular model embodied the proposals of Hoffmann and the authors made the following statement about the influence of the photoperiod :

"decreasing light photoperiod has two effects :

1. stimulation of gonadotrophin release

2. lowering of the intensity of the negative feedback of testicular androgens.
Conversely, increasing light photoperiod

1. is inhibitory or at least less stimulatory to the LH release,

2. increases the negative feedback effect of androgens on hypothalamo-hypophyseal activity."

These proposals of Pelletier and Ortavant are compatible with the results obtained in the present project. In fact the work

described in this thesis extends their hypotheses by providing positive evidence that the pineal gland is involved in the photoperiodic regulation of gonadotrophin secretion (see later for discussion).

In Experiments 5 and 6 the parameters affected most by photoperiod reversal were : plasma testosterone and prolactin levels, seminal fructose levels, testicular weights, epididymal weights, seminal vesicular weights, epididymal spermatozoal reserves, and the concentration and total content of fructose in the seminal vesicles. Significant effects of lighting regimes on the parameters listed above indicated that gonadal activity was regulated by daily photoperiod; no significant influences of lighting regimes were recorded for plasma LH levels, other semen characteristics, or the remaining autopsy data, largely because of the high degree of variability in these parameters, allied with the limited numbers of rams per group.

Although Experiments 5 and 6 did not clarify whether the effects of photoperiod on gonadal activity were mediated by changes in LH secretion patterns, French workers have recorded LH secretion patterns which would support such a conclusion (Pelletier, 1971; Pelletier and Ortavant, 1975a). It is now clear that since LH is secreted in a highly pulsatile manner, groups of experimental animals must be larger if significant differences in the patterns of secretion of this hormone are to be detected. Nevertheless the possibility remains that gonadotrophins other than LH may have been involved in photoperiodic effects on gonadal activity.

Results of research, mainly carried out with rats, has shown that FSH may not have any significant function in the control of spermatogenesis in adult animals (Ortavant, Courot and de Reviers, 1969; Courot, Ortavant and de Reviers, 1971). However, as it is not known to what extent this statement about FSH can be applied to rams,

the possibility that this hormone could be involved in responses to altered daily photoperiod can not be eliminated. A radioimmunoassay for ovine FSH was not available during the course of the present studies and as yet workers at other laboratories have not published information on the FSH secretion patterns of rams exposed to different lighting regimes. (In fact Lincoln (1976b) recently presented a paper which indicated that both plasma levels and frequency of episodic release of FSH in Soay rams were stimulated by decreasing daily photoperiods, and suppressed by reversed lighting changes.) In addition prolactin may function as a gonadotrophin in rams, and the present results have shown that plasma concentrations of this hormone in rams were very responsive to daily photoperiod.

Programmes involving active immunization of rams against specific protein hormones such as LH, FSH, or prolactin, or the use of long-acting specific antagonists to the secretion of these hormones, should provide useful data relating to their respective roles in seasonal reproductive changes. Long-term field studies with rams subjected to such treatments would thus be useful adjuncts to the experiments in this thesis.

3. REPRODUCTIVE FUNCTIONS OF PROLACTIN IN RAMS

Seasonal changes in plasma levels of LH and prolactin recorded in Experiment 3 indicated that both hormones were secreted maximally during midsummer, which suggested that their release was controlled on a seasonal basis by the same environmental factor, namely daily photo-period. French studies with rams subjected to artificial lighting regimes showed that although secretion of both prolactin and LH in rams was influenced by photoperiod, plasma prolactin levels were highest in rams subjected to long daily photoperiods (Pelletier, 1973) whilst LH levels were highest in rams subjected to short or decreasing daily

photoperiods (Pelletier, 1971; Pelletier and Ortavant, 1975a).
This apparent relationship between the timing of the maximal rates of
secretion of prolactin and LH was supported in Experiments 5 and 6 by
the timing of plasma prolactin and testosterone peaks. However,
plasma LH results in these two experiments were inconclusive, while
results from Experiment 3 indicated that maximal rates of LH secretion
occurred approximately one month before the highest peak of
testosterone secretion. In Experiment 6, sham-operated rams
subjected to the Normal lighting regime had the highest values for
parameters associated with gonadal and accessory sex gland activity at
autopsy even though these rams had the lowest plasma prolactin levels.

None of the above findings excluded the possibility that
prolactin may have been involved in reproductive seasonality in rams as
a "conditioning" hormone. Evidence from comparative studies of the
physiology of prolactin has shown that this hormone is involved in a
multitude of endocrine systems and also has a function in various
endocrine tissues "conditioning" their responsiveness to their specific
trophic hormones (Nicoll, 1973). The elevated plasma prolactin levels
recorded during exposure of rams to long daily photoperiods in the
experiments in this thesis may have conditioned their reproductive
organs to the influence of LH, so that peaks of reproductive organ
activity could follow during the period of decreasing daily photo-
periods. In other words, prior exposure of reproductive tissues to
high levels of prolactin may have been a pre-requisite for optimal
responses to gonadotrophic stimulation, even though the exposure to
raised plasma prolactin levels may have preceded plasma testosterone
and seminal fructose peaks by approximately two and three months,
respectively.

Since the period of elevated plasma prolactin levels recorded
from rams on pasture (Experiment 3) overlapped that of plasma LH,

the possibility that these two hormones act synergistically can not be overlooked. Evidence for such synergism between prolactin and LH, in male rats has been summarized in Chapter I.

4. PINEAL GLAND FUNCTION AND REPRODUCTION IN RAMS

Semen from grazing cranial cervical ganglionectomized rams studied in Experiment 4 exhibited much greater fluctuations in spermatozoal numbers, motility indices and percentages of unstained spermotozoa than that from control rams. Also, in the ganglionectomized rams, the normal seasonal patterns of change in plasma LH and prolactin levels were disrupted. At autopsy, testicular and epididymal weights of ganglionectomized rams were elevated, while seminal fructose levels were below those of the control rams. As discussed earlier, these effects of ganglionectomy probably could be attributed to altered pineal gland function.

Likewise, much of the plasma LH, testosterone and prolactin data from Experiments 6, 7.1, 7.2 and 7.3, revealed significant effects of pinealectomy, and significant Lighting Regimes x Operations interactions. These significant findings provided direct evidence that the pineal gland did influence reproductive function in rams, and also that this gland was an important regulator of prolactin secretion.

Because cranial cervical ganglionectomized rams were not studied under identical conditions to those used in the later experiments involving pinealectomized rams, definitive statements about the effectiveness of ganglionectomy in paralleling the effects of pinealectomy can not be made, even though cranial cervical ganglionectomy was effective in depressing pineal enzyme activity and cell volumes.

Support for the conclusion that the pineal gland is required for the expression of seasonal patterns of reproductive activity could undoubtedly be provided by studying grazing pinealectomized rams over

a period of one or more years. If such an experiment also included ganglionectomized rams, some of the questions raised by the present work should be answered. Fighting amongst rams which had undergone surgical removal of the pineal gland would have to be avoided by methods such as individual paddock grazing or tethering.

Most reports of the effects of the pineal gland on reproductive function in rats, golden hamsters, and ferrets have favoured the concept that this gland is antigonadotrophic (Reiter, 1973b). Consequently the onset of breeding activity in these animals must result from inhibition of pineal gland activity, which supposedly occurs during increased light exposure. However in an experiment utilizing long versus short daily photoperiods, and pinealectomized versus sham-operated female hamsters, significant Lighting Regimes x Operations interactions indicated that the pineal gland also could be progonadotrophic (Hoffmann and Reiter, 1966). A progonadotrophic function of the pineal gland of rams was suggested by data from Experiment 6, since in rams subjected to the normal lighting regime, parameters related to gonadal and accessory sex gland activity were higher in sham-operated than pinealectomized rams.

Research on pineal function in sheep has been very limited, but evidence from other species has indicated the possible existence of several mechanisms of action. As the pineal gland contains high levels of melatonin and serotonin, these and other closely related indoleamines are considered to be the most likely mediators of pineal antigonadotrophic actions (Fraschini, Collu and Martini, 1971). Serotonin suppressed the spontaneous LH release in castrated rams (Riggs and Malven, 1974). In ewes melatonin prevented the post-castration rise in plasma LH levels (Roche et al., 1970b), while both melatonin and serotonin blocked the pre-ovulatory peak of LH secretion and prolonged oestrous cycles (Domański et al., 1975). In

their report, Domański et al. described inhibition of LH release in
ewes with lesions of the anterior hypothalamic area (AHA), which
indicated that the inhibitory actions of melatonin and serotonin
occurred in the region of the medial basal hypothalamus. This
conclusion was based on the fact that these lesions blocked the normal
inhibitory influences of the AHA over GnRH secretion, so the effects
of the indoleamines must have occurred nearer to the site of GnRH
secretion.

Antigonadotrophic peptides from sheep pineal glands have been
studied using in vitro systems with pituitary tissue from rodents
(Moszkowska et al., 1974; Ebels, 1975); as yet comparable research
with ovine pituitary tissue has not been reported. Considering the
high antigonadotrophic potency of some of these pineal peptides, they
must be regarded as potential regulators of reproductive seasonality
in rams, and so merit further study. Such research could include
immunologic investigations, using antisera raised against these
peptides conjugated to proteins.

Since no potentially progonadotrophic substances had previously
been isolated from the pineal gland, the finding of high levels of
immunoassayable GnRH in sheep pineal glands (White et al, 1974) has
provided at least one possible explanation for the progonadotrophic
property of this organ.

Another possible mechanism of action of pineal principles
could involve direct effects on the gonads themselves. For example,
in rat testes Liu and Kinson (1973) described inhibitory effects of
melatonin on the production of testosterone, and of serotonin on
spermatogensis. These findings indicated the need for investigation
of pineal-gonadal interrelationships in rams. Evidence of gonadal
steroid regulation of pineal function has been presented in Chapter I.
This evidence was confined to studies with rodents because of the

paucity of similar studies in large domestic animals.

Results from Experiment 6 showed that the pineal gland altered the endocrine status of rams, probably entirely due to its own responses to changes in daily photoperiod. This finding was not surprising since the pineal gland has been described as a neuro-endocrine transducer, instrumental in producing endocrine responses to photic stimuli (Axelrod, 1974).

Possible interactions between photoperiod, the pineal gland, and olfactory function can not, however, be overlooked considering the disrupted seasonal patterns of hormone secretion recorded from olfactory bulbectomized and cranial cervical ganglionectomized rams, and also those subjected to both surgical treatments (Experiment 4). Interactions between lighting and olfaction in male rats were suggested by the fact that both blinding and olfactory bulbectomy were required to produce regression of their reproductive organs (Reiter et al., 1971).

In grazing rams although elevation of plasma LH levels preceded the rise in plasma testosterone levels, LH output remained high during the period of peak plasma testosterone secretion (Figure 3.7). As testosterone inhibited the release of LH by a negative feedback effect (Pelletier, 1970), the occurrence of high levels of both hormones at the same time of the year requires discussion. Pelletier and Ortavant (1975b) considered that decreasing daily photoperiods lowered the sensitivity of the hypothalamo-hypophyseal system to the negative feedback of androgens, so this change in sensitivity could account for the continued release of LH when plasma androgen levels were high. It is possible that the change in sensitivity to androgen feedback resulted from the production of pineal substances which altered hypothalamic function. The fact that the activity of the pineal gland could be modified directly by changes in daily

photoperiod supported this hypothesis. Acute studies need to be carried out to study the effect of testosterone injections on LH secretion in rams in the presence or absence of the pineal gland or some of its principles. Provided that the testosterone dose used produced physiological levels of this hormone, such studies could confirm or refute this suggested role of the pineal gland, and thus help establish the control mechanism for the seasonality of reproduction in rams.

The existence of complex physiological interrelationships between neural structures and endocrine glands confirmed the concept that the hypothalamus receives a wide variety of inputs and integrates these for the final expression of its regulation of anterior pituitary function. The individual role of any input to the hypothalamus is difficult to discern because mammalian endocrine systems show a remarkable degree of compensation. This point was illustrated by the fact that in the present studies even though some of the surgical treatments had marked effects on the patterns of hormone secretion, there was no evidence of a clear impairment of reproductive function. Thus further experiments will be required to elaborate the exact importance of the pineal gland and the olfactory system in the regulation of reproduction in rams.

5. POSSIBLE APPLICATIONS OF THE PRESENT FINDINGS

Knowledge of the photoperiodicity of reproduction in ewes has been used to increase reproductive rates by allowing a greater frequency of lambings (Williams, 1970; Ducker and Bowman, 1972; Newton and Betts, 1972). However the application of such knowledge must not be confined to ewes since the problems associated with deep-freezing ram semen have meant that high quality semen may not be

available throughout the year. Exposure to artificially decreased daylight improved libido in rams in Queensland, Australia, and thus assisted out of season semen collection for artificial insemination programmes (S.J. Miller, personal communication). However, the general applicability of photoperiodicity in sheep is severely limited because of the impracticability of manipulating daily photoperiod for large numbers of sheep.

Surgical removal of pineal glands from rams might alter seasonal breeding patterns and produce a relatively constant high level of reproductive activity throughout the year. Technical difficulties involved in this procedure would prohibit its general use, although cranial cervical ganglionectomy or chemical sympathectomy (for example, using 6-hydroxy dopamine), might provide alternative means of altering pineal function. On the other hand sustained production of high quality semen may not be possible, since Ortavant (1961) has shown that maximal spermatogenesis is maintained only for a limited period, and it is likely that the unknown limiting factors involved would operate in the same manner in pinealectomized rams.

More likely, expanded knowledge of neuroendocrine control of reproduction in sheep will assist the development of pharmacological techniques which have practical agricultural benefits. For example, the use of steroids, prostaglandins and gonadotrophins in such techniques as : induction of parturition or lactation, regression of corpora lutea, contraception, ovarian stimulation and superovulation, and treatment of infertility and impotence, has followed fundamental scientific studies on the endocrine control of reproduction. In the present case, the demonstration that the pineal gland of rams can exert a controlling influence over reproductive function, paves the way for further investigations to determine the chemical nature of the substances involved. Such research could lead to the discovery

of pharmacological agents which improve present day techniques of manipulating ovine reproduction.

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