STUDIES OF TESTICULAR AND REPRODUCTIVE ENDOCRINE PARAMETERS IN THE RAM WITH PARTICULAR REFERENCE TO SEXUAL MATURATION

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by PETER RAYMOND WILSON

Experiments in this thesis were designed to establish short-term and longitudinal patterns of secretion of LH, testosterone and prolactin, responses to GnRH administration, testicular and reproductive endocrine changes associated with sexual maturation in the ram, and to study endocrine factors which may influence reproductive development.

24 hour hormonal secretion profile studies employing 20 min sample collection intervals in 9 adult rams, and 8 h secretion profile studies in eight prepubertal, pubertal and early post-pubertal rams confirmed that LH was secreted in a pulsatile manner. Testosterone was secreted quantitatively following each episodic LH elevation in pubertal and older rams, but a consistent qualitative and quantitative secretory response was not observed in prepubertal rams. Plasma prolactin levels were stable and high during the summer, but low basal levels interspersed with pulsatile fluctuations occurred in winter sampling periods. Prolactin secretion profiles of prepubertal and sexually maturing rams were similar, and levels fluctuated markedly at each stage of development.

No circadian rhythms of LH, testosterone or prolactin secretion were evident in adult or sexually developing rams, but data was produced which supported the existence of a sampling-induced elevation of LH and prolactin in young rams: higher levels of both hormones were observed in early samples of sequential sampling studies employing venepuncture techniques.

Study of hormone levels of ram lambs bled each two weeks from birth to approximately 8 months of age showed that plasma LH content was low at birth, rose to a peak at approximately 6 weeks of age, and

declined during the period of major testicular development (10 - 18 weeks); plasma testosterone concentrations were low at birth and increased steadily throughout the period of study; while prolactin levels were low at birth, increased rapidly to reach a plateau from approximately 6 to 20 weeks, then gradually decreased to low levels by 32 weeks.

Testicular and epididymal development, seminiferous and epididymal tubule growth, and the onset of spermatogenesis during puberty followed patterns similar to those reported by previous authors. Regression analyses failed to distinguish major correlations between these developmental parameters and endocrine changes associated with sexual maturation.

Responses to intravenous GnRH injection and infusion to adult rams showed that LH output occurred in a dose-dependent manner but subsequent testosterone elevations were not significantly different. The LH responses of prepubertal rams to intravenous injections of GnRH (1 µg/Kg) were high, but progressively decreased at consecutive four-weekly injection and sampling routines from 6 to 32 weeks of age. Peak LH responses were progressively delayed during this period. Testosterone output following GnRH-induced LH elevations were low in prepubertal rams and increased progressively during sexual maturation. Maximal responses to repeated GnRH injections were observed in pubertal (14 week old) rams.

Weekly treatment of ram lambs with GnRH failed to alter any of the testicular or endocrine parameters assessed during sexual development whereas weekly androgen treatment depressed mean testis and epididymal weights, seminiferous and epididymal tubular diameters and epididymal spermatozoal reserves, as well as basal and GnRH-stimulated testosterone output.

Prenatal androgenization of female lambs resulted in masculinization of external genitalia. Postnatal basal levels of LH were depressed in both males and females from androgenized ewes, while LH responses to GnRH were unaltered. It was concluded that prenatal androgenization depressed hypothalamic hypophysiotrophic function.

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CHAPTER I

INTRODUCTION

The survival of a species requires that its individual members reproduce. Simplest life-forms reproduce by asexual means: fission, budding or fragmentation. Intermediate forms, some of which are hermaphrodites, undergo alternate sexual and asexual processes, parthenogenesis or external fertilization. In contrast, phylogenetically more advanced animals have developed accessory structures which allow not only the transfer of spermatozoa from male to female, but internal fertilization, implantation and embryo and foetal development. This form of procreation while obviating the need for production of large numbers of gametes, highlights the need for a precisely controlled system for both male and female gamete production, as well as for embryo and foetal management.

Accompanying the physiological evolution of reproductive processes has been a behavioural evolution necessary for environmental and seasonal adaptation, mating and also for later care of the young. This physiological and behavioural evolution has resulted in the establishment of highly complex neuroendocrine mechanisms for the regulation of reproductive functions.

Recent neurological, biochemical, microanatomical and endocrine investigations have provided more detailed understanding of neuroendocrine functions which have been adapted for practical purposes, not the least of which include hormonal contraception and oestrus cycle manipulation. Advances are also being made toward elucidation of neuroendocrine control concepts in male reproduction. An understanding of the physiology and control of reproduction is an essential prerequisite for improvement of reproductive efficiency, a factor of vital concern to agricultural science.

This review of literature is concerned primarily with current concepts of reproductive physiology and endocrinology in the male, with particular emphasis placed on the roles of gonadotrophin releasing hormone (GnRH), luteinizing hormone (LH), testosterone and prolactin as they affect puberty in the animal studied in this thesis, the ram. Aspects of hypothalamic involvement will be discussed, followed by an outline of pituitary gland function. Testicular hormones will then be

considered briefly and aspects of sexual maturation discussed.

1. NEUROENDOCRINE CONTROL OF REPRODUCTION.

Removal of the gonads has long been known to prevent the onset of puberty and render the adult sterile (Schally et al., 1972). Precocious puberty and other reproductive abnormalities often were noted to be associated with hypothalamic lesions, while yet other disturbances of reproduction were attributable to pituitary gland lesions (Harris, 1948). Moore and Price (1932) postulated "a new concept of hormone interactions" involving the pituitary gland-gonad complex, after experimentation with the newly available sex hormones "testis hormone" and "oestrin". This concept involved hypophyseal control of the gonads and reciprocal gonadal control of the hypophysis, and laid the foundation for future understanding and research into reproductive neuroendocrinology. This concept however, failed to recognise hypothalamic involvement.

Green and Harris (1947) demonstrated the hypothalamo-pituitary portal vascular link between the median eminence and pars distalis and correctly identified the direction of blood flow as being towards the pituitary. Harris (1948) summarised direct and indirect evidence supporting the concept of neuroendocrine control of the adenohypophysis: from anatomical considerations he postulated that ultimate control of this gland resided with hypothalamic neurons which released compounds into portal vessels and these compounds dictated the secretory activity of the adenohypophyseal cells.

LH releasing activity from crude hypothalamic extracts was first demonstrated by McCann et al. (1960), and a hypothalamic substance tentatively designated "LH-Releasing Factor" was proposed. Later, from the same group (Igarashi and McCann, 1964) came evidence of follicle stimulating hormone (FSH) releasing activity of hypothalamic origin, and thus the endocrine basis for regulation of LH and FSH became established.

(1) The Hypothalamus.

Although it is now generally accepted that the hypothalamus has an important role in regulation of reproduction, definition of its precise function was made difficult because of the multitude of direct and indirect neural afferent inputs from extrahypothalamic areas such as the amygdala, hippocampus, septum, anterior thalamus, pyriform cortex,

visual and auditory centres, and other influences, both chemical and endocrine (Donovan, 1970). There are many inherent difficulties involving methods of study of hypothalamic function, for example, lesion and isolation studies remove not only the local function but may interfere with nearby centres or with nerve tracts passing to other areas. Since the majority of experimentation has been carried out on the rat, one can only make predictions or assumptions as to hypothalamic involvement in regulating reproductive functions in the sheep.

(i) Anatomy of the hypothalamus. The anatomy of the hypothalamus of the sheep has recently been reviewed (Tarttelin, 1969; Daniel and Prichard, 1975). The hypothalamic area influencing anterior pituitary function, the "hypophysiotrophic area", consists of a number of physiological nuclei and tracts. The preoptic area (POA), anterior hypothalamic area (AHA) and suprachiasmic nucleus (SCN) are in an anterior situation and have been shown by lesion studies (Barraclough, 1973) and by anatomical isolation studies (Halasz and Gorski, 1967) in the rat to be specifically involved in regulating ovulation of females. However, similar studies performed on the monkey (Knobil, 1974) have shown that ovulatory cycles continue despite surgical isolation of anterior hypothalamic areas. Such observations highlight probable species differences in control mechanisms, and hence difficulties associated with making inter-species comparisons in lieu of experimental observations. The medial basal hypothalamus (MBH), consisting of the arcuate nucleus and ventromedial hypothalamus (VMH), regulates "tonic" release of gonadotrophins (Halasz and Gorski, 1967; Donovan, 1970), and is the area considered to contain the steroid negative feedback receptor sites (Smith and Davidson, 1974). When surgically isolated, the MBH is capable of maintaining normal male pituitary and gonadal structure and function whereas its removal abolishes normal sexual function (Davidson, 1966; Halasz, 1969). The median eminence (ME), immediately dorsal to the pituitary gland is considered to contain the final common pathway for the control of the adenohypophysis.

(2) Extrahypothalamic Influences.

Extrahypothalamic influences are considered to act as "bias-setters" or "homeostat" control centres for integrated hypothalamic function and are the subject of extensive reviews (Clegg and Doyle, 1966; Wurtman, 1966; Donovan 1971). Major afferent and efferent impulses pass via the

median forebrain bundle which is a neuronal bundle connecting the olfactory apparatus, septum, amygdala, hippocampus and caudate nucleus with the hypothalamus (de Groot, 1966).

(i) Amygdala. The stria terminalis, part of the forebrain bundle, serves as a primary link between the amygdaloid nuclei and the POA and AHA. In turn, the amygdala provides a functional link from the olfactory system, hippocampus, basal ganglia and septum to the hypothalamus (de Groot, 1966), as well as being a relay point for peripheral sensory input (Kawakami et al., 1969). The amygdala is concerned with emotions and can exert influences upon emotional and sexual behaviour of hypothalamic origin (Donovan, 1970). Amygdaloid lesions have been associated with male hypersexuality (Donovan, 1970), seminiferous tubule degeneration and aspermia.

In female rats, electrical stimulation of the amygdala results in LH and FSH output from the pituitary (Velasco and Taleisnick, 1969a; Barraclough, 1973), but such responses have not been recorded in the male (Donovan, 1971). Serum and pituitary prolactin levels decreased after amydaloid stimulation in females, but hippocampal stimulation had a converse effect (Kawakami et al., 1973). Donovan (1971) also reported a pre- and post-pubertal difference in LH and FSH responses to amygdaloid stimulation.

(ii) Hippocampus. The hippocampus appears to exert an inhibitory influence on the amygdala or directly on the hypothalamus, and generally has functions inverse of those of the amygdala (Velasco and Taliesnick, 1969b; Donovan, 1971). In the male rat, hippocampal stimulation has been shown to elicit erection, licking of genitalia and grooming behaviour (Donovan, 1971).

Though both the amygdala and hippocampus possess important modulating abilities, it is difficult to assess their importance in view of the fact that total surgical isolation does not interfere with oestrous cycling and ovulation in the female rat (Donovan, 1971). This result highlights the fact that the hypothalamus retains a high degree of physiological autonomy.

(iii) Olfactory System. Olfactory bulbectomy of lower species has been shown to abolish male sexual behaviour (Clegg and Doyle, 1966). Mating by anosmic rams was considered to be normal (Lindsay, 1965) though precopulatory behaviour was markedly altered. Olfactory

ablation also impaired the ram's ability to detect oestrus ewes (Lindsay, 1965; Clegg and Doyle, 1966). Barrell (Pers.Comm.) observed that mating by rams continued despite olfactory bulbectomy, while the regular seasonal variation in LH levels (but not of prolactin levels) of similarly treated animals was disrupted (Barrell, 1976). Ablation of the olfactory bulbs in ewes significantly reduced numbers mating out of season but not during the normal mating season (Morgan et al., 1972); these results suggested that the ram influenced the onset of oestrus in ewes through the olfactory receptors. Though olfactory influences in sheep have not been fully investigated, they do appear to influence sexual behavioural responses.

(iv) Visual Stimuli and Light. Blind girls attained puberty earlier than normal girls (Donovan, 1970) but blinded rats failed to show oestrus. No direct retino-hypothalamic tracts have been demonstrated and the full significance of visual stimuli remains to be assessed (Donovan, 1970).

The pineal gland appears to be important in modulating the light-related seasonal fluctuation of LH, testosterone and prolactin output in rams (Barrell, 1976).

(v) Peripheral Afferent Input. Copulation induced ovulation in rabbits and cats as a direct result of vaginal stimulation inducing gonadotrophin output (Clegg and Doyle, 1966). Little information is available as to the role of tactile and other peripheral stimuli in the ram, though Morgan et al.(1972) have shown that removal of hearing and touch senses did not interfere with mating in ewes.

(3) Hypothalamic Efferents.

Hypothalamic efferents connect with the medial orbitofrontal, septal and parolfactory regions, and thalamus and brain stem areas, and are particularly important in establishing sexual behaviour (Donovan, 1970). The hypothalamus with its many functions receives and transmits information to and from other major CNS centres (Davidson, 1966) aiding integration necessary for dispatch of information via the major efferent route, the functional link with the pituitary gland.

(4) Gonadotrophin Releasing Hormone.

Gonadotrophin Releasing Hormone (GnRH) has been the subject of many recent reviews (Schally et al., 1972a,b; Blackwell and Guilleman,

1973; Convey, 1973; Reichlin et al., 1976).

McCann et al. (1960) and Igarashi and McCann (1964) isolated hypothalamic extracts which released LH and FSH, respectively, and considered this as evidence for the existence of separate LH and FSH releasing hormones. However, Schally et al. (1971a,b) were unable to separate the LH and FSH releasing activities of highly purified porcine GnRH, while enzymatic breakdown destroyed both activities simultaneously. Subsequent isolation, structural determination and synthesis of the decapeptide confirmed the dual activity of the one molecule (Schally et al., 1972a).

However, there remains a degree of uncertainty about the existence of separate LH and FSH releasing hormones: asynchronous changes in plasma LH and FSH levels occured in normal physiological conditions and also following exogenous administration of GnRH (Mortimer et al., 1973); Johansson et al. (1973) reported the biosynthesis of a FSH-releasing hormone-like compound; Bowers et al. (1973) and Currie et al. (1973)demonstrated the chemical existence and partial purification of such a compound from the hypothalamus and reported that differing ratios of FSH and LH output followed administration of different fractions; and Sandow et al. (1975) and Fawcett et al. (1975) described chromatographic separation of two hypothalamic fractions possessing widely differing gonadotrophin releasing activities (though Sandow et al. (1975) suggested that this may have been an artifact due to structural modification during chromatography).

On the other hand, Spona (1973a,b) and Grant et al. (1973) offered an explanation for the apparent dual action of a single hormone. They both demonstrated the existence of differing GnRH binding sites on pituitary cell membranes in vitro. Spona (1973a) claimed that a low affinity site was responsible for LH release and a high affinity site for FSH release.

(i) Chemistry and Structure. Schally et al. (1971b) initially claimed that the GnRH molecule contained nine amino acids. However, Matsuo et al. (1971) ultimately determined the decapeptide structure of GnRH as being:

(pyro)Glu - His - Trp - Ser - Tyr - Gly - Leu - Arg - Pro - Gly(NH_2)

This structure has been shown to be identical in all species studied (Schally et al., 1972a).

(ii) Synthesis. Little research has been done on in vivo synthesis of GnRH (Reichlin et al., 1976). In vitro hypothalamic incubations with amino acids, including C^{14}-glutamic acid (Johansson et al., 1973) and requiring ATP and magnesium ions (Reichlin et al., 1976), has yielded bioactive releasing hormone. Synthesis is assumed to take place in the perikarya of neurons of the MBH. Production rates of GnRH have been estimated at between 1.5 and 15μg per day in man (Jeffcoate and Holland, 1974). Synthetic GnRH is now widely manufactured, and recently, incorporation of tritrated amino acids (Coy et al., 1973), iodination (Miyachi et al., 1973), and radioimmunoassay (Jeffcoate et al., 1974; Jonas et al., 1975) have been described.

(iii) Metabolism and Excretion. Peptidases from the hypothalamus abolish the immunoreactivity and bioactivity of GnRH by splitting off the C-terminal amino acid (Griffiths and Hooper, 1973; Griffiths et al., 1974). Though such enzymes may influence GnRH activity, they are relatively non-specific and their physiological significance is doubted (Reichlin et al., 1976).

The plasma half life of GnRH has been estimated to be 4 minutes in man (Redding et al., 1973; Miyachi et al., 1973; Virkkunen et al., 1974), between 5-10 minutes in rats (Miyachi et al., 1973), and approximately five minutes in sheep (Crighton et al., 1973).

In man, up to 97% of tritiated GnRH was recovered in urine within 24 hours of administration; it appeared as two fractions corresponding to a pyroglutamate-histidine dipeptide and the N-terminal octapeptide (Redding et al., 1973). Since the radiolabelled GnRH was concentrated in the liver, kidney and pituitary gland, it was likely that breakdown occurred in liver and/or kidney, and excreted in the urine.

From its behaviour during gel filtration, Virkkunen et al. (1974) concluded that GnRH was free in plasma rather than bound to plasma proteins.

(iv) Location of GnRH.

(a) Hypothalamus. GnRH has been located in many parts of the hypothalamus (Reichlin et al., 1976). Extracts from the arcuate nucleus,

ME (Watanabe and McCann, 1968) and SCN (Crighton and Schneider, 1969) all elicited gonadotrophin output from the adenohypophysis.

Zimmerman et al. (1974) found immunohistochemically active GnRH only in arcuate neuronal perikarya and ME tanycytes of mice. On the other hand, Silverman (1976) found the decapeptide in the POA, AHA, SCN, and arcuate neurons, ME nerve endings and in tracts between these centres in the guinea pig, while Gross (1976) localised the hormone only in parts of the POA and ME axons. Thus it is difficult to specify precisely where in the hypothalamus the compound is synthesised and stored in view of these conflicting reports.

In attempts to show the intracellular location of GnRH, Pelletier et al. (1974) adapted immunohistochemical methods to electron microscopy and identified GnRH in rat ME nerve ending granules and Bennett and Edwardson (1975) demonstrated the release of hypophysiotrophic hormones from ME synaptosomes of sheep in response to electrical or ion stimulation. Small and large intra-axonal microtubules have been described in the hypothalamus by Bergland (1972), who postulated that these carried releasing hormones and catecholamines respectively, but Kizer et al.(1975) considered that though some large axonal granules did contain catecholeamines, it was unlikely that both this compound and GnRH were found in the same axons. Thus GnRH appears to be stored in axonal endings, but the intracellular site of synthesis and transport mechanisms remain unknown.

(b) Third Ventricle. Third ventricular administration of GnRH elicited the release of gonadotrophins from the anterior pituitary (Ondo et al., 1973; Ben-Jonathan et al., 1974; Cramer and Barraclough, 1975). GnRH may be transported by tanycytes, modified ependymal cells, from the third ventricle to the ME (Ondo et al., 1973). However, GnRH did not appear in CSF after peripheral administration, possibly since it could not cross the blood-brain barrier, and when mixed with CSF in vitro, lost much of its activity (Cramer and Barraclough, 1975). Only a small portion of GnRH given intraventricularly can be recovered from hypothalamo-hypophyseal vessels (Ben-Jonathan et al., 1974) and its action was considerably delayed compared to similar doses given intravenously. Such evidence suggested that the ventricular system was unlikely to have played an important part in the distribution of GnRH.

Reichlin et al. (1976) summarised the current concepts of

releasing hormone production and output by suggesting that GnRH may be synthesised in the POA, AHA and particularly in the arcuate neurons, then is passed by axonal transport to the median eminence where it is stored and from which it is released into the portal vessels for transport to the adenohypophysis.

(v) Release from Storage Sites. Appropriate neurochemical and electrical stimulation of the ME can elicit GnRH release. Dopamine, norepinephrine and serotonin were found in the hypothalamus (Schneider and McCann, 1969; Anton-Tay and Wurtman, 1971; Cuppola, 1971) and these amines, particularly dopamine were concentrated in the median eminence (Kavanagh and Weisz, 1973). Dopamine has been shown to augment stalk-median eminence extract induced output of LH (Schneider and McCann, 1970) and FSH (Kamberi et al., 1970) and currently is considered to be the neurochemical transmitter agent responsible for GnRH release. Dopamine may cause the propulsion of granules and/or expulsion of releasing hormones from axonal storage sites into pericapillary spaces of the primary capillary beds of the pituitary portal blood system, from where they are transported to the adenohypophysis.

(vi) Mechanism of Action. Current theories on the mechanisms by which GnRH exerts two functions, gonadotrophin release and synthesis, have recently been reviewed (McCann, 1971; Jutisz et al., 1972).

(a) Gonadotrophin Release. In vivo GnRH administration resulted in a rapid but shortlived release of pituitary LH and FSH in many species (Schally et al., 1972a; Convey, 1973), indicating that there was a rapid association of GnRH with gonadotroph receptors. Ultrastructural studies have shown that extrusion of gonadotroph secretory granules occurred within one minute of intracarotid GnRH injection (Mendoza et al. 1973).

Gonadotrophin release may be due to altered cell membrane permeability, followed by calcium uptake, which in turn results in expulsion of GnRH secretory granules (McCann, 1971). GnRH increased cyclic AMP activity in pituitary tissue (McCann, 1971; Borgeat et al., 1972; Jutisz et al., 1972; Makino et al., 1974), and when administered to pituitary incubates alone, cAMP will cause gonadotrophin release. Thus cAMP may enhance calcium ion entry into, and granule content expulsion from, gonadotrophs. Glycogenolysis increased simultaneously with LH release to provide the necessary energy for this process (Makino et al., 1974).

(b) Gonadotrophin Synthesis. When incubated in vitro with pituitary tissue, GnRH increased the total quantity of gonadotrophins, signifying stimulation of synthesis (Jutisz et al., 1972), possibly as a result of increased cAMP activity. In vivo release of LH following high doses of GnRH followed a biphasic pattern; the first elevation has been attributed to immediate LH release and the second elevation has been attributed to further release of the gonadotrophin following GnRH-stimulated LH synthesis (Borgeat et al., 1972). Thus available evidence strongly suggests a dual secretory and synthetic function for GnRH.

(vii) GnRH Analogues. Some synthetic analogues of GnRH possessed gonadotrophin releasing activity (Guilleman et al., 1972, 1973) possibly due to a capacity for binding to less specific low affinity binding sites (Grant et al., 1973). Other analogues competitively suppressed LH output in a dose dependent manner (Guilleman et al., 1972; Coy et al., 1973; Vilchez-Martinez et al., 1974) and may prove effective in the future as contraceptive agents.

(viii) Clinical Applications for GnRH. In man, GnRH has been used in investigations of numerous clinical conditions ranging from anovulatory sterility to use in tests of pituitary function (Marshall and Besser, 1973; Zanartu et al., 1974). Many dose rates and routes of administration have elicited gonadotrophin output (Gonzalez-Barcena et al., 1973) including the intranasal route (London et al., 1973).

GnRH caused ovulation in ewes (Crighton, 1973a) but the subsequent luteal function was abnormal (Haresign et al., 1973; Hoffman et al., 1975). The releasing hormone is potentially useful in induction of multiple ovulation following oestrus synchronization, and in the treatment of follicular cysts (Hoffman et al., 1975). Convey (1973) proposed its potential use to advance puberty, manipulate spermatogenesis and libido, and possibly increase the quantity of spermatozoa produced, particularly in animals used in artificial insemination programmes. However, GnRH does not possess some of the desirable features of gonadotrophins and steroids for some of the above applications and its use as a clinical agent may in fact prove to be limited.

(5) Control of Prolactin Output.

Pituitary lactotrophs, the source of prolactin, possess a high degree of autonomy in vitro or after pituitary stalk section and it is therefore considered that hypothalamic control of prolactin output is

primarily inhibitory in nature (Nicoll, 1971), though evidence does exist for a dual control involving a prolactin releasing factor. Control concepts have been studied in many reviews (Apostolakis, 1968; Donovan, 1970; Nicoll, 1971; Frantz et al., 1972; Meites et al., 1972; Blackwell and Guilleman, 1973; Horrobin, 1973; Reichlin et al., 1976).

(i) The Hypothalamus and Prolactin Output. Knowledge of the hypothalamic nuclei controlling prolactin output is fragmentary. ME lesions were followed by elevated prolactin output, whereas VMH and POA stimulation has been reported to either raise or lower output (Meites et al., 1972; Horrobin, 1973). Recently Malven (1975) produced an elevation of prolactin output after anterior median eminence stimulation in sheep, but a drop in output following posterior ME stimulation, showing the likely importance of the ME in mediating prolactin output. Prolactin output follows in vitro application of hypothalamic extracts to the pituitary of the rhesus monkey, a result which supports the concept of the existence of a prolactin releasing factor in addition to an inhibitory factor (Horrobin, 1973). Though it is clear that the hypothalamus regulates prolactin secretion, there is insufficient evidence to allow conclusions to be reached about the mechanisms involved.

(ii) Prolactin Inhibitory Factor (PIF) Output. Dopamine released PIF when applied directly to the hypothalamus or to the third ventricle (Meites et al., 1972; Reichlin et al., 1976) while L-Dopa has been used clinically to suppress prolactin output (Frantz et al., 1972). Further evidence for the role of dopamine as an inhibitor of prolactin release stems from the in vivo demonstration that dopamine blocking agents induced prolactin output in rats (Ojeda et al., 1974) and sheep (Davis and Borger, 1973). In addition to the dopaminergic inhibitory influence, there also appeared to be a serotoninergic stimulatory component in the control of PIF output since serotonin administration raised prolactin output (Chen and Meites, 1975; Reichlin et al., 1976).

(iii) Prolactin Release and Inhibition. Attempts have been made to isolate PIF activity from porcine hypothalamic tissue (Takahara et al., 1974) but though an inhibitory effect was observed, this effect could have been due to catecholeamines which were found to be present. PIF is likely to be a small molecule, presumably peptide in nature, similar to other hypothalamic hormones (Meites et al., 1972).

A prolactin releasing factor (PRF), the major prolactin control factor in birds (Apostolakis, 1968), also has been proposed to exist in mammals (Reichlin et al., 1976). Thyrotrophin releasing hormone (TRF) released prolactin in cattle (Convey, 1973) and in other species (Debeljuk et al., 1973) but this compound was not considered to be of major physiological significance in regulating prolactin output. Furthermore, Valverde et al. (1972) and Szabo and Frohman (1976) found prolactin releasing activity in hypothalamic extracts even after the charcoal adsorption or chromatographic removal of TRF. Malven (1975) proposed that the anterior ME in the sheep produced a prolactin releasing factor (See earlier) though stimulation of this area may have inhibited the release of PIF rather than causing the release of PRF. The amino acids arginine, leucine and phenylalanine released prolactin after intravenous infusion into sheep (Davis, 1972), but the physiological significance of this result is not clear. Phenothiazine derivatives raised prolactin levels in sheep, presumably by inhibiting PIF output (Bryant et al., 1968) while bromergocryptine, which is known to stimulate dopaminergic receptors, also inhibited prolactin secretion by releasing PIF (Fluckiger, 1975).

Suckling raised plasma prolactin levels in cattle (Karg and Schams, 1974) and prolactin levels were found to decrease in post-menopausal woman and to increase in similarly aged men (Vekemans and Robyn, 1975). An important consideration in prolactin studies is the stress-induced output. Restraint, surgery, exercise and disease have been shown to raise plasma prolactin levels in man (Frantz et al., 1972), goats (Bryant et al., 1968) and cattle (Raud et al., 1971), while venepuncture and restraint raised plasma prolactin levels in adult and immature sheep within 15 - 30 minutes (Davis, 1972).

(iv) Mechanism of Action of PIF. It is considered that PIF reduced the calcium influx to the lactotrophs thus preventing the spontaneous membrane depolarisation and subsequent prolactin release (Nicoll, 1971). Cyclic AMP can overcome the inhibitory effects of PIF, thus raising the possibility that PIF may inhibit the adenyl cyclase system whereas PRF may stimulate it in a manner similar to GnRH and other releasing hormones. Potassium ions raised lactotroph membrane permeability to calcium and this resulted in prolactin output (Nicoll, 1971).

(6) Sexual Differentiation.

(i) Hypothalamic Differentiation. The pre-differentiation hypothalamus of both males and females possesses an inherent cyclic ability; in females this is manifest at puberty as oestrous cyclicity. The male gonad secretes testosterone at a "critical age" and this abolishes the inherent cyclic ability in favour of a "tonic" secretory capability of the male hypothalamus (Everett, 1969; Jost, 1970; Gorski, 1971; Jost et al., 1973). In rats this critical age was up to five days neonatally, in the human between the 11th and 17th weeks of in utero life (Reyes et al., 1973), and between 25 - 120 days (Alifakiotis et al., 1976) or 20 - 60 days of intrauterine life (Short, 1974) in sheep. Androgens (or their metabolite, oestrogen) are thought to modify input pathways to the POA, the area responsible for female hypothalamic cyclicity, abolishing its cyclic potential without affecting the tonic control influences of the medial basal hypothalamus and the remaining hypophysiotrophic area (Everett, 1969; Gorski, 1971). Though the physical or biochemical basis for this phenomena has not yet been determined, sexual dimorphism of axosomatic junctions of amygdaloid and non-amygdaloid origin has been observed in the preoptic area (Raisman and Field, 1971), suggesting perhaps an anatomical basis for hypothalamic differentiation.

(ii) Gonadal Differentiation. Histological signs of differentiation of the foetal gonads become evident 34 - 35 days after conception in sheep (Sapsford, 1962; Attal, 1969; Geir and Marion, 1970), but determination of gross anatomical differentiation is possible only after 45 days. However, ovine foetal testicular testosterone and androstenedione were measurable from day 30 (Attal, 1969) and throughout foetal life. The testicular content of steroids tends to fall in the last 80 days in utero, but quantities vary enormously between individuals of the same age (Attal, 1969; Pomerantz and Nalbandov, 1975). Attal (1969) found that the foetal testicular testosterone:androstenedione ratio changed after 46 days, with relatively more androstenedione, and again at 65 - 70 days, to a ratio similar to that before 46 days. The significance of these changes in steroid ratios is unknown.

The early development and function of Leydig cells is considered to be under gonadotrophic stimulation (Jost, 1970) since early hypophysectomy interfered with organ development and subsequent sexual dimorphism. Thus, gonadal differentiation occurs early and male gonadal

steroidogenic function is initiated immediately.

(iii) Genital Tract Differentiation. If testosterone is not present at a critical period of intra-uterine life, Mullerian structures continue to develop thus expressing the intrinsic female nature of the genital tract primordia (Jost et al., 1973). Testosterone stimulates development of Wolffian duct structures in genetic males. This organ differentiation occurs prior to hypothalamic differentiation; for example male rat genitalia differentiate at 18 days in utero whereas CNS differentiation occurs postnatally (Jost et al., 1973).

(iv) Sexual Behaviour Differentiation. Sexual behavioural dimorphism also is androgen dependent. Androgen treatment of neonatal female rats abolished adult lordosis exhibition, whereas neonatal castration of males resulted in display of lordosis later in life (Gorski, 1971). Androgens appear to exert an imprinting influence, possibly on hypothalamic centres, to direct the form of sexual behaviour exhibited in later life.

(v) Interference With Sexual Differentiation. Androgens administered at appropriate ages and dose rates mimic testicular androgens in inducing central nervous and external genital tract masculinizing effects (Jost, 1970). The importance of using appropriate timing and dosage of androgen to produce such masculinizing effects has been shown by the fact that androgen administration to foetal sheep after 12 weeks gestation, or post partum, failed to interfere with differentiation (Przekop et al., 1974) whereas earlier (days 20 - 60 of gestation) administration did (Short, 1974). Post partum treatment of heifers, gilts and bitches produced no effects, neither did androgen administration to foetal calves older than 82 days (Zimbleman and Lauderdale, 1973).

Short (1974) found gross genital masculinization of female lamb foetuses exposed to androgen from 20 - 40 days in utero. Externally these offspring were identical to males except that they did not possess palpable testes, while internally the uterus and ovaries apparently were normal. Lambs exposed at 60 or 80 days showed only mild genital masculinization. Treatment on days 20, 40 or 60 abolished later oestrous behaviour, while micturition patterns were typically male in all androgenized offspring (Short, 1974). Recently, from the same group, has appeared confirmation of the initial observations of Short, and further studies of oestrous behaviour in androgenized offspring (Clarke et al.,

1976). Short (1974) measured plasma progesterone levels in his masculinized ewes and was able to show some luteal activity, though these animals showed abnormal fluctuations in progesterone levels. The 20 - 60 day treated intersex animals failed to exhibit the normal ovulatory LH peak in response to oestrogen administration, suggesting that the hypothalamic positive feedback system had been abolished.

Recently, Alifakiotis et al. (1976) described a similar induction of intersexuality in sheep after treatment between 25 - 120 days of intrauterine life. These authors found epididymides, ductuli defferentia, seminal vesicles, prostates and bulbourethral glands, in addition to normal female internal organs.

2 PITUITARY HORMONES

The hypothalamo-pituitary portal vascular system carries the hypothalamic releasing and inhibiting factors responsible for regulating anterior pituitary gland function. Current concepts of pituitary control were the subject of an extensive review by Daniel and Prichard (1975). The present review concentrates on regulation of LH and prolactin secretion, with only brief mention of FSH.

(1) Luteinizing Hormone.

(i) Structure and Chemistry. LH is a glycoprotein of M.W. 30,000 approximately, consisting of two dissimilar 15,000 M.W. peptide chains (Greep, 1973a). The αchain also is common to FSH and TSH, but the more strongly antigenic βchain is hormone specific. LH is a globular non-helical molecule which is stabilized by cystine disulphide bridges (Frieden and Lipner, 1971; Ward et al., 1973). The structure and chemistry of ovine LH has recently been reviewed (Butt, 1971; Sairam et al., 1972; Ward et al., 1973; Papkoff et al., 1973).

(ii) Metabolism. LH and its subunits are found free in plasma (Laburthe et al., 1973), though LH carrier proteins have been reported (Rajanieme and Vanha-pertulla, 1973).

LH disappears from plasma in an exponential manner and has a half life of 136 minutes in man (Marshall et al., 1973). However, most authors have described a biphasic semi-logarithmic decay pattern; ovine I^{125}-LH has consecutive half lives of 22.3 and 174 minutes in the rat, and a first half life of 30 minutes in sheep (Geschwind, 1972). These

estimates correspond closely with half-life estimates of 26.7 - 32 minutes reported by de Kretser *et al*.(1973) for LH in adult sheep, 28 - 30 minutes in foetal sheep (Foster *et al*., 1972c), and 21 and 65 minutes for the two half life phases in the bull (Monkonpunya *et al*., 1972). The biphasic plasma disappearance pattern has been attributed to diffusion and distribution to and from body compartments (Geschwind, 1972). FSH on the other hand has longer half-lives of 107 and 149 minutes (Geschwind, 1972). Because of rapid clearance of LH, it has been recommended that the interval between plasma sample collection be less than the half-life of the hormone (Geschwind, 1972) and as frequently as every 2 - 3 minutes in the rat (Gay and Sheth, 1972).

I^{125}-LH has been located autoradiographically in renal proximal convoluted tubules (de Kretser *et al*., 1973), from where it can pass into the urine. However, the precise site of degradation has not yet been elucidated.

(iii) *Actions*.

(a) *Steroidogenesis*. LH binds to specific receptors on the cell membranes of Leydig cells and stimulates the adenyl cyclase system to produce cyclic AMP (Greep, 1973a). cAMP subsequently converts cholesterol stores to pregnenolone (Eik-nes, 1971; Dorfman and Unger, 1972) and possibly also aids *de novo* synthesis of cholesterol from acetate. The generally accepted steroidogenic chain is:

pregnenolone ⟶ dehydroepiandrosterone ⟶ androstenediol (or androstenedione) ⟶ Testosterone. (Yanaihara and Treon, 1972)

Steroidogenesis takes place in the mitochondria once the precursors are available. An alternative pathway, from desmosterol, a cholesterol precursor, to pregnenolone has also been suggested (Dorfman and Unger, 1972).LH also promoted Leydig cell protein synthesis, which was possibly a prerequisite for steroidogenesis, or for steroid storage and release (Irby and Hall, 1971).

(b) *Testosterone Release*. Administration of LH *in vivo*, or the endogenous rise in LH after GnRH, results in rapid secretion of testosterone by Leydig cells. The topic of testosterone secretion will be dealt with more fully in a later section.

(c) *Feedback Effects*. It has been postulated that LH inhibits the production or release of GnRH by a direct negative feedback on the

hypothalamic hypophysiotrophic centres (Greep, 1973a).

(2) Follicle Stimulating Hormone.

FSH had no demonstrable steroidogenic action (Dorfman and Unger, 1972) but passed into the seminiferous tubules of many species, including the ovine (Setchell and Wallace, 1972), where it was assumed to influence Sertoli cell function (Greep, 1973b). FSH increased fluid secretion by the testis, increased tubular size (Setchell et al., 1973) and enhanced tubular amino acid uptake (Means and Hall, 1967).

Although significant correlations have been reported between plasma FSH levels and numbers of spermatogonia, primary spermatocytes, and early and late spermatids in men (de Kretser et al., 1974), other authors have shown that no such relationship existed (Paulsen et al., 1972). The precise role of FSH is difficult to assess and no definite conclusions can yet be made about its functions, though recent work suggested a role in Leydig cell receptor formation in young rats (Odell and Swerdloff, 1976) and in formation of androgen binding proteins by the Sertoli cells (Means et al., 1976).

The control of FSH release also remains undetermined. GnRH elicited FSH output from the pituitary and a post-castration rise in plasma FSH occurred, but it is not clear whether the negative feedback was due to testosterone (Crim and Geschwind, 1972b; Burger et al., 1972) or to another compound (Odell et al., 1973). Destruction of tubular elements but not Leydig cells, by irradiation, resulted in increased FSH output. Steroid-free extracts of ovine (Nandini et al., 1976) and bovine (Keogh et al., 1976) testes selectively suppressed post-castration elevation of FSH levels in the rat and ram, respectively. It has been postulated that a tubular substance termed "Inhibin" is responsible for feedback control of FSH, possibly acting in conjunction with testicular steroids (Nandini et al., 1976).

(3) Pituitary Gonadotrophin and Gonadal Steroid Secretion Patterns.

Rapid estimation of hormones, facilitated by radioimmunoassay techniques, has led to a reappraisal of conventional "steady state" negative feedback control mechanisms and have provided a means whereby the short-term dynamics of hormone output have been examined quantitatively and qualitatively (Geschwind, 1972).

(i) Pulsatile Variation. LH was released in a pulsatile manner in

rams (Bolt, 1971; Geschwind, 1972; Sanford et al., 1974a; Katongole et al., 1974; Lincoln, 1976a; Barrell, 1976), wethers (Riggs and Malven, 1974), and ewes (Geschwind, 1972; Nett et al., 1974; Coppings and Malven 1975, Baird et al., 1976). Although Bolt (1971), Geschwind (1972) and Katongole et al. (1974) concluded that LH was released in a pulsatile manner, it was Sanford et al. (1974a) who first reported a detailed investigation of LH output in the ram, after sampling each 20 minutes for 24 hours and measuring the LH and testosterone content in each sample. In their study on Finnish Landrace and crossbred rams, Sanford et al. (1974a) found basal levels of LH to be low, though 3 - 5 peaks of up to almost 10 ng/ml were observed at irregular intervals during the 24 hour period. The peaks were of rapid onset and rapid disappearance, consistent with the short half life of LH. An identical plasma sampling frequency was employed by Barrell (1976) who reported a similar episodic release of LH in Romney rams. Geschwind (1972) and Lincoln (1976a) suggested that virtually no LH was released between episodic peaks in the rat and ram respectively, hence explaining the low between-peak levels.

Similar short-term fluctuations in plasma LH levels have been reported in other species including the boar (Sanford et al., 1976), bull (Katongole et al., 1971; Gombe et al., 1973b), man (Nankin and Troen, 1971; Elwood et al., 1973; McNeilly et al., 1975), rat (Gay and Sheth, 1972) and the domestic cockerel (Wilson and Sharp, 1975).

Plasma FSH also showed irregular fluctuations in concentration in rams but these were less spectacular than those of LH and elevations had a more prolonged existence than the LH peaks (Sanford et al., 1976).

(ii) Seasonal Variation. In sheep, short-term hormone fluctuations were superimposed on seasonal variations. In rams, plasma LH levels were highest in summer months prior to the breeding season (Katongole et al., 1974; Barrell, 1976). The seasonal peak preceded the seasonal testosterone peak by 10 weeks in the Soay breed, a breed noted for its marked seasonal changes in reproductive phenomena (Lincoln, 1976a). Lincoln (1976a,b) clearly showed that increases in both frequency of episodic LH and testosterone peaks, as well as a marked elevation in both peak and basal levels of LH, occurred as daily photoperiod decreased and the testes increased in size. By comparison, Romney rams displayed a markedly smaller seasonal fluctuation in semen characteristics and reproductive hormone levels (Barrell, 1976).

(iii) Circadian Variation. Although episodic fluctuations were found in plasma concentrations of all anterior pituitary hormones studied (Weitzman et al., 1975), and circadian variations in secretion of ACTH and cortisol are widely accepted (Resco and Eik-nes, 1966; Weitzman et al., 1975), there is conflicting evidence as to the occurrence of circadian variations of gonadotrophin and testosterone secretion. Demonstration of such rhythms is confounded by the widely varying plasma collection routines and sampling frequencies reported in the literature. A circadian or "about the day" rhythm implies a consistent pattern from day to day (Boon et al., 1972), a pattern which exists for many other functions, such as eating and drinking, which also are controlled by the hypothalamus. Single unit discharges of hypothalamic neurons have a circadian pattern (Schmitt, 1973) indicating that rhythms in hypothalamic activity do exist.

Work with humans has yielded conflicting results. On one hand some authors have recorded circadian variations of testosterone (Barberia et al., 1973; Smals et al., 1974) and LH plasma levels (Bodenheimer et al., 1973), yet Boon et al. (1972) and Elwood et al. (1973) found no such rhythm in secretion of testosterone, while de Lacerda et al. (1973) reported no such rhythm of LH output. Peterson et al. (1968) showed no circadian rhythms in either LH or FSH. However, many authors are in agreement that there was a sleep-related elevation of LH in pubertal boys associated with the REM stage of sleep (Judd et al., 1974; Weitzman et al., 1975; Kulin et al., 1976).

Bolt (1971), Falvo et al. (1973), Sanford et al. (1974a), Carr and Land (1975) and Barrell (1976) all concluded that no circadian variation of either LH or testosterone levels occurred in the ram. Chamely et al. (1973) did not record circadian variations in secretion of prolactin or growth hormone (GH), but data from Barrell (1976) suggested the existence of a consistent elevation of plasma prolactin levels at approximately 22.00 hours.

(iv) Hormonal Interrelationships. GnRH elicits an immediate dose-related output of pituitary LH and FSH (Schally et al., 1972a). However, an attempt to demonstrate a relationship between peripheral plasma levels of GnRH and subsequent LH elevations at oestrus in the ewe failed (Nett et al., 1974); this negative result may reflect GnRH assay insensitivity, technical deficiencies in sample collection, or a poor correlation between hypothalamo-hypophyseal portal and peripheral plasma GnRH levels

due to the high degree of dilution of GnRH in peripheral plasma.

Although LH stimulation of testosterone output is well established, the qualitative and quantitative relationships between the two are not always repeatable. Elwood et al. (1973) showed that testosterone output usually followed LH spikes by one hour in man, but not all LH elevations were followed by testosterone output. Both de Lacerda et al. (1973) and Murray and Corker (1973) also showed disparity between the number of LH and testosterone peaks, while Rowe et al. (1975) summarized the situation in man by stating that a testosterone peak may precede, follow, or be totally unrelated to a LH peak.

Katongole et al.(1971) recorded testosterone peaks 40 minutes after episodic LH peaks in bulls, but Smith et al.(1973) found that only 64% of LH elevations (greater than 1 ng/ml) resulted in elevations of testosterone in the same species. The interval between LH peaks and testosterone peaks approximated one hour in the latter case.

Sanford et al.(1974a), Barrell (1976) and Lincoln (1976a) recorded close time relationships between LH and testosterone secretory patterns in rams, with an interval of 40 - 75 minutes between the LH and testosterone peaks. Testosterone levels usually began to increase within 20 minutes of the LH peak. No quantitative relationship between LH and subsequent testosterone peaks has been reported, but Lincoln (1976a) reported a difference between LH and subsequent testosterone levels related to daily photoperiod. The testosterone output was greater and more rapid when testicular activity of his Soay rams was highest.

(4) Feedback Influences on Gonadotrophin Output.

Testicular removal abolished the endogenous testosterone negative feedback on LH output in the ram (Bolt, 1971; Pelletier, 1974). Testosterone administration lowered the post-castration rise in LH (Riggs and Malven, 1974) but LH administration caused no alteration in frequency of LH peaks or in LH levels in ewes (Coppings and Malven, 1975). Exogenous testosterone delayed and lowered the LH response of wethers and intact rams to GnRH administration (Galloway and Pelletier, 1975). The inhibition of LH secretion in wethers followed a biphasic pattern (Pelletier, 1974; Galloway and Pelletier, 1975), the primary inhibition being attributed to direct depression of LH release and the second (a delayed effect) to a temporary decrease in GnRH systhesis. These results confirmed earlier work (Pelletier, 1970) which showed that

pituitary LH content rose after testosterone propionate administration to wethers, whereas two days post-treatment, hypothalamic GnRH activity was reduced. It is apparent that testosterone had negative effects both on the pituitary, inhibiting output of LH, and in the hypothalamus, lowering endogenous GnRH production and output.

(5) Nutritional Effects.

Mann and Rowson (1957) found that underfeeding of bulls delayed puberty and lowered sperm density as compared to results from twin control animals. Gombe and Hansel (1973) reported lower plasma progesterone but not LH levels in underfed heifers, but Gombe et al. (1973a) reported a reduction of gonadotrophin-producing cells in calves suffering from poor nutrition, offering a possible explanation for poor reproductive performance in underfed animals. Drymundsson (1973) stressed the importance of nutritional factors in determining the onset of puberty in rams.

(6) Stress Effects.

Anaesthetic and surgical stress resulted in elevation of plasma LH and testosterone levels for up to 24 hours (Carstensen et al., 1973). Ether stress abolished a 20.00 hour circadian peak of LH but increased the mean LH level in male rats (Dunn et al., 1972). Other authors have confirmed that similar stress does not lower plasma LH levels (Neill, 1970). In rats, severe blood volume depletion raised plasma LH concentrations (Seyler and Reichlin, 1973) an effect attributed to neurally mediated GnRH output, rather than to altered pituitary function, haemoconcentration or LH clearance and metabolism. Social subordination stress decreased testosterone concentrations in the male rhesus monkey (Rose et al., 1972). Roche et al. (1970) determined that no alteration of plasma LH levels followed different methods of blood collection from sheep although in this species, prolactin does rise after stress (Davis, 1972). Thus a variety of stressful procedures result in LH increase in many species, but no reports of stress-induced alteration of LH levels in sheep appear in the literature.

(7) Prolactin.

Ovine prolactin is a protein hormone of M.W. 20,000 approx., and is almost identical to caprine prolactin (McNeilly and Andrews, 1974). Synthesis and secretion is from anterior pituitary lactotrophs

(Horrobin, 1973) and the control of secretion has been discussed (see p. 10). Prolactin possesses diverse activities including stimulation of the synthesis of all major components of milk during lactation (along with oestrogen, progesterone and other hormones), fluid and electrolyte maintenance, and possibly involvement in mental illness (Horrobin, 1973). In this review an attempt has been made to outline functions of prolactin which may affect the male reproductive system.

Prolactin is carried free in plasma and has a half-life in man of between 15 and 20 minutes (Horrobin, 1973) or 30 minutes (Frantz et al., 1972). As with the other hormones examined, prolactin showed very wide within and between-animal variations in plasma concentrations (Davis et al., 1971; Buttle, 1974).

(i) Prolactin Output.

(a) Short-term Patterns. Plasma prolactin levels fluctuated during the day in female, castrated male(Hart, 1973) and entire male goats (Buttle, 1974). Buttle (1974) believed that these fluctuations may have been due to a variety of stress factors such as feeding and sampling. Chamley et al.(1973) showed similar fluctuations in rams subjected to continuous blood sampling. Barrell (1976) collected blood samples each 20 minutes for 26 hours from 4 normal rams and recorded fluctuating plasma levels with a well defined nocturnal peak at 22.00 - 24.00 hours. This peak was not stress-induced as blood samples were collected at a remote sampling station and by means of indwelling catheters; the circadian pattern of secretion was abolished after pinealectomy.

Episodic output of prolactin has been observed in normal and pregnant women (Boyar et al., 1975) and a sleep-related rise has been seen in both men and women (Sassin et al., 1972).

(b) Seasonal Patterns. Pelletier (1973) found that plasma prolactin levels of intact and castrate Ile de France rams were positively correlated with daily photoperiod, when the animals were subjected to artificial lighting regimes. A similar relationship between length of daylight and prolactin levels was recorded from Romney rams grazed on pasture or housed under artificial lighting regimes (Barrell, 1976). Barrell (1976) also showed that pinealectomy abolished this pattern, indicating a pineal gland modulation of seasonality of prolactin output in rams. Recently Ravault et al. (1976) reported a "16th hour" photosensitive phase for prolactin secretion in rams; one hour light

pulses given 16 hours after dawn will counteract the shortened daylight effect of intervening periods of darkness.

Similar seasonal fluctuations in prolactin secretion were recorded from goats by Buttle (1974) who suggested that elevated prolactin levels may aid salt retention during summer, or stimulate spermatogenesis in anticipation of the breeding season.

(c) Pubertal Patterns. Oxender et al.(1972) detected prolactin in the serum of very early foetal calves; these levels increased almost twenty-fold towards parturition, then fell rapidly shortly after birth. In foetal sheep, prolactin could not be detected before 122 days, then subsequently levels were high (Moger and Geschwind, 1971).

Male rats produced increasing amounts of prolactin 25 days after birth, with peak levels at 70 - 90 days (Dohler and Wuttke, 1975); this pattern paralleled ventral prostate growth (Negro-Vilar et al., 1973). Before puberty, autumn born rams exhibited a small rise in prolactin concentrations at two weeks of age; this was followed by a dramatic rise at 70 days of age and lasted 1 - 2 weeks, before plasma levels dropped to earlier values (Courot, 1974; Ravault and Courot, 1975). Though these rams were born outside the normal breeding season, the normal seasonal prolactin elevation occurred as summer approached. The 70 day peak occurred at the period of maximal testis growth and the commencement of spermatogenesis. Presently, the pattern of prolactin output from spring born lambs has not been reported.

(ii) Role of Prolactin in Male Reproduction. As yet, no definite role has been ascribed to prolactin in the regulation of reproductive function in rams and there is a paucity of information about the role of prolactin in males in general. However, in the female, prolactin does influence reproduction since oestrous cycling can be initiated by the use of prolactin suppressants such as bromergocryptine (Kann and Martinet, 1975). Kann and Denamur (1974) suggested that the preovulatory prolactin rise may have been responsible for steroidogenesis and luteinization in ewes, but Karsch et al.(1971) failed to maintain the corpus luteum of sheep with prolactin alone.

Specific prolactin binding sites have been found in many rat tissues, particularly liver, testes, epididymides, seminal vesicles and prostates (Aragona and Friesen, 1975). Testicular binding sites increase with age from 20 - 70 days in this species. Prolactin acted synergistic-

ally with testosterone to raise the DNA and RNA content of castrate rat ventral prostates (Thomas and Manandhar, 1975). Prolactin raised testicular 17- β-OH steroid dehydrogenase levels in enzyme deficient dwarf mice (Musto et al., 1972). This enzyme increased in quantity with the onset of puberty, raising the possibility that prolactin may have a role in the onset of puberty. In the prostate tissue, prolactin stimulated adenyl cyclase activity, a mechanism of action common to many hormones (Golder et al., 1972).

Bartke (1971b) has shown that together, prolactin and LH increased testicular cholesterol ester stores, whereas LH tended to diminish such stores in favour of testosterone synthesis, and prolactin alone had no effect. After hypophysectomy of rats, prolactin produced a small rise in spermatid numbers, but together with LH completely restored spermatogenesis; an effect greater than that achieved by LH alone (Bartke, 1971a). Testosterone alone had a spermatogenic function in this situation, thus Bartke (1971a) concluded that prolactin may have aided testosterone synthesis or output. Hafiez et al. (1972) supported this hypothesis and showed that prolactin alone stimulated testosterone output to a limited extent in rats; similar prolactin augmented output of testosterone has been recorded in man (Rubin et al., 1976). Thus one can only speculate as to the precise role of prolactin on the reproductive system in males, particularly in the domestic species.

3. SEXUAL STIMULI AND HORMONE OUTPUT

Amongst domestic animals, sexual stimuli have been shown to produce a variety of hormonal responses. Following sexual stimulation of two bulls, Katongole et al. (1971) noted an immediate LH and subsequent testosterone elevation. Other authors (Convey et al., 1971; Smith et al., 1973) were unable to show such an elevation after false mounts or "sexual preparation", but a small non-significant elevation occurred after ejaculation. Although Gombe et al. (1973b) did not record a rise in LH in bulls, Convey et al. (1971) showed a marked rise in plasma prolactin concentrations after ejaculation.

Sanford (1974) exposed three young, two yearling and two mature rams to a single mating, continued mating and to the stimulation of observing an oestrous ewe. Of the seven rams, one young ram responded to all three stimuli by releasing LH and testosterone, one yearling responded to mating, and one young and one mature ram responded to the

observation of an oestrous ewe. In a subsequent experiment Sanford (1974) found that when two mature rams were placed together with oestrous ewes, their normal twenty four hour LH and testosterone secretory patterns were markedly altered. The majority of the 44 and 24 mounts undertaken by respective rams occurred in the first 12 hours, during which time peaking of LH and testosterone in plasma continued but at a greater frequency than normal. During the following 12 hours LH and testosterone peaks virtually ceased, resulting in a dramatic fall in mean hormone output.

4. TESTOSTERONE.

Testosterone, its precursors dehydroepiandrosterone, androstanediol and androstenedione, and its metabolite 5 α-dihydrotestosterone (DHT) are found in plasma. Approximately 99% of circulating androgen is bound to one of three proteins: the highly specific sex hormone binding globulin (SHBG), albumin, or the less specific cortisol binding globulin (Ismail, 1976). It was believed that only the unbound steroid was biologically active, but it is now considered likely that the bound fraction is also capable of biological activity (Ismail, 1976).

Testosterone synthesis is the function of the Leydig cells (Ismail, 1976), but van der Molen and De Bruyn (1971) and Lacey et al. (1972) believe that Sertoli cells also produced testosterone, under the stimulus of FSH. This seminiferous tubular production may be responsible for direct maintenance of the germinal epithelium.

(1) Metabolism of Testosterone.

Testosterone is largely metabolised in the liver to androstenedione and other weak androgens, though a second pathway involving changes in the A-ring does occur and yields more potent androgen by-products (Ismail, 1976). These metabolites are then conjugated as glucuronides or sulphates and excreted in the bile.

In peripheral tissues possessing androgen receptors, testosterone is metabolised by the enzyme 5 α-reductase to become DHT (Djosland et al., 1973; Mainwaring and Mangan, 1973; Minguell and Sierralta, 1975). Such tissues include the epididymis and accessory glands. The 5 α-reduced form is considered to be the active metabolite of testosterone and is found in plasma in a consistent ratio with testosterone (Vermuelen, 1976). Androstenedione possesses only 20% of the activity of

testosterone.

Though some authors believe DHT to be the neuroactive metabolite of testosterone (Martini, 1970; Sholiton, 1972), Naftolin et al. (1975) presented evidence supporting the hypothesis that in fact oestrogens were the active metabolites in neuroendocrine tissues. These authors have shown that testosterone-induced CNS sexual differentiation was mimicked by oestrogen and indeed, oestrogens did exert a negative feedback on gonadotrophin output in the male of many species, including sheep (Bolt, 1971; Riggs and Malven, 1974). MacDonald et al. (1971) suggested that the peripheral oestrogen found in the male was a product of target organ metabolism of testosterone.

Testosterone has been ascribed three plasma half-life phases: 0.11, 0.55 and 3.41 hours respectively (Ismail, 1976).

(2) Actions of Testosterone.

Discussion of the mechanisms of sex steroid actions is outside the scope of the present review, but is the subject of two recent comprehensive reviews (Minguell and Sierralta, 1975; Gorski and Gannon, 1976).

Testosterone in rams was concentrated in the testis by a counter-current exchange between the pampiniform plexus and spermatic artery (Ginther et al., 1974). It was found in significant quantities in seminiferous tubules and rete testis fluid and primarily was active in promoting spermatogenesis and spermatozoal maturation (Cooper and Waites, 1975).

(i) Spermatogenesis. Though it is difficult to isolate the functions of the individual hormones involved in spermatogenesis, it is clear that both gonadotrophins and testosterone are necessary for complete spermatogenesis in the adult after hypophysectomy, provided testicular atrophy has not taken place (Steinberger, 1971; Ahmad et al. 1973). Testosterone also was necessary for epididymal spermatozoal maturation, since when the testes were removed and the epididymides left, the remaining epididymal spermatozoa became incapable of fertilization (Dyson and Orgebin-Crist, 1973). Rete testis fluid had a high content of testosterone, but relatively more DHT was found in the epididymis and the rest of the reproductive tract (Ganjam and Amann, 1973). Seminiferous tubule androgen was bound to a protein possibly formed

following FSH stimulation of Sertoli cells (Hansson et al., 1974).

Androgen administration in low doses suppressed spermatogenesis indirectly in intact adult males, but high doses were capable of maintaining seminiferous tubular epithelium and spermatogenesis by direct action at the testicular level (Steinberger, 1971). Androgen was noted as early as the 1930's to prevent the onset of spermatogenesis of the prepubertal testis through suppression of pituitary gonadotrophin secretion (Moore and Price, 1932). Gonadotrophins on the other hand, were stimulatory to the onset of spermatogenesis (Lunenfield and Weissing, 1972). Localised spermatogenesis has been noted in a prepubertal human subject adjacent to an androgen secreting Leydig cell tumor, further confusing the role of androgen in the initiation of spermatogenesis (Steinberger et al., 1973).

(ii) Accessory Reproductive Structures. Mann (1974) described various aspects of the specific dependence of the bovine accessory reproductive organs on testosterone for both structural and functional normality. Fructose and citric acid output by these glands was androgen dependent, and after castration the production of these could be maintained by testosterone or DHT replacement therapy. Atrophy of accessory structures after castration is well known. The role of testosterone in determining reproductive anatomical development has been discussed (see p13).

(iii) Testosterone Feedbacks. Testosterone suppressed the post-castration rise in gonadotrophins (Davidson, 1966; Pelletier, 1974) and maintained normal pituitary gonadotroph structure. Negative feedback receptors have been localised in the MBH (Davidson, 1966) and in the ME (Davidson et al., 1968). Others (Sar and Stumpf, 1973; Perez-Palicios et al., 1973) also have demonstrated the presence of steroid receptors in the arcuate nucleus, VMH, POA, Stria terminalis, lateral septum, hippocampus and amygdala, areas involved in regulation of gonadotrophin secretion and sex behaviour. Pituitary uptake of androgen was high Perez-Palicios et al., 1973) and androgens can suppress GnRH stimulated gonadotrophin output from pituitaries in vitro (Kao and Weisz, 1975) and in vivo, both in man (von zur Muhlen and Koberling, 1973) and the ram (Pelletier, 1974; Galloway and Pelletier, 1975).

(iv) Sexual Behaviour. Initiation and maintenance of male sexual behaviour is totally androgen dependent, and the role of steroids in this phenomenon has been discussed in many reviews (Phoenix et al., 1966;

Lisk, 1966; Davidson and Levine, 1972).

The POA, amygdala and possibly other hypothalamic areas form an androgen sensitive behavioural control system (Davidson and Levine, 1972). These behaviour centres are more "dose-sensitive" than peripheral androgen sensitive tissues. Oestrogen possessed activity akin to testosterone in initiation of sex behaviour in hamsters and rats, whereas no peripheral androgenic effects of oestrogen were seen (Coniglio et al., 1973; Sodersten 1973). Yet other authors (Davidson and Levine, 1972; Luttge and Hall, 1973; Morali et al., 1974) have claimed that oestrogen was more effective in stimulation of sexual behaviour than DHT or other non-oestrogen androgen metabolites. These findings support those of Naftolin et al. (1975) who suggested that CNS tissues convert testosterone to oestrogen, the active metabolite in this tissue.

5. GONADAL AND REPRODUCTIVE TRACT DEVELOPMENT

(1) Foetal.

The testes develop from undifferentiated gonads on the urogenital ridges, and in sheep become distinguishable from the female gonad with the appearance of the tunica albuginea at days 34 - 35 (Sapsford, 1962; Geir and Marion, 1970). Primordial germ cells, presumably from yolk sac endoderm, were present as early as 29 days (Sapsford, 1962), and later were seen to be included in ingrowing aggregations of coelomic epithelial cells in clumps or groups, which become the sex cords. Interstitial cells, derived from mesenchyme, are distinguishable within two days of gonadal differentiation.

At 42 days, shortly after differentiation, the testis consists of sex cords which are the solid precursors of seminiferous tubules; between these is fibrous connective tissue containing blood vessels and interstitial cells (Sapsford, 1962). The testis cords contain peripheral indifferent cells (future Sertoli cells) and central gonocytes, the future gametogenic stem cells. Each cord is surrounded by a complex series of membranes (Steffert, 1971).

Indifferent cells constitute the majority of the cord cells and cytoplasmic processes extend into the centre. Cord diameter increases with indifferent cell and gonocyte division, and indifferent cell growth. Though indifferent cell boundaries are indistinct and have a syncytial

appearance, Sapsford (1957) believed they were in fact individual units.

Descent of the testes occurs as the gubernaculum contracts and the surrounding tissues grow, drawing the testes and associated ducts toward the inguinal canal, after which further contraction of the gubernaculum and intra-abdominal pressure results in passage into the scrotal sac. Testicular descent is completed in foetal rams by day 80 (Geir and Marion, 1970).

(2) Postnatal.

Sapsford (1964) described the development of the gonocyte of the ram in detail. Gonocytes of the early foetus undergo many morphological changes. Usually centrally placed and enveloped by indifferent cell cytoplasm, gonocytes are easily distinguishable by having a large round nucleus with two to four nucleoli and well defined cytoplasm. Mitotic figures signify continuing gonocyte division through the prepubertal stage, though some abnormally large degenerating cells may also be noted. Mature gonocytes, the second phase gonocytes present at birth, show finer nuclear chromatin and fewer nucleoli, in contrast with the earlier clumped chromatin and multiple nucleoli. Nuclear enlargement and disappearance of all but one of the nucleoli distinguishes the third phase in gonocyte development, the prospermatogonia. These cells resemble the adult type A spermatogonia, but are generally larger. They contain rounded nuclei, and usually retain a central position in the sex cords. The prospermatogonia appear as the ram approaches a body weight of 15 Kg.

Testicular growth was slow up to about 70 days (Steffert, 1971), then followed a period of more rapid growth which was concurrent with the onset of spermatogenesis. This period of accelerated growth occurred at different ages in different breeds, being earlier in high prolificacy breeds such as the Finnish Landrace, and even more so in cross-breeds (Land, 1973). When body weight reached approximately 21 kg in the Merino (Sapsford, 1962) and Romney (Steffert, 1971), or when the testis reached 6g in the Ile de France (Courot, 1962) or 10g in the Romney (Steffert, 1971), the sex cords enlarged further and lumina began to appear. Prospermatogonia assume their adult position on the basement membrane and transform into type A spermatogonia, which have a flattened appearance and distinct oval nuclei. In the Ile de France breed, as the testis approaches 12g, primary spermatocytes appear, spermatids appear

when the testis is 30g, and mature spermatozoa when 65g. In Suffolks, Skinner et al. (1968) found primary spermatocytes at 70 days of age, spermatids at 105 days, and spermatozoa in the 100g testis at 112 days. Data of Carmon and Green (1952) for the Southdown breed and Skinner et al.(1968) for the Suffolk breed have highlighted between-breed differences in the timing of onset of spermatogenesis, even though the sequence of events was the same. Most authors (Dun, 1955; Watson et al., 1956; Courot, 1962; Steffert, 1971) have found that testicular development was closely related to body weight rather than age, during the pubertal phase. Seminiferous tubular diameter increased from 40μ at birth, to 60μ at 56 days, and 185μ at 112 days in Suffolks, after which the diameter remained reasonably constant until at least 168 days (Skinner et al., 1968). A similar increase was recorded in Merinos as testis weights approached 300g (Watson et al., 1956).

Concurrent with final gonocyte maturation, indifferent cells become Sertoli cells with tongue-like cytoplasmic projections penetrating toward the tubular lumen to provide points of attachment for spermatids. Leydig cell numbers increase throughout foetal life (Sapsford, 1962) appearing singly, in cords or in clumps. Many foetal and prepubertal interstitial cells have distinct eosinophilic cytoplasmic granules which increase in number until the 70th day of foetal life, then as maturity approaches, granulation diminishes. In the adult, Leydig cells are situated in the triangular areas between adjacent seminiferous tubules.

Not all tubules commence spermatogenesis simultaneously, thus tubules of many developmental stages may appear in the one testis. Those tubules containing free spermatozoa are considered mature, and are structurally and functionally similar to those of adult rams. The adult spermatogenic cycle commences immediately, though quantitatively, spermatogenesis increases toward adulthood (Courot, 1962; Watson et al., 1956). Steffert (1971) found completion of spermatogenesis in all Romney rams with body weights of over 27Kg.

Epididymal weights paralleled the biphasic pattern of testicular growth (Watson et al., 1956; Skinner et al., 1968), with a sharp increase at the time that spermatozoa appeared in the epididymal tubule. The basic structure of the seminal vesicle of the young resembles that of the adult, but as puberty progresses, the lumen of secretory lobules increase in size and secretion commences (Skinner et al., 1968). The

pituitary gland also increases in weight up to 98 days in Suffolk rams Skinner et al., 1968) and 112 days in Dorper rams (Skinner, 1971).

Prior to puberty the urethral process is adherent to the penis and the glans penis to the prepuce (Johnstone, 1948). The urethral process becomes free at 112 days in Dorper rams (Skinner, 1971) while urethral process and preputial adhesions are broken down between 105 and 140 days in the Merino (Johnstone, 1948). Dun (1955) claimed that collection of semen was not possible while these adhesions were present, though Skinner and Rowson (1968) collected semen earlier.

6. PREPUBERTAL NEUROENDOCRINE MECHANISMS

Foetal pituitary tissue contains an increasing amount of bioactive LH over the final 30 days of intra-uterine life (Foster et al., 1972a) and a further elevation occurs postnatally (Skinner et al., 1968). Castration of rams 1 - 4 days after birth resulted in a rise in plasma LH levels, but unlike the situation in adult rams, pituitary LH content did not increase (Foster et al., 1972b). Between 30 and 150 days of age the post-castration rise in plasma LH levels can be suppressed by testosterone propionate (Crim and Geschwind, 1972b) demonstrating the existence of prepubertal steroid negative feedback mechanisms. Pituitaries of 126 - 138 day foetal, and 3, 11 and 68 day postnatal rams responded to both crude hypothalamic extract and purified porcine GnRH in a manner similar to adults, but the 3 and 11 day animals produced less LH than the other animals, a finding which was consistent with data on pituitary LH content (Foster et al., 1972c). LH releasing activity could not be shown in the hypothalamus of 30 - 147 day foetal lambs but was present in 14 -18 day neonatal rams (Foster et al., 1972d). The apparent absence of GnRH from foetal hypothalamic tissue may have been due to assay insensitivity.

Using the hypophysectomised prepubertal ram as a model, Courot (1967) examined endocrine control of the testis. Following pituitary removal, testis and seminal vesicular weights dropped, and sex cords were reduced in diameter. Supporting cell numbers were halved, but gonocytes were unaffected. However, pituitary extracts, or LH and FSH replacement therapy restored gametogenic activity. If given alone, very high levels of testosterone were necessary for the maintenance of testicular growth. Thus prepubertal testes, like the adult, required LH and FSH for structural and functional maintenance.

Thus the majority of available evidence suggests that the neuroendocrine control mechanisms and hormonal requirements of the gonad prior to puberty are similar to those of the adult.

7. THE ONSET AND CONTROL OF PUBERTY

Puberty is a phase in sexual maturation during which the onset of spermatogenesis occurs and there is accelerated growth and development of the gonads and accessory reproductive structures leading to the attainment of the potential to reproduce.

Though the control of the onset of puberty has been the subject of many and extensive publications and reviews (Critchlow and Bar-Sela, 1967; Kraght and Masken, 1972; Ramirez, 1973; McCann, 1976) the exact nature of the factors involved in governing this phenomenon remain an enigma. A brief summary of current concepts is presented.

Low prepubertal levels of gonadotrophins, sex steroids and possibly prolactin probably relate to an "immaturity" of hypothalamic feedback receptor sites, or of the cells involved in feedback mechamisms. Such feedback systems are highly sensitive to androgen when compared to those of the adult. As a result, gonadotrophins are secreted in quantities insufficient to stimulate gonadal growth and development. Intrahypothalamic implants of testosterone delay male puberty, ME implants being the most successful (Smith and Davidson, 1967). However, the dose of testosterone necessary for suppression of gonadotrophin output prepubertally was significantly less than that required for the parallel effect in adults. Smith and Davidson (1967) suggested that this indicated a decreasing sensitivity of steroid receptor sites in the hypothalamus as puberty progressed. Changes other than to receptor sites may also occur. Alteration of hormone transport forms (McCann, 1976), as well as altered intraneuronal activity, may affect feedback systems. Ramirez (1973) refuted the hypothesis that steroid metabolism altered during puberty, but concurred with the view that hypothalamic steroid sensitivity changed in some way. Hypothalamic catecholamine content increased during puberty (Kraght and Masken, 1972), a factor possibly associated with increased GnRH output. However, Ramirez (1973) emphasised the paucity of data allowing formulation of concepts of the control of puberty in the male.

Alteration of the behaviour of extrahypothalamic limbic structures suggested that extrinsic modulating influences may act on the

hypothalamus, in conjunction with intrinsic changes, to influence the course of puberty (Teresawa and Timiras, 1968). Lesions of the amygdala in female rats advanced the onset of puberty suggesting an inhibitory or delaying influence of this centre.

Current concepts of the major neuroendocrine events occurring during puberty, and discussed in the reviews mentioned above, can be summarized:

As the intrinsic sensitivity of hypothalamic feedback integrators decreases, the release of GnRH gradually increases. The resulting greater output of gonadotrophins stimulates gonadal steroidogenesis and the onset of spermatogenesis. Subsequently, testosterone affects androgen sensitive tissues causing anatomical development of the male reproductive system as well as maintaining a modulating influence on gonadotrophin output. With time, the hypothalamic feedback centres reach another "set point" or level of sensitivity, after which no further sensitivity changes occur and the neuroendocrine axis has reached its adult level, producing greater quantities of hormones than in the prepubertal subject.

This theory however, falls short of explaining the many varied endocrine changes which occur from birth to maturity, as discussed below for the sheep. Since most of the evidence upon which this theory is based was from the rat, it is not possible to extend its implications to other species. It is likely also that the hypothesis will be altered in the future as more intense fundamental neuroendocrine, anatomical and biochemical research is done and therefore the hypothesis as presented must be regarded as tentative.

8. ENDOCRINE PATTERNS THROUGH PUBERTY IN RAMS

(1) Luteinizing Hormone.

Plasma LH concentrations show a steady rise from birth until approximately 70 days, whereupon levels become variable (Courot 1974; Cotta et al., 1975; Courot et al., 1972; Lee et al., 1976a), and this pattern occurs regardless of the season of birth (Courot et al., 1975). The initial increase in LH levels correlated closely with the testosterone pattern, but was unrelated to the testis growth spurt and the onset of spermatogenesis.

Sanford (1974) compared LH levels in 2 to 7 month old, yearling

and adult rams and found highest levels at 2 - 3 months of age; unfortunately he did not measure LH levels in younger rams. Thimonier et al. (1972) observed that the rise in LH secretion which occurred between 4 and 11 weeks was greater in ram lambs of high prolificacy breeds.

Pituitary LH content rose sharply from 20 - 80 days and remained high thereafter (Skinner et al., 1968; Foster et al., 1972a; Courot et al., 1975) indicating greater storage capacity prior to the onset of spermatogenesis.

(2) Follicle Stimulating Hormone.

Between 30 and 150 days of age plasma FSH concentrations in rams remained relatively constant (Crim and Geschwind, 1972b). However, Lee et al. (1976a) recently reported that FSH levels of ram lambs rose from 11 - 22 ng/ml at birth, to a mean peak of 47 ng/ml at five weeks, then dropped again to 30 - 40 ng/ml thereafter. FSH levels were seen to display episodic elevations but these were not as marked as those of LH.

(3) Testosterone.

Peripheral plasma levels of testosterone were low at birth, but rose at between 3 and 5 months in Ile de France rams (Attal et al., 1972) and at up to 41 weeks in the Merino (Lee et al., 1976l). Cotta et al. (1975) and Courot (1974) described a gradual rise in plasma testosterone concentration from birth to approximately 100 days in Ile de France ram lambs, which was followed by a relative stabilization of levels up to 150 days. These authors emphasised that this pattern was not specifically associated with the onset of puberty. Crim and Geschwind (1972a) found an age-related rise in spermatic vein testosterone levels up to 30 weeks, with highest levels occurring once the initiation of spermatogenesis was complete.

(4) Prolactin.

Patterns of prolactin levels in plasma during puberty have been discussed in an earlier section (p23).

(5) Response to GnRH.

Foster et al. (1972c) found that pituitary LH responses to exogenous GnRH were higher in 68 day old, rather than in 3 or 11 day

old rams, a finding which was consistent with known increases in pituitary LH content during this period (Foster et al., 1972c). Galloway and Pelletier (1974) injected GnRH into young rams and observed that the LH response rose between days 7 and 40, and between days 20 and 60 in two groups of animals. Lee et al. (1976b) found that response to intracarotid injection of GnRH was low in 2 and 4 week old rams, but significantly higher in rams of 2 to 3 months of age. FSH did not follow the LH output pattern, and no change in response was seen with age.

9. THE EJACULATE AT PUBERTY

Skinner et al. (1968) attempted to collect electroejaculates from 30 days of age onward, but were successful only after 42 days in spring born lambs, and 63 days in lambs born out of season. Spermatozoa appeared in ejaculates at 115 - 146 days. Breed differences in the time of appearance of spermatozoa in ejaculates, summarized by Skinner and Rowson (1968), range from 103 days in Suffolk crossbreds to 213 days in Merinos. The age at which spermatozoa first appear in ejaculates was markedly affected by the season of birth and was most rapid in spring born rams (Alberio and Colas, 1976). Epididymal spermatozoal reserves were lower in immature rams, and an increasingly large number of spermatozoa per ejaculate were noted throughout puberty (Skinner and Rowson, 1968; Courot, 1976). The latter author noted that this increase in production of spermatozoa was accompanied by an improvement in motility and morphology, while Skinner and Rowson (1968) noted that seminal fructose and citric acid levels, indicative of androgen levels, also rose during pubertal development.

10. SPERMATOGENESIS IN THE ADULT

Spermatozoal production is a cyclical event subdivided on the basis of seminiferous tubule cellular content into eight stages, or if regard to the spermatozoal acrosomal development is included, into 14 stages (Courot et al., 1970). A detailed description of the dynamics of spermatogenesis in the ram is to be found in reviews by Ortavant et al. (1969) and Courot et al. (1970).

Type A spermatogonia divide to provide a replacement spermatogonium and an intermediate spermatogonium. Type B spermatogonia are produced subsequently and divide to produce up to 16 diploid cells, the primary

spermatocytes. Meiotic division of these cells results in the production of X and Y chromosome bearing secondary spermatocytes. Secondary spermatocytes are short lived and a further mitotic division produces the round spermatids. Metamorphosis of spermatids to become spermatozoa (Spermiogenesis) is the sum of nuclear and cytoplasmic changes including nuclear elongation, acrosome formation, mitochondrial aggregation and tail formation.

Though the immediate post-pubertal testis produces spermatozoa by a cyclical process identical to the seminiferous cycle of the adult, continued testicular growth to adult weight is responsible for increased spermatozoal production. The duration of the spermatogenic cycle of the ram was found to be 49 days (Ortavant et al., 1969) and epididymal passage usually required 11 - 15 days. Spermatogenic cycles of the pubertal ram were longer than those of the adult due to cellular degeneration and delayed cellular processes, and the quality of semen produced following the first appearance of spermatozoa in the ejaculate improved dramatically during the first 3 months of production (Courot, 1976).

The yield of spermatozoa was influenced by many factors, many of which influence the primary stages of spermatogenesis, the spermatogonial divisions (Courot et al., 1970). Many spermatocytes degenerated, particularly under influences such as elevated scrotal temperature, and hence limited the efficiency of spermatogenesis (Ortavant et al., 1969). Total spermatozoal numbers produced by the adult depend largely on the testicular volume (Lino, 1972). Large photoperiod-induced changes in testicular volume of rams have been reported (Lincoln, 1976a) together with a decrease in spermatozoal production per unit weight of testis (Barrell, 1976), were responsible for decreased spermatozoal output in the non-breeding season.

11. PURPOSE OF THE PRESENT STUDY

The investigations described in this thesis involved the use of radioimmunoassay procedures for measurement of peripheral plasma levels of ovine LH, testosterone and prolactin in order to study aspects of their secretion and physiological functions in the New Zealand Romney ram.

The study was designed to establish the patterns of secretion of

these hormones from birth to sexual maturation in the ram, and to attempt to relate certain testicular and epididymal developmental parameters to endocrine parameters. In order to achieve this, it was considered necessary to: examine closely the short-term secretion patterns of these hormones at various stages of development including the adult; to study the longitudinal pattern of changes in hormone levels with maturation; and to employ techniques such as GnRH administration to delineate changes of hormone output specific to the pituitary gland and gonad.

A continuation of the study was to examine the use of various hormonal treatments in manipulating hormonal patterns during the onset of puberty, and to this end GnRH and androgen were given to developing rams and studies on prenatal androgen administration were undertaken.

CHAPTER II

MATERIALS AND METHODS

1. ANIMALS

Animals of the New Zealand Romney breed were used in experiments described in this thesis. In addition, four Dorset ram lambs were used in Experiment 3.1, a pilot study for Experiment 3.2. All animals were subject to general clinical and reproductive system examinations prior to acceptance for experimental procedures.

2. ANIMAL MANAGEMENT

(1) On Pasture

Lambs used in Experiments 3, 4 and 5 were maintained with their mothers for the first three months of life on ryegrass and white clover dominant pasture. Ewes were given an anthelmintic drench ("Wormguard" TVL (N.Z.)) and vaccinated with a multiple clostridial vaccine ("Covax-5" TVL (N.Z.)) prior to lambing. Lambs were vaccinated for enterotoxaemia and tetanus ("PK-antitet", TVL (N.Z.)) at tail docking one to two weeks after birth, were weaned at approximately three months of age, and drenched with anthelmintic ("Nilverm", ICI (N.Z.) Limited) at weaning and periodically thereafter. Six weeks after shearing at approximately four months of age animals were sprayed for ectoparasites with diazinon. Lambs were weighed at each sample collection period.

Ram lambs used in Experiment 4 (1975 - 1976) remained healthy throughout. However, during 1974 - 1975, those used in Experiments 3.2, 3.3, 7.1 and 7.2 encountered the following animal health problems: a minor outbreak of contagious ophthalmia occurred in January 1975; ryegrass staggers occurred in March 1975 and the few animals severely affected were temporarily excluded from the experiments; a serious outbreak of facial eczema occurred toward the conclusion of the study in April 1975, and resulted in termination of the study two weeks earlier than planned. Seriously affected animals were culled from sampling routines. Some masculinized females described in Chapter 7 were predisposed to urine scald and were treated prophylactically with diazinon to avoid cutaneous myiasis.

(2) Indoors.

Adult rams used in Experiment 1 were housed and artificial lighting corresponding to the natural daylight length was provided. Those used in Experiment 2 were housed in a room subject to natural lighting. All these rams weighed 70 - 80 Kg and were held in individual crates and fed a maintenance diet of chaffed hay and concentrates, and water ad libitum. Feeding was undertaken at 0900 h each day. Rams were allowed approximately one week to adjust to the new environment and diet before sampling, then were returned to pasture between experimental procedures.

3. SURGICAL PROCEDURES

(1) Catheterization.

Polythene (Portex Ltd.) or siliconised rubber tubing ("Silastic", Dow Corning, Australia) of approximately 2 mm diameter was used for indwelling jugular cannulae. Catheters were inserted into both jugular veins to minimise sample collection failure due to thrombus formation. The jugular groove was prepared for surgery and after injection of subcutaneous local anaesthetic (2% xylocaine), the catheters were inserted via 10 gauge hypodermic needles. Occasionally rams were lightly sedated for catheterization using 0.3 ml of xylazine hydrochloride (2% (w/v) "Rompun", Bayer, Germany). A protective horizontal mattress suture was placed over the catheter at the skin puncture site, then transfixing sutures through surgical adhesive tape around the catheter, were used to fix the catheter to the skin immediately anterior to the skin puncture, caudal to the ear, and along the dorsum. Heparinized (15 I.U./ml) sterile 0.9% saline was infused continuously at 1 cm^3 per hour to maintain catheter patency. As required, blood samples were withdrawn and GnRH injected via three-way taps at the infusion pump, and catheters were flushed with heparinized saline immediately after these procedures. For Experiment 1 a remote infusion and sampling station was established outside the animal accommodation room so that the rams were not disturbed during bleeding procedures.

(2) Hypophysectomy.

One adult ewe was hypophysectomised to provide gonadotrophin and prolactin-free plasma for use in radioimmunoassays. Anaesthesia was induced using sodium thiamylal ("Surital", Parks Davis (N.Z.) Limited)

and maintained with a mixture of approximately 1.5% Halothane vapour ("Fluothane", ICI, U.K.) in oxygen, administered via endotracheal tube.

A midventral incision was made caudally from the level of the rami of the mandible and the larynx retracted laterally after blunt dissection. Parapharyngeal blunt surgical dissection was employed to expose the ventral aspect of the sphenoid bone. Entry to the pituitary fossa was made using a dental burr, and a midline dural incision made to expose the hypophysis. Pituitary tissue was removed by a suction curette after gentle maceration then the fossa was packed with silver nitrate-impregnated absorbable gelatin sponge ("Spongostan", Ferrosan, Denmark). Parapharyngeal tissues were repositioned using absorbable sutures and the skin incision was closed with monofilament nylon. An intravenous electrolyte solution was administered throughout.

Post-operatively, penicillin and streptomycin were given prophylactically while 12 mg of dexamethasone trimethly acetate ("Opticortinol" Ciba-Geigy, (N.Z.) Limited) was administered subcutaneously each week. 400 ml of blood was collected by jugular venepuncture each week, plasma was separated by centrifugation, then withdrawn and stored frozen until required. No recognisable pituitary tissue was found upon post mortem histological examination.

(3) Castration.

The scrotal sac and ventral abdominal area were prepared for surgery and local anaesthetic (2% xylocaine) injected into the neck of the scrotum and spermatic cords. A mid-ventral incision was made and the testes exposed through the tunica vaginalis. Testes were removed after severing the spermatic cord with emasculators. Extraneous tissue was removed then testes and epididymides were carefully separated and weighed individually.

4. BLOOD COLLECTION

For Experiments 1 and 2, blood was withdrawn via catheters as described above, then was immediately heparinized (15 I.U./ml). All other blood samples were collected into heparinized (150 I.U.) evacuated glass tubes after jugular venepuncture. Samples were centrifuged immediately after collection and plasma withdrawn and frozen over solid CO_2 (Experiments 3, 4 and 5) or in a freezer (Experiments 1 and 2) and stored at -20°C until required for assays.

5. HISTOLOGICAL PROCEDURES

5 mm thick equatorial sections of testicular or epididymal tissue were carefully cut and fixed in Bouin's fluid for 24 hours before embedding in paraffin wax. Two 5 µm sections of each specimen were stained with haematoxylin and eosin and examined under a light microscope.

Estimates of seminiferous and epididymal tubule diameters for each specimen were made as the mean recorded from 10 circular seminiferous tubules and up to 10 circular epididymal tubule sections (where available), measurements being made on a microscope fitted with an eyepiece micrometer.

6. EPIDIDYMAL SPERMATOZOAL RESERVES

Both epididymides from each animal were processed together; hence results presented represent the total epididymal spermatozoal reserves for each animal. Spermatozoal reserves were estimated after preparation in a manner similar to that described by Lino (1972): after sectioning into small pieces, epididymides were homogenized in 150 ml of 0.9% saline. Then homogenates were strained through coarse gauze, increased in volume to 200 ml with 0.9% saline, and aliquots were diluted to appropriate concentrations for duplicate counting in a haemocytometer. Total epididymal spermatozoal reserves were comparable with those reported by Barrell (1976) who used essentially the same technique.

7. 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, respectively, in PBS.

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

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

PBS-0.1% BSA and PBS-3.0% BSA were solutions of 0.1% (w/v) and 3.0% (w/v) bovine serum albumin (Sigma Chemical Company, U.S.A.) respectively, in PBS.

Organic solvents generally were technical grade and were distilled prior to use. Ethanol was refluxed over m-phenylenediamine then distilled twice before use.

Scintillation fluid consisted of toluene-triton X-100 (2:1) containing 3 g of 2,5 diphenyloxazole (PPO) and 100 mg of (1,4-bis(2-(5-phenyloxazolyl)) benzene; Phenyl-oxazolylphenyl-oxazolyl-phenyl) (POPOP) per litre.

(2) LH Radioimmunoassay.

A double antibody radioimmunoassay technique similar to that described by Niswender et al. (1969) was employed to measure ovine-LH concentrations.

(i) Radioiodination of Ovine-LH with ^{125}I. Radioiodination of ovine-LH was performed using a modification of the chloramine T method of Greenwood et al. (1963). One mCi of iodine-125 (100 mCi/ml, The Radiochemical Centre, Amersham, U.K.) was added to 2 µg of highly purified LH (LER-1374A) 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, then two minutes later, 500 µg of sodium metabisulphite in 50 µl of 0.05 M phosphate buffer, pH 7.5, was added to stop the reaction. After addition of 100 µl of transfer solution (1% (w/v) potassium iodide, 0.01% (w/v) bromophenol blue and 16% (w/v) sucrose in distilled water) the mixture was transferred 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 vial was rinsed with 100 µl of a second transfer solution containing potassium iodide and bromophenol blue as above, and 8% sucrose, which also was transferred to the column. 1 ml fractions eluted from the column with PBS-1% EW were collected into tubes containing 1 ml of PBS-1% EW and counted for radioactivity. The highest number of counts usually appeared in tube 5, and this was frozen until required for the assay.

(ii) Preparation of Precipitating Antibody. Antisera against rabbit gamma globulin was raised in adult sheep by intramuscular injection of 25 mg of rabbit gamma globulin ("Pentex"fraction II, Miles Laboratories, U.S.A.) in an emulsion of 2.5 ml of complete Freunds adjuvant and 2.5 ml of sterile 0.9% saline. Four booster injections of 10 mg or 25 mg of rabbit gamma globulin were administered in an emulsion of incomplete Freunds adjuvant and saline at approximately three-weekly intervals. Six weekly 500 ml blood samples were collected following the third booster injection and serum withdrawn then titrated against non-immune rabbit serum (NRS) to determine optimal dilutions for both (Figure 2.1). Anti-gamma globulin sera was used diluted 1:7 with 0.5 M EDTA-PBS in the LH assay.

(iii) Radioimmunoassay Procedure. 11 x 75 mm polystyrene tubes were used for all phases of the radioimmunoassay procedure and both unknown plasma samples and assay standards were assayed in triplicate.

Ovine-LH standards (NIH-LH-S18) corresponding to a range of 0 - 8 ng/ml of plasma were prepared in 200 µl of PBS-1% EW. 200 µl of hypophysectomised ewe plasma was added to each standard tube and equivalent quantitites of unknown plasma added to sample tubes. Both standard and sample tubes were adjusted to 0.5 ml with PBS-1% EW then 200 µl of rabbit anti-ovine-LH serum (#15, courtesy of Dr G.D. Niswender, Colorado State University, U.S.A.) diluted 1:80,000 with 0.05 M EDTA-PBS containing NRS (1 in 400) was added. After mixing and incubation at 4^0C for 24 h, approximately 50,000 cpm of radioiodinated ovine-LH in 100 µl of PBS-0.1% BSA were added to each tube, then a further 24 h incubation followed.

200 µl of precipitating antiserum was then added and after a 72 h incubation at 4^0 C for 30 min, the supernatant was removed by aspiration. The precipitate was then counted for radioactivity in a Packard Auto-Gamma Spectrometer (Model 5285).

Each assay contained a triplicate of tubes with the first antibody excluded as a check for non-specific binding of ^{125}I-LH.

An IBM 1620 computer was used to calculate plasma LH concentrations by the method of Burger et al. (1972). With this technique a least mean square regression was calculated for the standard curve and values and 95% confidence intervals of unknown samples computed. A representative standard curve is shown in Figure 2.2.

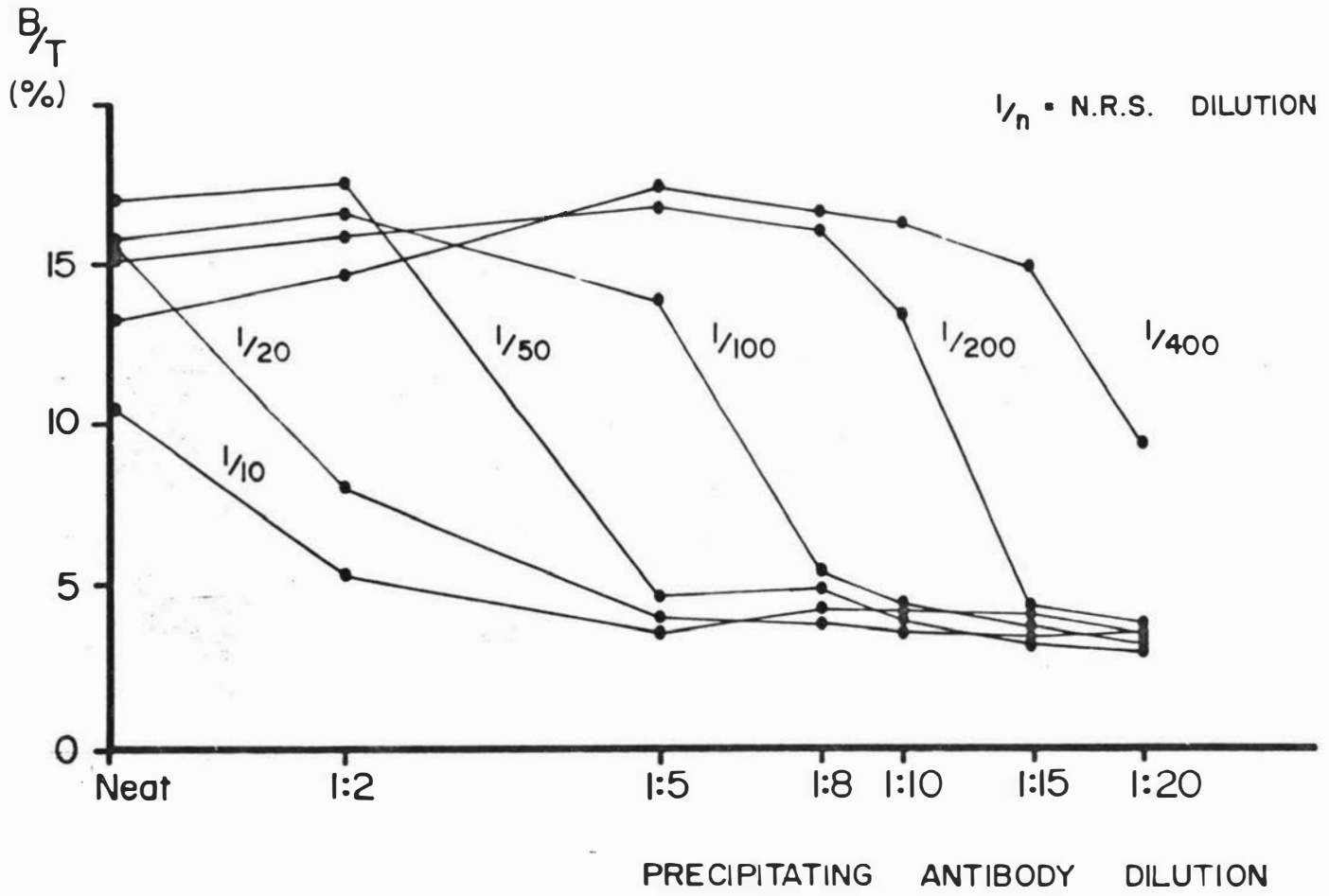


Figure 2.1: Binding of ^{125}I-labelled LH following titration of various dilutions of precipitating antiserum (No. 160) and non-immune rabbit serum (NRS).

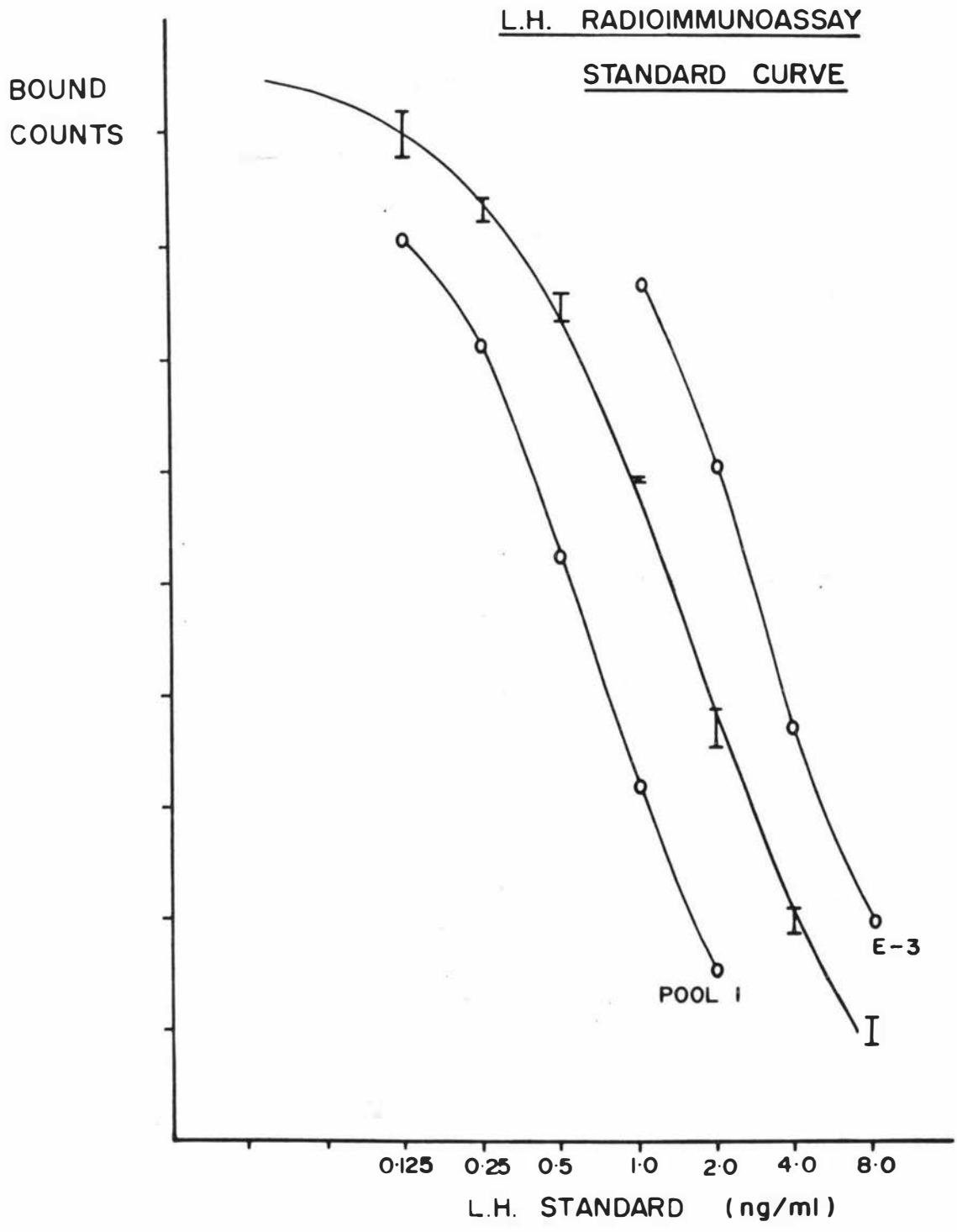


Figure 2.2: Representative standard curve for the LH assay computed by the method of Burger et al. (1972a), and doubling dilutions of reference plasma pools in hypophysectomised sheep plasma.

(iv) Validation of Ovine-LH Radioimmunoassay. The specificity of the ovine-LH antiserum has been determined by Niswender et al. (1969) who showed that estimates of LH potency were unaffected by high levels of FSH, TSH, growth hormone or prolactin.

Hypophysectomised sheep plasma added to standard tubes in place of PBS-1% EW as used by Niswender et al. (1969) considerably increased binding of ^{125}I-LH (Figure 2.3) and its use consequently eliminated the occurrence of false negative LH levels for unknown plasma samples. The lowest detectable plasma LH concentration, defined as the level at which the 95% confidence interval of estimates intercepted zero level (Burger et al., 1972) was between 0.04 and 0.11 ng/ml in 35 assays.

Reduction of the quantity of unknown plasma did not influence estimates of LH concentrations when the total plasma volume in each tube was made up to 200 µl with hypophysectomised sheep plasma (Table 2.1). Results demonstrated close parallelism of unknown sample dose-response curves and standard curves (see also Figure 2.2). Results also indicated that concentrations of LH up to 128 ng/ml of plasma could be measured accurately if sample plasma was diluted with hypophysectomised sheep plasma.

Reproducibility of assay results was determined by repeated estimations of reference plasma pools in consecutive assays. Coefficients of variation were determined after analyses of variance of hormone levels in these reference plasma pools (transformed to logarithms) and are presented in Table 2.2.

On the basis of the validation procedures performed it was considered that this assay provided reliable estimates of plasma ovine-LH concentrations.

(3) Testosterone Radioimmunoassay.

Plasma samples were assayed for testosterone concentrations using a radioimmunoassay based on the method of Smith and Hafs (1973) but incorporating modifications described by Terqui and Thimonier (1974).

(i) Extraction Procedure. Single 500 µl plasma aliquots were extracted with 9.0 ml of toluene-hexane (1:2) in 16 x 125 mm screw-capped glass culture tubes, by vigorous shaking in a laboratory shaker for 10 minutes. The aqueous fraction was frozen then the solvent decanted into 16 x 100 mm glass tubes and evaporated to dryness under air. The walls of each tube were rinsed with 1 ml of dichloromethane

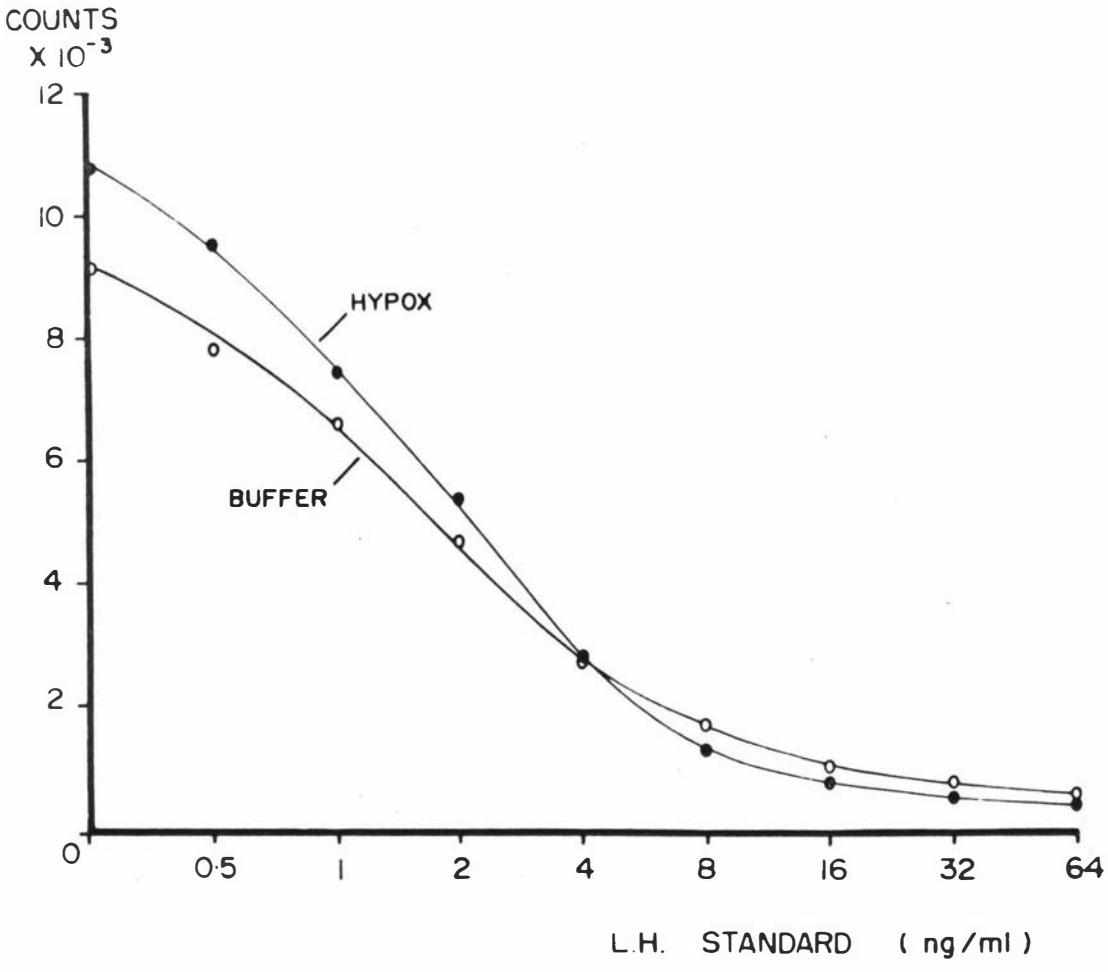


Figure 2.3: Bound counts observed in LH standard curves with and without addition of hypophysectomised sheep plasma.

TABLE 2.1.

Effect on estimates of plasma LH concentrations of dilution of reference plasma samples with hypophysectomised sheep plasma.

Dilution	Mean Plasma LH Concentrations (ng/ml ± SEM)				
	E-3 (n=4)	P-1 (n=5)	228 (n=13)	W-1 (n=13)	W-2 (n=1)
Undiluted	4.39 ± 0.16	4.26 ± 0.23	4.41 ± 0.14	5.91 ± 0.18	5.38
1:1	4.14 ± 0.26	4.22 ± 0.36			5.18
1:3	3.85 ± 0.37	4.06 ± 0.36	4.03 ± 0.16	6.26 ± 0.28	4.92
1:7	4.14 ± 0.35	4.10 ± 0.28			4.84
1:15					5.28

TABLE 2.2.

Coefficients of variation (CV) for the ovine LH assay based on repeated estimates of reference plasma samples.

Reference Plasma	Number of Assays	Replicates Within Assays	Mean LH Concentration (ng/ml)	Between Assay CV (%)	Within Assay CV (%)
E-3	35	5	4.30	13.1	4.6
50	4	5	0.15	12.7	7.5
228	5	5	4,41	8.8	4.7
W-3	6	5	5.91	13.2	7.3

and tubes again dried under air. Recovery of tritiated testosterone added to plasma, prior to extraction was 77.56 ± 0.32%.

(ii) Radioimmunoassay Procedure. Plasma extracts were redissolved in 500 µl of ethanol and three 100 µl aliquots transferred to 11 x 75 mm polystyrene tubes for assay. Testosterone (Mann Research Laboratories, U.S.A.) at appropriate concentrations in 100 µl of ethanol, was added to triplicate tubes to provide a series of standards corresponding to plasma testosterone concentrations of 0-50 ng/ml.

After evaporation under air, 400 µl of a solution containing 20,000 cpm of 1, 2, 6, 7-H^3 testosterone (84 Ci/mmole, The Radiochemical Centre, Amersham,U.K.) and rabbit antiserum to testosterone-3-(O-carboxymethyl)-oxime-bovine serum albumin (S250, courtesy of Dr G.D. Niswender, Colorado State University, U.S.A.) at a 1:50,000 dilution in PBS-1% gelatin and NRS (1:800) which had equilibrated at room temperature for 30 minutes, was added to each tube. An initial incubation for 30 min at 40°C was followed by a 2 h incubation at 4°C.

Free steroid was precipitated by addition of 300 µl of dextran-coated charcoal (1% (w/v) Dextran T-70, Pharmacia, Sweden; and 0.5% (w/v) Darco G60 charcoal, Atlas Chemical Industries, U.K., in distilled water), and underwent a 10 min incubation at 4°C before centrifugation at 1900g for 10 min at 4°C. 0.5 ml aliquots of supernatant were transferred to glass scintillation vials and 5.0 ml of scintillation fluid added. The radioactivity was counted in a Beckman (Model LS-350) liquid scintillation counter or in a Packard Tri-carb scintillation spectrometer (Model 3375), and hormone levels computed as described above for the LH assay.

(iii) Testosterone Radioimmunoassay Validation. The specificity of the testosterone antiserum has been determined by Dr V.L. Gay, University of Pittsburg, U.S.A., (pers. comm.). Oestradiol and androstenedione cross-reacted less than 1%, 3α and 3 β- androstanediol cross-reacted 14 and 22% respectively, and dihydrotestosterone displayed a 69% cross-reactivity. All other androgen precursors examined, progesterone, oestrone, corticosteroids, and cholesterol failed to show cross-reactivity greater than 0.1%. Since testosterone is the major androgen in ram lambs (Skinner et al., 1968), dihydro-testerone is present in plasma in concentrations proportionate to those of testosterone (Vermuelen, 1976), and since other androgens possess very low biological potencies (Ismail, 1976), these cross

reactions were considered to be of minimal significance to the determinations of testosterone concentrations.

Serial dilution of reference plasma samples with wether plasma did not influence the quantity of testosterone measured (Table 2.3), and demonstrated close parallelism of unknown sample dose-response curves with standard curves. Reproducibility of assay results was by measurement of coefficients of variation as described for the LH assay and are presented in Table 2.4

These validation procedures showed the testosterone radioimmunoassay provided reliable estimates of plasma testosterone concentrations.

(4) Ovine Prolactin Radioimmunoassay

(i) Radioiodination of Ovine Prolactin with ^{125}I. Radioiodination of ovine prolactin was performed using the method of Greenwood et al. (1963) with modifications described by Langley (1974).

One mCi of iodine-125 (100 mCi/ml, The Radiochemical Centre, Amersham, U.K.) was added to 5 µg of highly purified ovine prolactin (LER-860-2, courtesy of Dr L.E. Reichert Jr., Emory University, U.S.A.) in 10 µl of 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 and the reaction allowed to proceed for one minute before addition of 20 µg of sodium metabisulphite in 20 µl of 0.5 M phosphate buffer, pH 7.5, to stop the reaction. The reagents were mixed for a further 60 seconds, then a transfer solution consisting of 30 µl of 1% (w/v) potassium iodide, 0.01% (w/v) bromophenol blue and 16% (w/v) sucrose in 0.5 M phosphate buffer, pH 7.5, plus 30 µl of hypophysectomised sheep plasma, was added. The mixture was then transferred to a 0.7 x 18 cm polyacrylamide gel column (Biogel P60, Biorad Laboratories, U.S.A.) which had been equilibrated with 15 ml of 0.2 M barbital buffer, pH 8.6, containing 20% (w/v) acetone, and 2.0 ml of the same buffer containing 5% (w/v) bovine serum albumin. One millilitre fractions were collected into tubes containing 1 ml of PBS-0.1% gelatin and aliquots counted for radioactivity. The fraction containing the highest counts was further purified on a 1 x 25 cm dextran gel column (Sephadex G100, Pharmacia, Sweden) which had been equilibrated with 25 ml of 0.2 M barbital buffer, pH 8.6, containing 20% acetone (w/v), and 2.0 ml of the same buffer containing 5% bovine serum albumin. One millilitre fractions were collected into 1 ml of PBS-0.1% gelatin and the fraction containing

TABLE 2.3.

Effect of dilution of reference plasma samples with wether plasma on estimates of Testosterone concentration.

Dilution	Mean Testosterone Concentration (ng/ml)			
	10 (n=2)	13 (n=6)	18 (n=1)	228 (n=1)
Undiluted	4.03	11.43	6.69	6.25
1:1	4.08	12.96	6.26	6.60
1:3	3.80	13.20	6.63	7.45

TABLE 2.4.

Coefficients of variation (CV) for the testosterone assay based on repeated estimates of reference plasma samples.

Reference Plasma	Number of Assays	Replicates Within Assays	Mean Testosterone Concentration (ng/ml)	Between Assay CV (%)	Within Assay CV (%)
18	13	3	9.50	5.54	7.04
50	26	3	0.30	22.0	27.6
228	26	3	5.50	13.36	8.41

the highest counts was used in the radioimmunoassay.

(ii) Preparation of Prolactin Antiserum. Antiserum was kindly provided by Prof D.S. Flux, Massey University. Anti-ovine prolactin serum was raised in a rabbit following intradermal injection of 4 mg of ovine prolactin in complete Freunds adjuvant, and three subsequent boosters of 2 mg of ovine prolactin at two week intervals. Two weeks later, blood was collected and serum withdrawn and titrated for its ability to bind ^{125}I-labelled ovine prolactin.

(iii) Radioimmunoassay Procedure. 11 x 75 mm polystyrene tubes were used for all phases of the radioimmunoassay procedure and standards and unknown samples were assayed in triplicate.

Standard solutions of ovine prolactin (NIH-P-S11) in PBS-3% BSA were prepared to provide a standard curve corresponding to plasma prolactin concentrations of 0 - 160 ng/ml. 200 µl of standard, 200 µl of hypophysectomised sheep plasma, and 100 µl of 0.01 M EDTA-PBS-0.1% gelatin, were added to standard tubes, while 200 µl of unknown plasma and 300 µl of 0.01 M EDTA-PBS-0.1% gelatin were added to sample tubes. 200 µl of rabbit anti-prolactin serum, diluted 1:12,500 in 0.02 M EDTA-PBS containing NRS (1:400), was added to each tube. Reagents were mixed prior to incubation at 4^{0}C for 24 h. Radioiodinated prolactin (approximately 25,000 cpm in 100 µl of 0.01 M EDTA-PBS-0.1% gelatin) was added to each tube and a second 24 h incubation at 4^{0}C followed. 200 µl of precipitating antibody, similar to that described for the LH assay and which had previously been titrated against NRS (see Figure 2.4), diluted 1 in 30 with 0.01 M EDTA-PBS-0.1% gelatin, was then added. After a 72 h incubation at 4^{0}C, precipitation was completed by centrifugation (1900g) for 30 min and the supernatant was removed by aspiration. Bound radioactivity was counted in a Packard Auto-Gamma Scintillation Spectrometer (Model 5285).

Non-specific binding and plasma prolactin concentrations were determined as described for the LH assay.

(iv) Validation of Ovine Prolactin Assay. Anti-ovine prolactin serum was found to be free of cross-reactivity with the ovine hormones LH, FSH, ACTH, growth hormone and TSH (Figure 2.5).

Assay sensitivity ranged from 2.0 - 4.5 ng/ml in eight assays, and non-specific binding was less than 10% of the zero standard bound count.

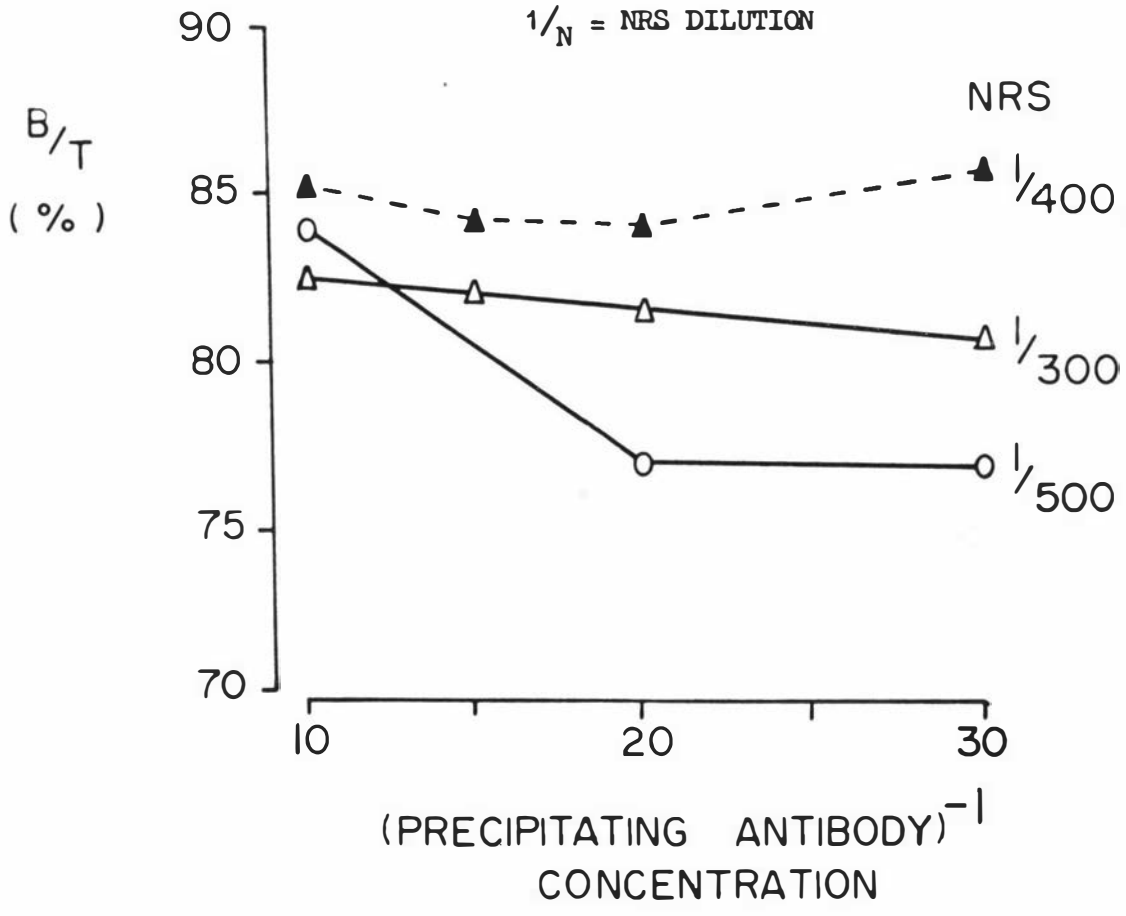


Figure 2.4: Binding of ^{125}I-labelled prolactin following titration of various dilutions of precipitating antiserum (No. 161) and non-immune rabbit serum (NRS).

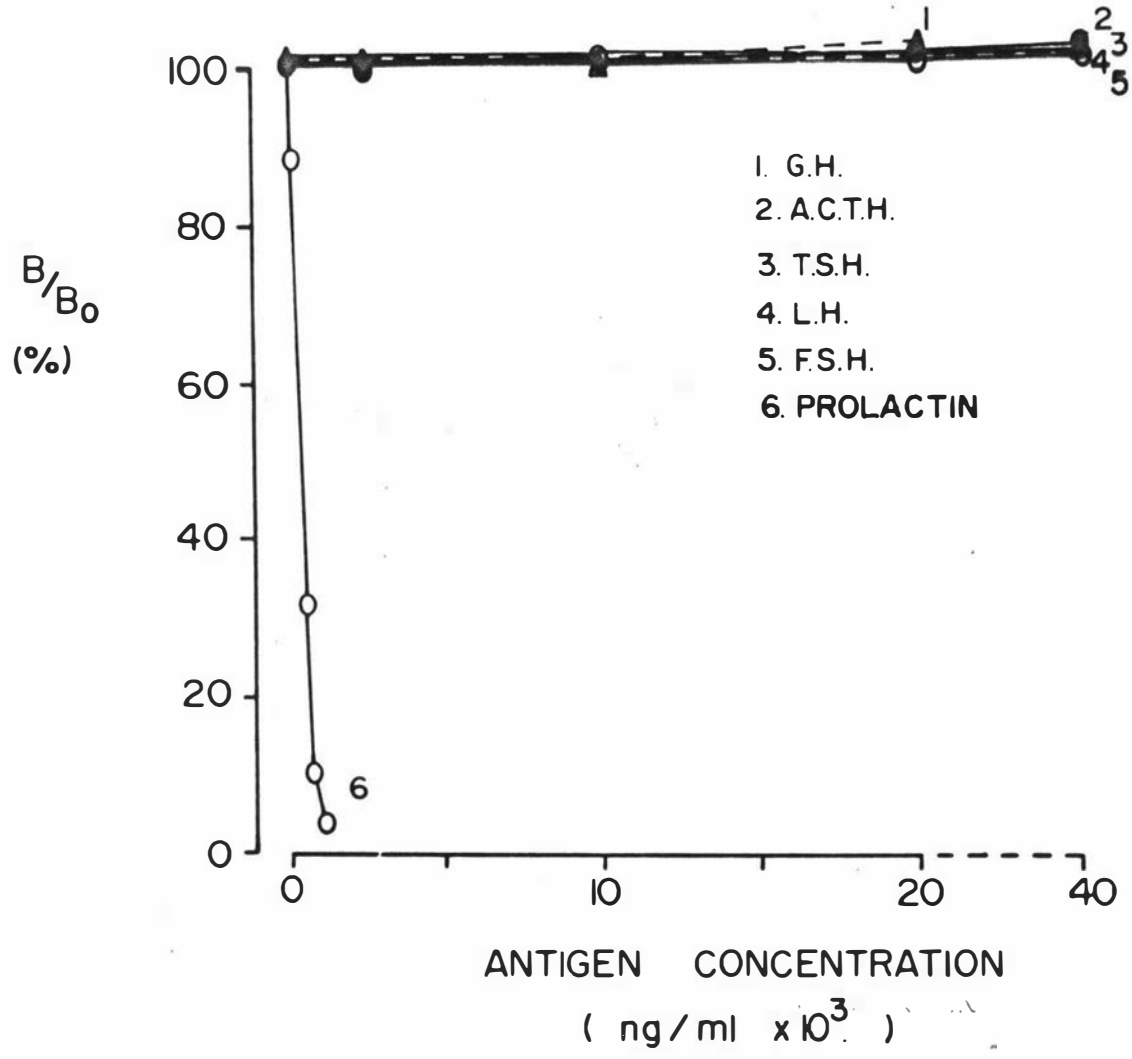


Figure 2.5: Cross-reactivity of anterior pituitary hormones in the ovine prolactin radioimmunoassay.

Dilution of reference plasma samples with hypophysectomised sheep plasma (Table 2.5) confirmed parallelism between sample dose-response curves and standard curves. Reproducibility of results was tested by repeated estimations of reference plasma samples (Table 2.6) and was satisfactory. Prolactin levels measured corresponded closely to levels in the same reference plasma samples measured in a similar assay described by Barrell (1976).

On the basis of the validation procedures undertaken, it was concluded that this assay provided accurate assessments of ovine plasma prolactin concentrations.

Table 2.5 Effect of dilution of reference plasma samples with hypophysectomised sheep plasma on estimates of plasma prolactin concentration (ng/ml ± SEM)

Dilution	Mean Prolactin Concentrations (ng/ml)		
Plasma	50 (n=24)	228 (n=24)	W - 1 (n=16)
Undiluted	13.15 ± 0.40	22.80 ± 0.77	50.91 ± 0.75
1:1	13.58 ± 0.82	22.99 ± 1.02	52.78 ± 2.88

8. EXPERIMENTAL DESIGN AND ANALYSIS

Details of the experimental designs and statistical analyses used for individual studies are given in appropriate sections of each chapter.

(1) Analysis of Variance.

The analysis of variance applied to each of the variables measured repeatedly in Experiments 2, 3, 4 and 5 could be represented by the following model:

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

where each individual observation, or mean of several observations made on each animal in each treatment or age, was assumed to be the sum of a number of parameters:

μ - the general population mean, and the deviations due to the following effects:

TABLE 2.6.

Coefficients of variation (CV) for the ovine prolactin assay based on repeated estimates of reference plasma samples.

Reference Plasma	Number of Assays	Replicates Within Assays	Mean Prolactin Concentration (ng/ml)	Between Assay CV (%)	Within Assay CV (%)
50	6	4	11.61	5.10	4.21
25	6	4	7.45	9.86	7.38
228	6	4	23.00	12.64	2.29
114	6	4	19.23	7.76	4.11
W-1	4	4	52.29	2.00	1.76
W-2	4	4	67.30	11.02	5.54

α_i - the "main effect" of the ith age or treatment

β_j - the "main effect" of the jth animal or treatment

$(\alpha\beta)_{ij}$ - the effect of the interaction of the ith age or treatment on the jth animal or treatment

ξ_{ijk} - a random error from a distribution with zero mean and homogeneous variance, estimated by the variance within subclasses.

A further error variance, between repeated observations from individual rams within sampling periods, which could be estimated for hormone concentrations, seminiferous or epididymal tubule diameters, and for epididymal spermatozoal reserve data, was not included in the analysis.

The main effects 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 which were constructed to test specific hypotheses. Individual coefficients were weighted for differences in numbers of animals between groups. Contrasts were constructed *a priori* and the number of comparisons for any effect did not 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 tables showing summaries of analyses of variance contain discrepancies between the total number of degrees of freedom and the number of single degrees of freedom contrasts computed. Contrast numbers in the text refer only to contrasts which are summarised 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.

For hormone data, occasional missing measurements were evident. Where unequal subclass analyses were not applied, missing data were substituted by the mean of the preceding and succeeding measurements.

(3) Transformations.

All estimates of hormone concentrations except those used in calculations of GnRH induced LH and testosterone output curves, were transferred to logarithms prior to statistical analyses, using the relationship:

logarithm of hormone concentration = $100 \log_{10} (x + 1 \cdot 1)$

where x is the hormone concentration in ng/ml.

This relationship 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) Analysis of LH and Testosterone Response Curves Following GnRH administration.

(i) Estimation of Total Hormone Output. The areas under individual (Experiments 2, 3 and 4) or mean (Experiment 5) LH and testosterone response curves were calculated as a measure of total hormone output in response to GnRH administration. Areas were calculated by integrating hormone concentrations in samples with time intervals between samples, and the parameter transformed to $\log_{10}$ for use in statistical analyses.

Throughout the text of this thesis, response curve areas thus calculated were referred to as the "total" hormone output.

Coefficients of regressions of various parameters (e.g. total testosterone output on total LH output) were calculated for each age (Experiments 3 and 4) or for each dose of GnRH (Experiment 2) and analyses of variance of regression coefficients were performed to determine the relationships between LH and testosterone output following GnRH administration. This form of analysis is referred to in this thesis as analyses of variance of regression coefficients.

(ii) Time Sequence of Hormone Output. An estimate of the time sequence of hormone output following GnRH administration was made by calculating linear, quadratic and cubic components of regressions of log hormone level with time after GnRH administration. This was performed on individual (Experiments 2, 3 & 4) or mean (Experiment 5) response curves, and the regression coefficients provided estimates of the shapes of the response curves. Regression coefficients were used as parameters in analyses of variance or *t* tests to examine differences in timing of hormone output in response to GnRH

administration.

Throughout the text of this thesis, regressions thus calculated are referred to as "regression fits" to hormone response curves.

(5) Sequential Sampling Experiments.

Hormone profile data in Experiments 1 and 4 was analysed by a cumulative regression technique. For each ram, log transformed values were progressively summed in numerical order of sampling, to form a series of cumulative totals. The linear, quadratic and cubic components of this cumulative distribution against time was used as parameters in analyses of variance to determine age effects (Experiment 4) or in a Student's t test to determine seasonal effects on hormone secretion profiles (Experiment 1). It was considered that the linear gradient provided a parameter which reflected hormone output more comprehensively than the arithmetic mean of hormone concentrations, while the non-linear components of regression provided estimates of circadian variations in hormone output (Experiment 1).

(6) Computation.

All calculations of totals, regression fits, cumulative regressions, transformations to logarithms, and analyses of variance and regression, were performed using an IBM 1620 computing system. Some small programmes used were compiled by the author with the assistance of Prof R.E. Munford, but general variance and regression analyses used statistical programme packages compiled at Massey University by Dr F.R.M. Cockrem and subsequently modified and extended by Prof R.E. Munford.

CHAPTER III

LH, TESTOSTERONE AND PROLACTIN SECRETION PROFILES OF ROMNEY RAMS

1. INTRODUCTION

At the outset of this study, the only report of intensive monitoring of plasma hormone levels in the ram was that of Bolt (1971) who stated "... the basal level of plasma LH is not well defined...", and he concluded that rams "... may release LH in brief surges rather than at a continuous rate".

With such a paucity of information about the character of endocrine secretion in the ram, it was considered essential to undertake a detailed study of plasma hormone levels in rams. This investigation was performed to determine the plasma levels of LH, testosterone and prolactin throughout the day, and to examine their interrelationships. This experiment was designed to provide base data required to enable appropriate interpretation of the hormonal measurements made during the course of subsequent investigations.

2. EXPERIMENT 1: MATERIALS AND METHODS

(1) Animals .

Six two-year old N.Z. Romney rams were chosen for this study. Rams were managed as described in Chapter 2.

(2) Experimental Procedures.

A summary of the experimental design is presented in Table 3.1. Blood was collected at a remote sampling station via jugular catheter each 20 min for 24 h, then plasma was withdrawn and stored for LH, testosterone and prolactin assay as described in Chapter 2. All samples from individual animals at each sampling period were assayed in the same assay.

(3) Statistical Analysis.

(i) Cumulative Regression. Cumulative regression curves were fitted to hormone output data from each animal at each sampling period, as described in Chapter 2 (p 61). Significant departures from linearity were interpreted as indicating the existence of circadian variations in hormone secretion, while linear coefficients provided estimates of total hormone output. Comparisons between seasons of sampling were made by *t* tests using individual regression coefficients

TABLE 3.1.

Sampling dates and animals used in 24-hour studies.

	Date	Ram
Summer	31.10.73	168(1)
	"	174
	13.11.73	184(1)
	5.12.73	37(1)
	"	168(2)
Winter	21.5.74	37(2)
	"	99
	"	184(2)
	"	228

NOTE: Numbers in parentheses indicate first or second sampling of animals sampled on two occasions.

as parameters.

(ii) LH and Testosterone Peak Relationships. For computation of the relationship between LH and testosterone peaks, LH peaks were defined as the highest LH level recorded singly or in a series of levels greater than 0.5 ng/ml, during pulsatile secretory episodes, and testosterone peaks were represented by the mean of the three testosterone estimates made on samples collected following the LH peak. Hormone levels used in regression analyses to represent concentrations prior to pulses were the means of the three levels preceding the LH peak. Using the data thus defined the regressions of testosterone peaks on LH peaks, and of peak levels on hormone levels prior to the pulses, were calculated.

3. RESULTS.

(1) Secretion Profiles.

See Figures 3.1 - 3.10 and Tables 3.2 and 3.3.

(i) LH . Plasma LH levels showed marked pulsatile fluctuations in each of the nine 24 h sampling periods. LH concentrations ranged from levels below the limit of sensitivity of the LH assay to over 10 ng/ml. The number of LH peaks ranged from 1 to 10 in 24 h, with a mean of 4.55. The overall mean level of LH peaks from all rams was 2.64 ng/ml.

Peaks of LH occurred irregularly, from 40 min to over 21 h apart. The highest level in each peak usually was reached within one sample interval (20 min), though in larger peaks the maximal level often was observed only after the second sample interval. After each peak the decline in LH levels was rapid, resembling a logarithmic decay curve.

Though the season of sampling (summer or winter) did not influence the episodic nature of LH secretion, comparison of linear cumulative regression coefficients (Table 3.3) showed a significantly lower total LH secretion in the winter sampling period (t_7 = 6.07, $P < 0.001$) (See Figure 3.10). The mean LH level in the summer period was 0.58 ng/ml, and in the winter period, 0.23 ng/ml. Cumulative regression components all were essentially linear (correlations 0.84 - 0.99) indicating the absence of circadian variations in LH secretion.

TABLE 3.2.

Summary of plasma hormone levels (ng/ml) observed during 24-hour sampling periods.

Ram	LH			Testosterone			Prolactin	
	Number of peaks	Mean peak level	Range of peak levels	Number of peaks	Mean peak level	Range of peak levels	Number of peaks	Range of plasma levels
SUMMER								
174	5	2.84	0.54 - 3.63	6	2.25	1.87 - 3.32	0	46.1 - 62.2
168	7	1.95	0.70 - 3.95	7	3.56	2.40 - 4.60	0	55.9 - 77.6
	10	1.94	1.06 - 3.37	9	4.43	2.70 - 5.91	0	48.5 - 63.5
184	1	1.14		1	3.48		0	47.5 - 65.9
37	7	6.39	3.46 - 10.85	7	5.24	4.24 - 6.80	0	29.0 - 55.1
Mean concentration during summer sampling periods		0.58			1.45			55.1
WINTER								
37	2	4.91	4.10 - 5.37	2	3.35	3.05 - 3.65	7	8.7 - 56.4
184	1	0.54		4	4.80	3.67 - 6.86	7	4.1 - 53.6
99	4	0.69	0.51 - 0.85	5	1.66	1.22 - 2.36	5	3.0 - 57.3
228	4	1.22	0.50 - 1.67	5	3.64	2.46 - 5.06	0	3.0 - 10.3
Mean concentration during winter sampling period		0.23			0.95			14.2

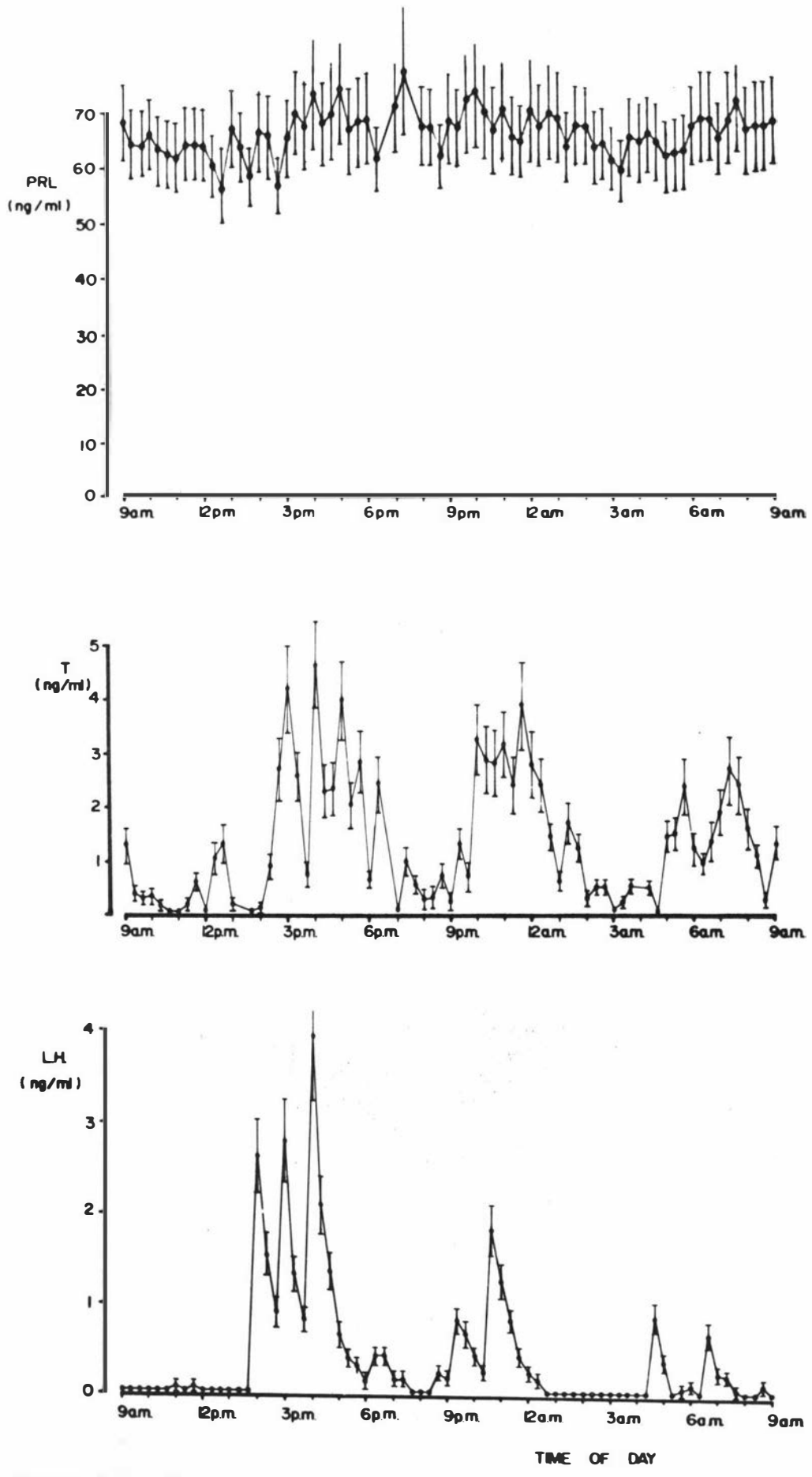


Figure 3.1: LH, testosterone and prolactin 24 h secretion profiles. Ram 168, sampled 31.10.73. (Lights off 7.20p.m. - 5.20a.m.)

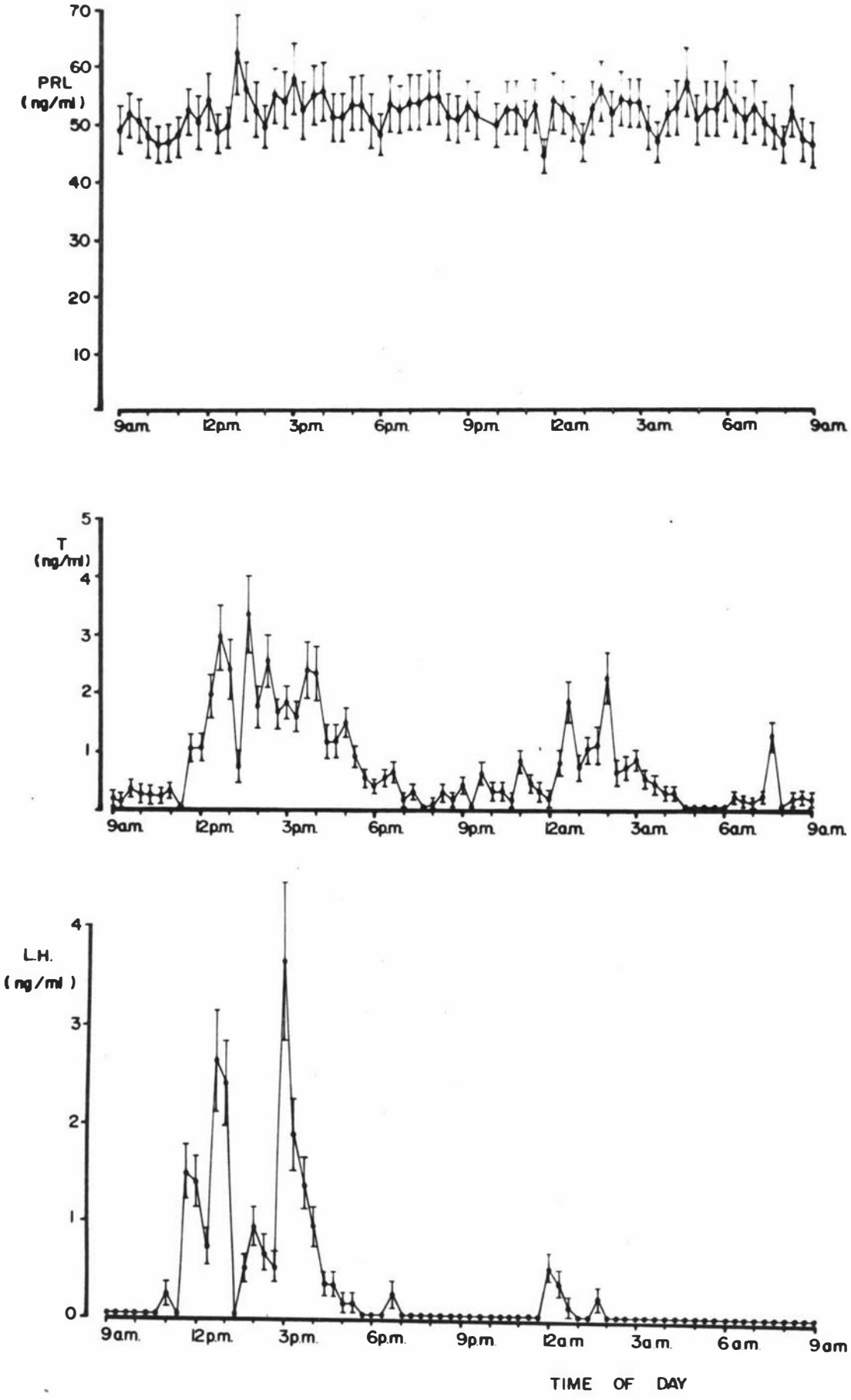


Figure 3.2: LH, testosterone and prolactin 24 h secretion profiles. Ram 174, sampled 31.10.73. (Lights off 7.20p.m. - 5.20a.m.)

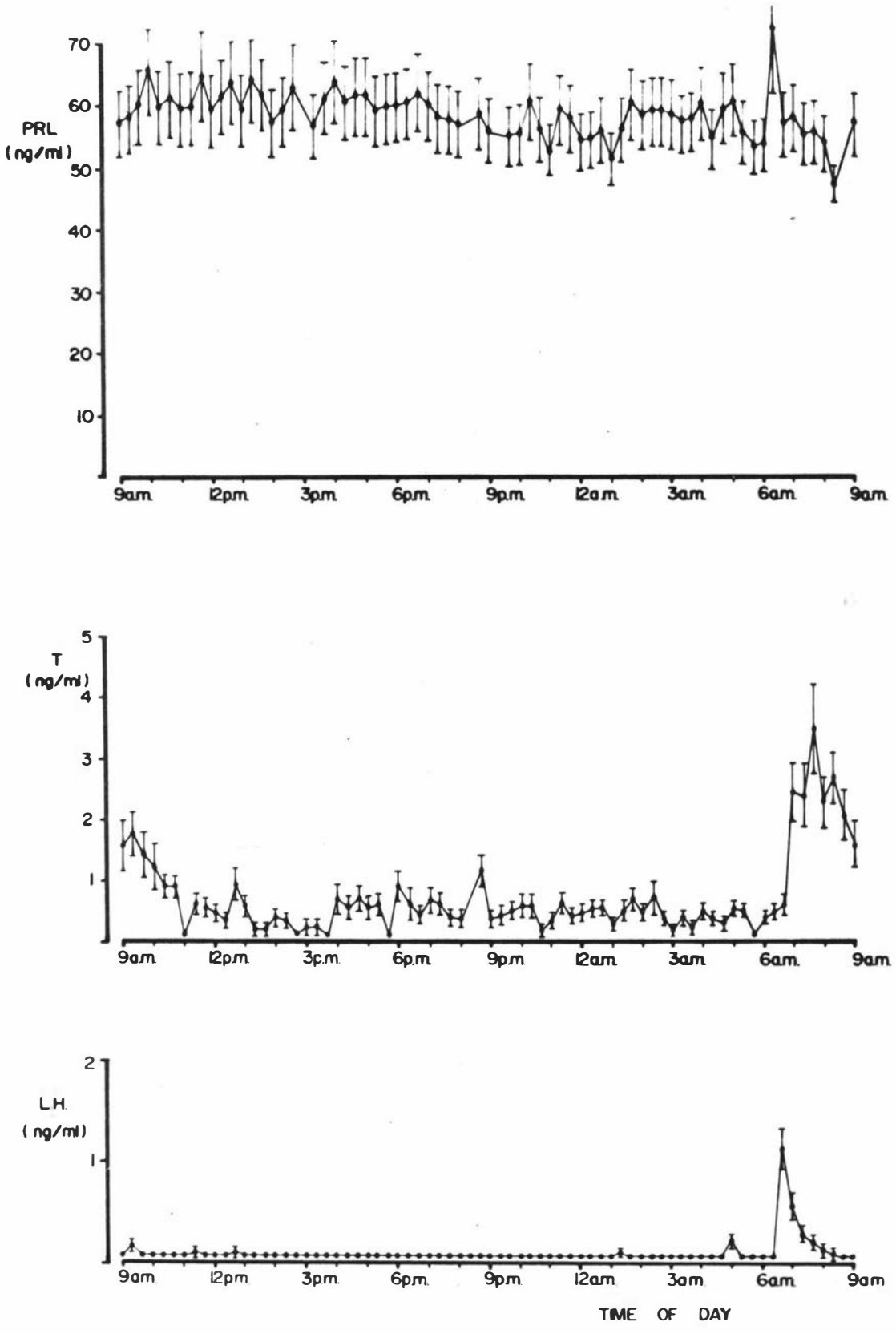


Figure 3.3: LH, testosterone and prolactin 24 h secretion profiles. Ram 184, sampled 13.11.73. (lights off 7.40p.m. - 4.40a.m.)

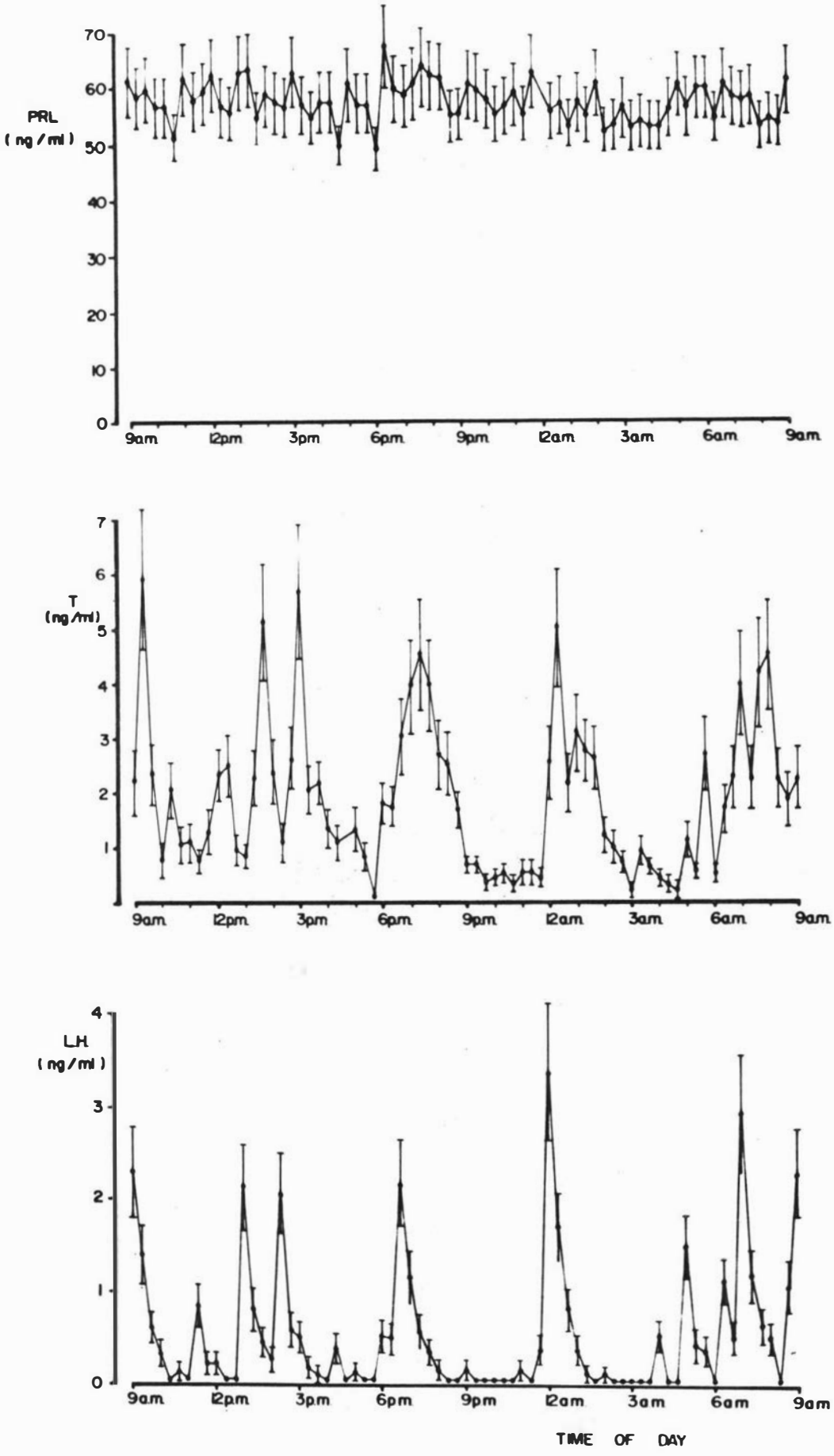


Figure 3.4: LH, testosterone and prolactin 24 h secretion profiles. Ram 168, sampled 5.12.73. (Lights off 8.00p.m. - 4.30a.m.)

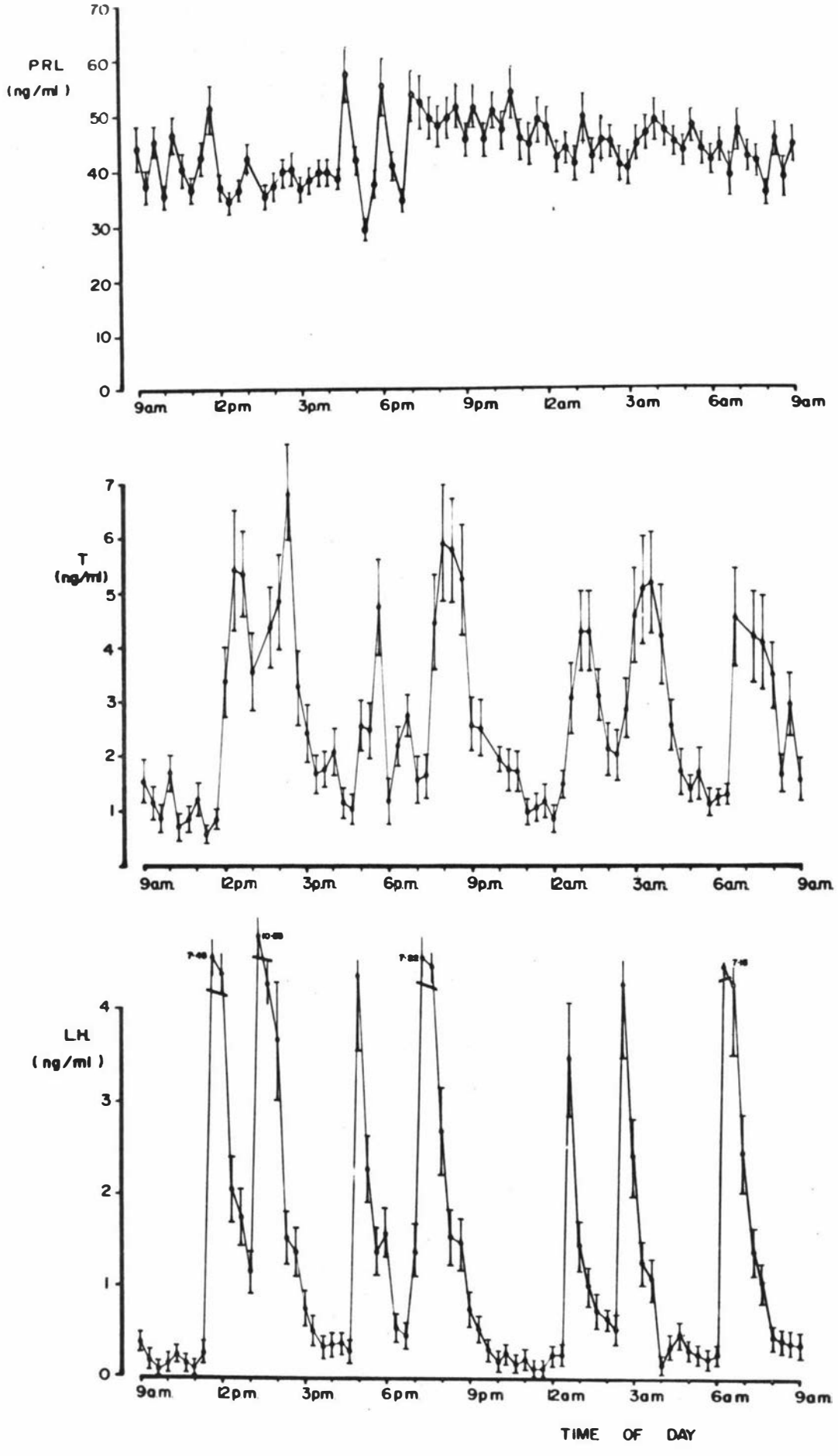


Figure 3.5: LH, testosterone and prolactin 24 h secretion profiles. Ram 37, sampled 5.12.73. (Lights off 8.00p.m. - 4.30a.m.)

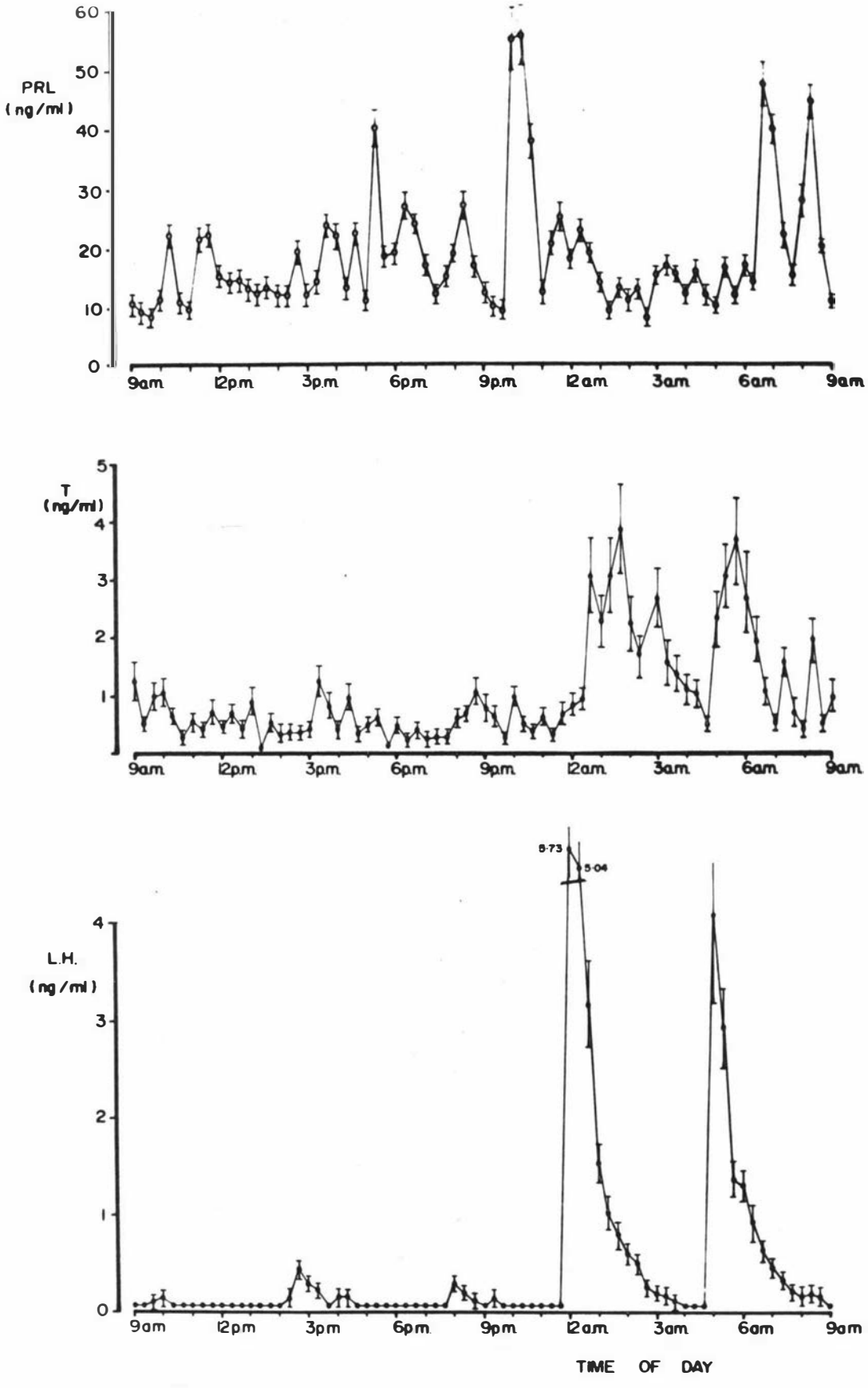


Figure 3.6: LH, testosterone and prolactin 24 h secretion profiles. Ram 37, sampled 21.5.74. (Lights off 5.40p.m. - 7.00a.m.)

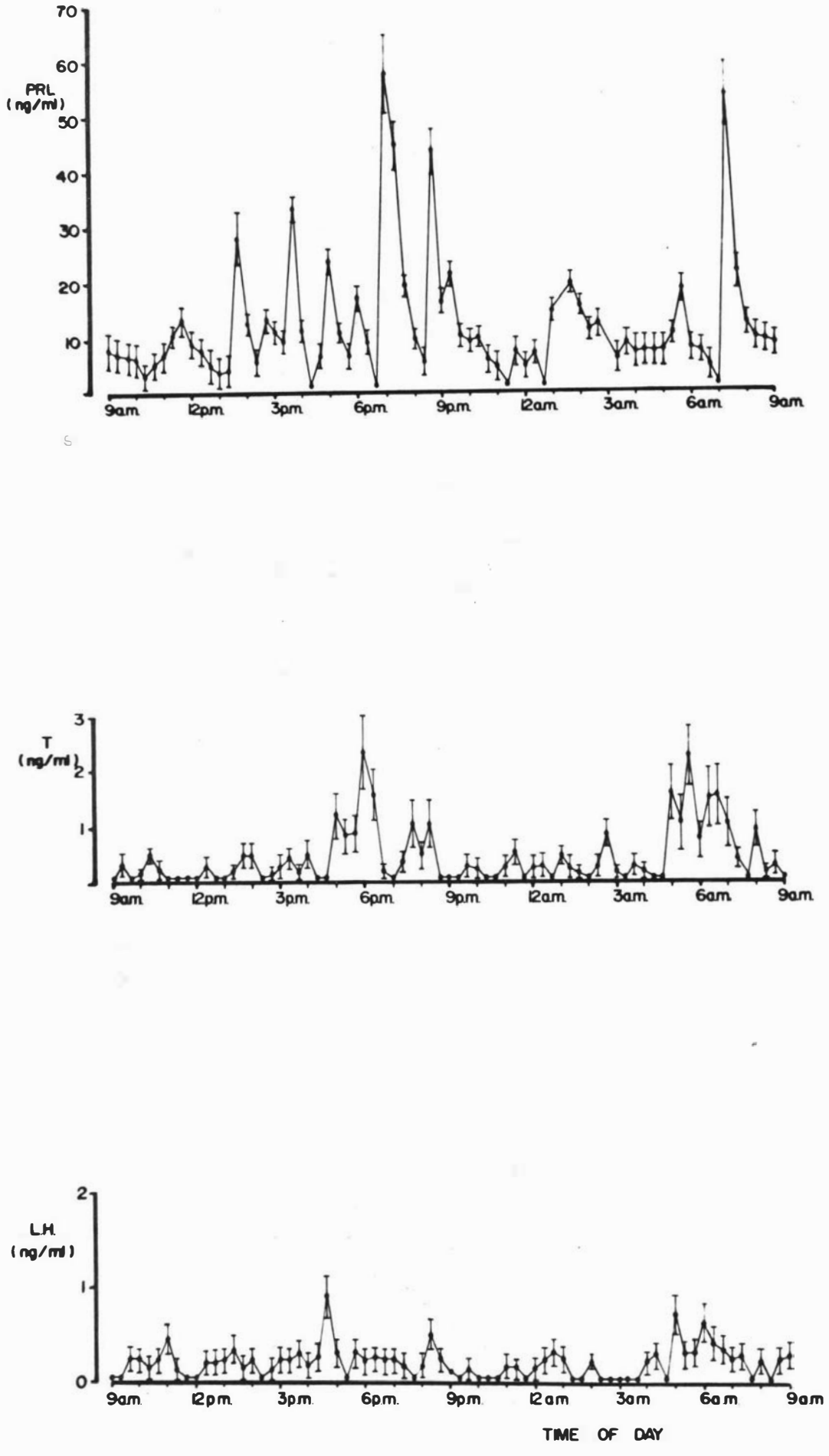


Figure 3.7: LH, testosterone and prolactin 24 h secretion profiles. Ram 99, sampled 21.5.74. (Lights off 5.40p.m. - 7.00a.m.)

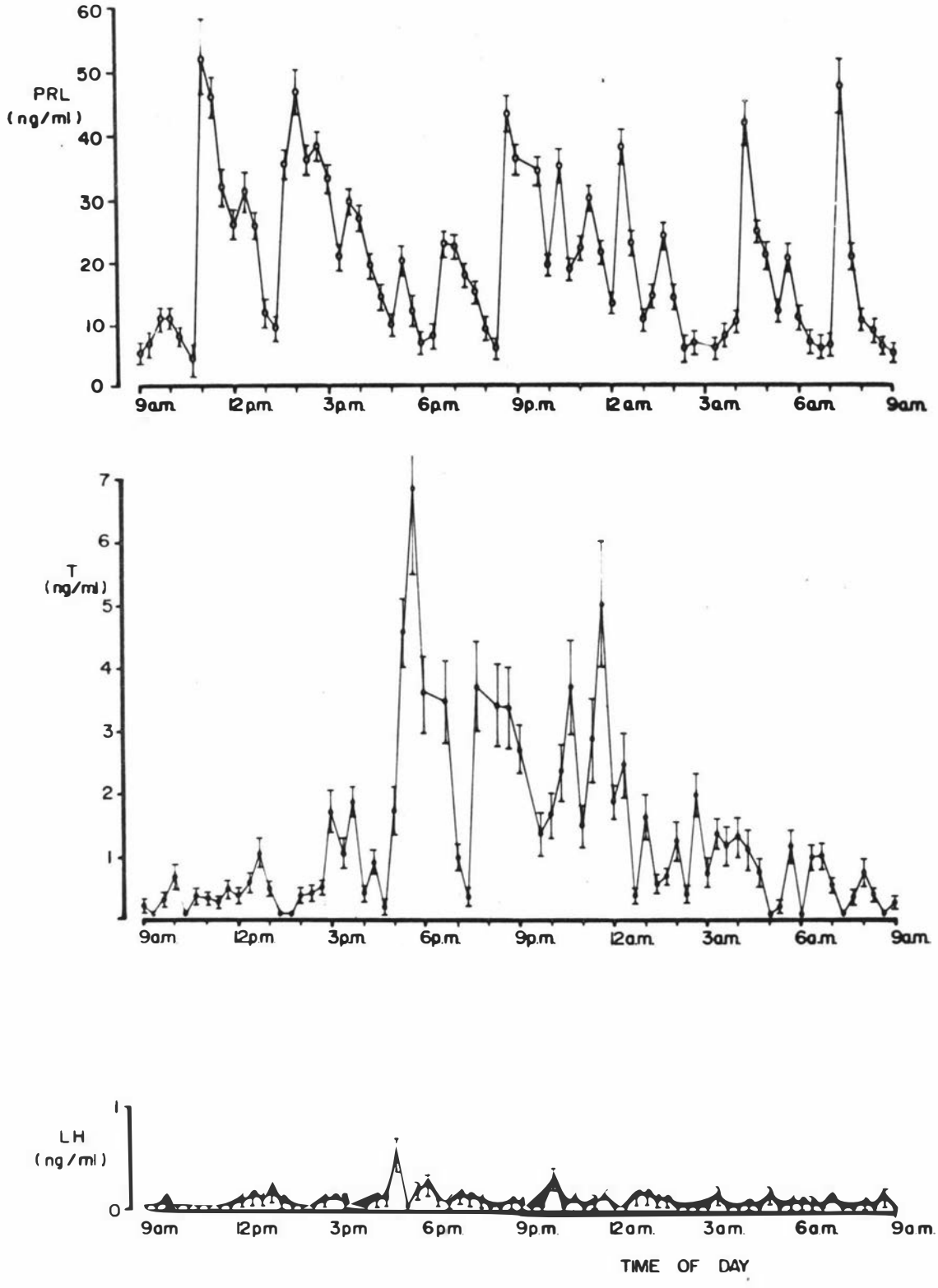


Figure 3.8: LH, testosterone and prolactin 24 h secretion profiles. Ram 184, sampled 21.5.74. (Lights off 5.40p.m. - 7.00a.m.)

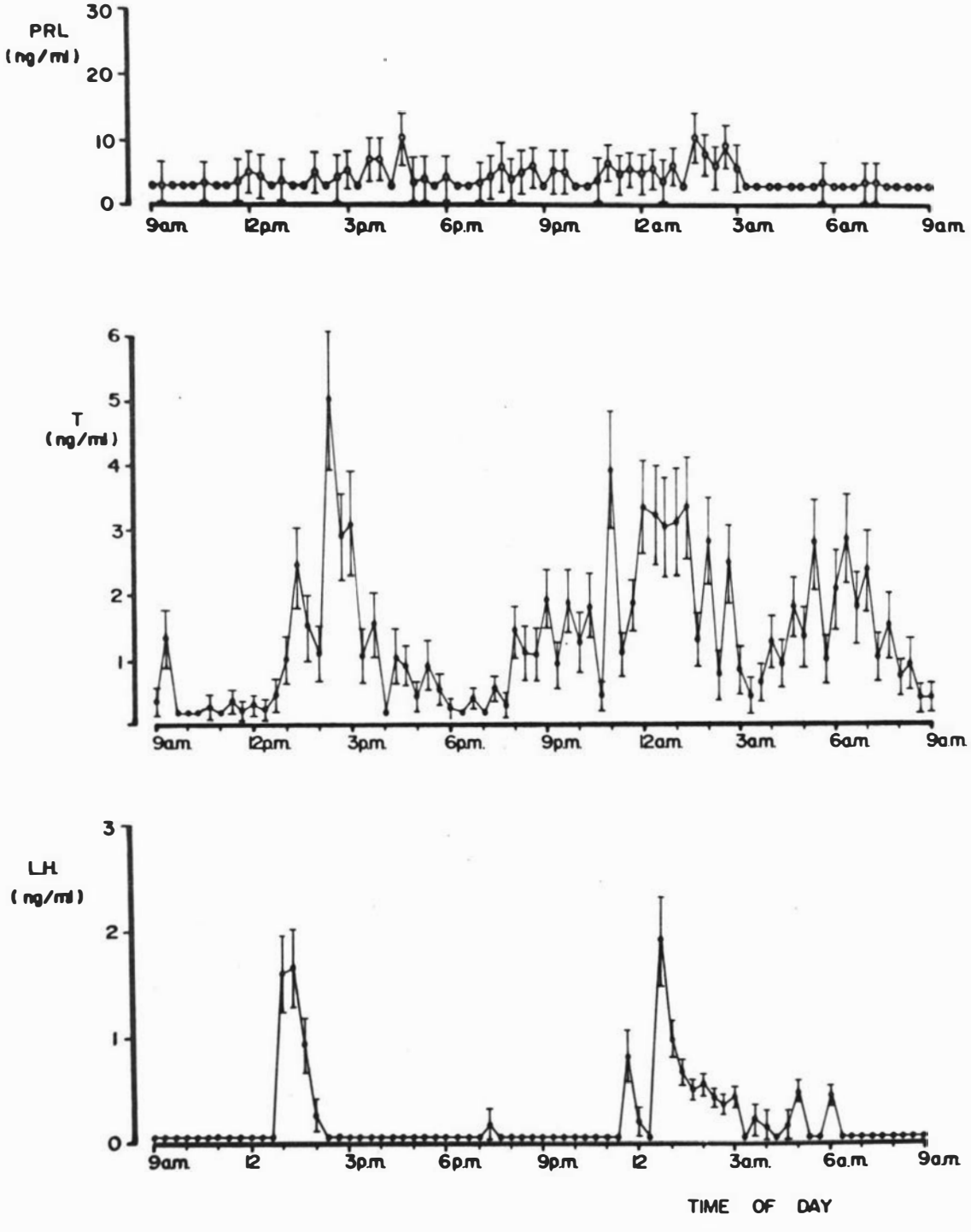


Figure 3.9: LH, testosterone and prolactin 24 h secretion profiles. Ram 228, sampled 21.5.74. (Lights off 5.40p.m. - 7.00a.m.)

TABLE 3.3.

Linear regression coefficients of curves representing cumulative hormone output against time.

Ram	LH	Testosterone	Prolactin
Summer			
174	0.1170	0.2472	1.7236
168	0.1751	0.3653	1.8216
	0.1594	0.4125	1.7654
184	0.0540	0.1946	1.7746
37	0.3449	0.5560	1.6541
Winter			
37	0.1501	0.2888	1.2629
184	0.0682	0.3641	1.2941
99	0.1068	0.1623	1.0217
228	0.0993	0.3629	0.5523

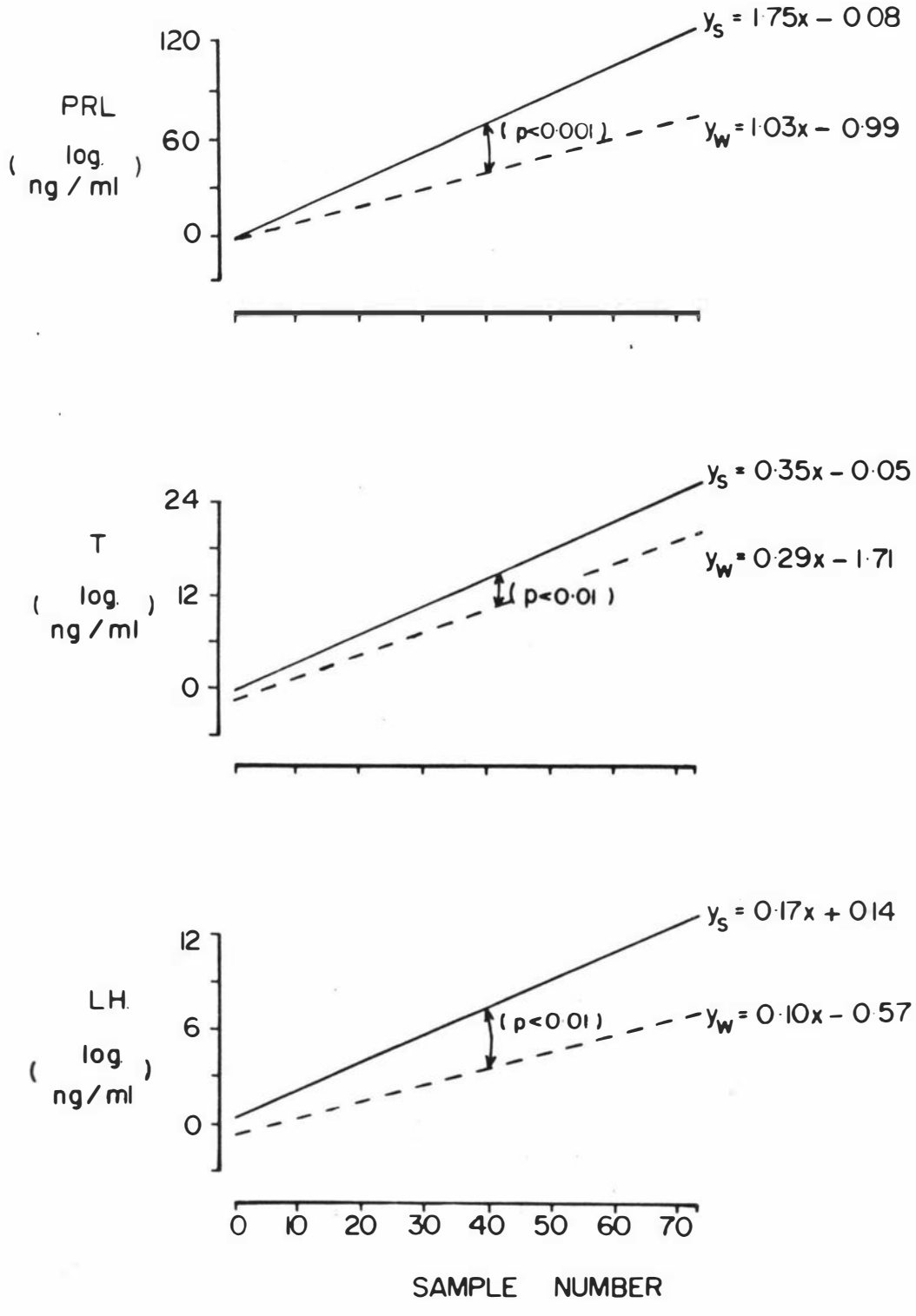


Figure 3.10: Mean linear cumulative regressions of 24 h secretion profile data against time to show differences in hormone output between summer(y_s) and winter (y_w) samplings.

(ii) Testosterone. Plasma levels of testosterone fluctuated in an episodic manner from low basal levels (0.06 - 1.0 ng/ml) to peak levels of up to 6.87 ng/ml. The character of testosterone peaks varied from being short-lived spikes to prolonged elevations of over 2 h duration.

The number of testosterone peaks during the nine 24 h sampling periods ranged from 1 - 9, with a mean of 5.11. Highest levels during episodic elevations for individual rams ranged from 1.22 to 6.87 ng/ml, and averaged 3.77 ng/ml.

Comparison of linear cumulative regression coefficients (Table 3.3) showed a significantly lower total testosterone secretion in the winter sampling period (t_7 = 3.94, $P < 0.01$, Figure 3.10). The overall mean testosterone level in the summer period was 1.45 ng/ml and in the winter peiod, 0.95 ng/ml. Cumulative regression components were essentially linear (correlations 0.96 - 0.99) indicating the absence of a circadian variation in testosterone secretion.

(iii) Prolactin. Five rams sampled during the summer period showed uniformly high plasma concentrations of prolactin (mean = 55.1 ng/ml, Figures 3.1 - 3.5). For each animal, levels rarely fluctuated above or below mean levels by more than 10 ng/ml. Conversely, during the winter sampling period (Figures 3.6 - 3.9) prolactin levels fluctuated dramatically in three subjects, while the fourth (228 Figure 3.9) exhibited uniformly low concentrations. In the first three of these rams, peak pulse levels ranged from 21.0 to 57.3 ng/ml, while basal levels in all four were low (approx 3 - 10 ng/ml). Overall mean prolactin concentration during the winter sampling was 14.2 ng/ml. Analysis of linear cumulative regression coefficients (Table 3.3) confirmed that the mean prolactin levels in summer were very highly significantly greater than those recorded in winter (t_7 = 18.79, $P < 0.001$, Figure 3.10). No circadian variation in plasma prolactin levels was evident.

(2) Relationship Between LH and Testosterone.

Each LH peak was followed by an elevation of testosterone concentrations within the period studied (i.e. excluding one LH peak which occurred in the last sample collected from ram 168, see Figure 3.4). Some testosterone peaks which followed LH peaks showed a biphasic appearance, but in one animal (184, Figure 3.8) testosterone

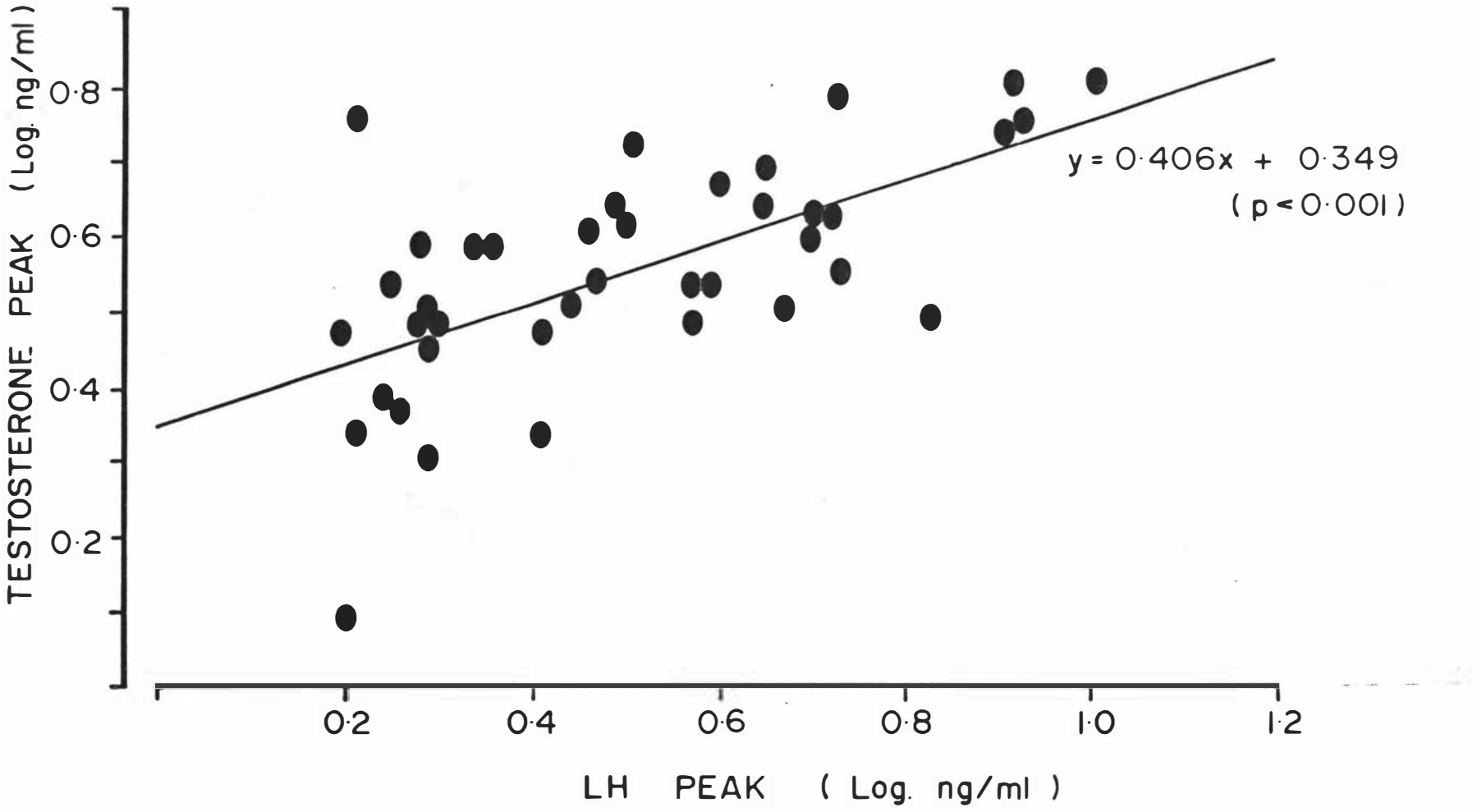


Figure 3.11: Regression of testosterone output following episodic LH peaks on the episodic LH peak levels.

levels rose independently of any LH elevation at both 7.40 p.m. and 10.40 p.m. No spontaneous testosterone peaks were observed when the same animal was sampled earlier (Figure 3.3). For those cases in which LH and testosterone peaks were related, approximately 20% of testosterone peaks occurred in the first sample after the causative LH peak; 46% occurred in the second sample, and 34% in the third sample (60 min). Also for instances where testosterone peaks followed LH peaks, the linear regression of testosterone peak on LH peak height, was highly significant ($P < 0.001$), (see Figure 3.11) indicating a quantitative output of testosterone in response to LH peaks.

The regressions of LH and testosterone peaks on respective hormone levels prior to peaks were significant ($P < 0.001$), but the regression of testosterone peaks on previous LH levels was not significant.

4. DISCUSSION

Blood collection at a remote sampling station obviated disturbance of rams other than during feeding, which itself caused no detectable alteration of hormone levels.

(1) Hormone Secretion Profiles.

(i) LH. Studies of LH levels in ram plasma have been reported extensively since this investigation commenced (Sanford et al., 1974a; Katongole et al., 1974; Barrell, 1976; Lincoln, 1976a,b; Schanbacher and Ford, 1976), and there is now general agreement as to the episodic nature of LH secretion in rams. Similar fluctuations in plasma levels of LH have been recorded in wethers (Riggs and Malven, 1974), ewes (Geschwind, 1972; Nett et al., 1974; Coppings and Malven, 1975; Baird et al., 1976), boars (Sanford et al., 1976; Flor Cruz, 1977), bulls (Monkonpunya et al., 1972; Katongole et al., 1972; Gombe et al., 1973b; Thibier, 1976) and man (Nankin and Troen, 1971 and 1972; Elwood et al., 1973; Murray and Corker, 1973; McNeilly et al., 1974).

Because of the nature of LH peaks, with sharp elevations of plasma levels being followed by an exponential decline, it was important to have employed a frequent sampling regime in studies of its secretory profiles. Geschwind (1972) recommended sampling intervals equivalent to or less than the half-life of the hormones under study; for LH, the half-life is approximately 30 minutes (Geschwind, 1972). Sampling intervals used in studies of ram LH secretion have ranged from 15 to

60 minutes (Sanford et al., 1974a; Barrell, 1976; Lincoln, 1976a; Schanbacher and Ford, 1976). Use of longer sampling intervals (Bolt, 1971; Katongole et al., 1974; Purvis et al., 1974) generally has resulted in poor definition of the true pulsatile character of LH and testosterone secretion.

The demonstration that pituitary LH output increases with pituitary portal blood GnRH levels (Ben-Jonathan et al., 1973), and the abolition of pulsatile LH release in severely stressed cockerels (Wilson and Sharp, 1975), indicated that ultimately pulsatile pituitary hormone output is determined by hypothalamic or higher central nervous centres. Episodic fluctuations persist despite the absence of the gonads in rams (Riggs and Malven, 1974), thus while testicular steroids restrict the output of pituitary LH (Hopkinson et al., 1974; Pelletier, 1974) they do not prevent pulsatile release of this hormone. The fact that infusion of LH into ovariectomised ewes failed to abolish or alter the frequency of pulsatile releases of endogenously produced LH (Coppings and Malven, 1975), also suggested some hypothalamic involvement in pulsatile hormone release mechanisms.

(ii) Testosterone. Fluctuations in plasma levels of testosterone similar to those observed in this experiment also have been reported recently for rams (Purvis et al, 1974; Katongole et al., 1974; Sanford et al., 1974a; Barrell, 1976; Lincoln, 1976a; Schanbacher and Ford, 1976), bulls (Thibier, 1976), boars (Flor Cruz, 1977; Sanford et al., 1976) and men (Boon et al., 1972; Murray and Corker, 1973; Barberia et al., 1973). Basal levels fluctuated only slightly while episodic elevation of testosterone levels occurred in response to pulsatile output of LH, as is discussed in the section on LH-testosterone interrelationships. Peaks of testosterone secretion were of longer duration (up to 2 hours) than those of LH, despite the similar half-life of the two hormones (Ismail, 1976). This suggested that Leydig cell receptor binding of LH is prolonged, and that testosterone continues to be secreted throughout the period of LH-receptor interaction.

(iii) Prolactin. Profiles of secretion of prolactin differed between the summer and winter sampling periods: levels in summer were uniformly high, but in winter basal levels were low and in three of four rams, marked pulsatile elevations of plasma levels occurred. Barrell (1976) also reported only minor variations in levels during

the daytime, but recorded a large nocturnal peak commencing at 22.00 h in rams sampled in December. Prolactin was secreted in erratic pulses in women (Boyar et al., 1975), and lactating cows (Koprowski et al.,1972), but in bulls, prolactin levels in blood fluctuated only slightly throughout the day (Monkonpunya et al., 1972).

Since the predominant regulation of prolactin secretion in mammals is claimed to be inhibitory (Reichlin et al., 1976), the persistently high levels observed in summer sampling periods suggested tonic suppression of Prolactin Inhibitory Factor (PIF) release (see p.11). Conversely, low levels observed during the winter sampling indicated inhibitory control. However, the secretory pulses observed in winter may have been responses to periodic cessation of PIF release, or may have been due to secretion of an as yet undetermined prolactin releasing factor such as that which Malven (1975) suggested to occur in sheep. The latter suggestion is attractive in view of the similarity in character of pulsatile prolactin elevations and LH episodic peaks, which are likely to be a direct result of periodic release of hypothalamic GnRH.

(2) LH and Testosterone Interrelationships.

The trophic effect of LH on testosterone secretion in the ram has been demonstrated clearly by the present data and by the results of other authors (Sanford et al., 1974a; Barrell, 1976; Lincoln, 1976a; Schanbacher and Ford, 1976). However, although each peak of LH was followed by an elevation of testosterone levels, some testosterone peaks were not preceded by a peak of LH. Wetteman and Desjardins (1973) and Schanbacher and Ford (1976) also described apparently spontaneous elevations of testosterone levels. The latter authors suggested that the preceding LH peak may have been missed between samples. The highly significant regression of testosterone peak levels on LH peak levels, indicated that the substantial testosterone output observed in these spontaneous peaks should have been preceded by a considerable rise in plasma levels of LH, a rise unlikely to have been missed between samples collected at 20 minute intervals. Disparities between the occurrence of LH and testosterone peaks also have been observed in bulls (Smith et al., 1973), boars (Flor Cruz, 1977) and men (Elwood et al., 1973; de Lacerda et al., 1973; Murray and Corker, 1973; Rowe et al., 1975).

The interval between the recording of LH peak levels and the following testosterone peak varied between 20 and 60 min, though the start of the elevation of testosterone levels always occurred within 20 min of the LH rise. Similar between-peak intervals were found by others (Sanford et al., 1974a;Barrell, 1976; Schanbacher and Ford, 1976) though slightly longer intervals (45 - 75 min) were recorded by Lincoln (1976a) in the Soay breed, suggesting that breed differences may exist.

The significant positive regression of testosterone peak levels on LH peak levels indicated a quantitative effect of LH on testosterone release. Other authors have not attempted mathematical determination of the relationships between LH and testosterone peaks in rams (Sanford et al., 1974a; Lincoln, 1976a; Schanbacher and Ford, 1976), but data presented in graphical form by Lincoln (1976a) suggested that peaks of testosterone in the Soay breed were highest when LH peaks were lowest, although abrupt changes in lighting regimes may have interfered with normal endocrine interractions. Vermeulen et al. (1973) reported that there was no direct correlation between LH and testosterone peaks in men.

(3) Circadian Hormone Variation.

Circadian variations in plasma levels of LH, testosterone and prolactin were not observed in this experiment. Though the mathematical technique employed to ascertain the occurrence of circadian rhythms may not have been sensitive enough to depict minor rhythms, none were obvious in a visual appraisal of results. The conclusion reached regarding LH and testosterone rhythms is in agreement with those of other authors (Sanford et al., 1974a; Barrell, 1976; Schanbacher and Ford, 1976). No circadian rhythm of LH output existed in bulls (Monkonpunya et al., 1972; Smith et al., 1973; Thibier, 1976) though in man the existence of circadian rhythms of LH and testosterone has been the subject of many conflicting reports (Petersen et al., 1968; Boon et al., 1972; Barberia et al., 1973; Bodenheimer et al., 1973; Elwood et al., 1973; de Lacerda et al., 1973; Nankin and Troen; Leymarie et al., 1973; Smals et al., 1974). However, there is general agreement about the existence of a circadian LH variation in pubertal human subjects (Weitzman et al., 1975).

Chamely et al (1974) was unable to distinguish a circadian rhythm of prolactin secretion in rams, but with the pooled-sample blood

collection technique utilized, minor rhythms may have been obscured. Conversely, Barrell (1976) showed a clear nocturnal elevation of prolactin plasma levels in sham-operated, but not pinealectomized, rams when using remote-sampling experimental conditions. This result suggested a light-dark modulation of prolactin control mediated by the pineal gland. In adult men and women, a consistent sleep-induced rise in plasma levels of prolactin occurred (Sassin et al., 1972; Weitzman et al., 1975).

(4) Seasonal Effects on Hormone Secretion Profiles

Seasonal and lighting-induced differences in plasma concentrations of LH and testosterone, such as seen in Experiment 1, have been recorded previously (Sanford et al., 1974b; Katongole et al., 1974; Barrell, 1976; Lincoln 1976a). On the other hand Schanbacher and Ford (1976) detected no difference in mean LH levels or in the number and magnitude of LH peaks between breeding and non-breeding seasons in Hampshire-Suffolk rams, though baseline levels of both LH and testosterone were signifcantly higher during the breeding season. Further, Lincoln (1976a) showed that during periods of long artificial daily photoperiod when testicular activity was low, LH levels were low and peaks few. During periods of short daily photoperiod, baseline levels of LH rose and the frequency of LH peaks increased, but peak levels were lower. Testosterone peak levels did not increase greatly but the frequency increased with LH peak frequency. Though seasonal changes in LH and testosterone have occurred in all breeds for which data is available, few have displayed the striking light-induced changes of the Soay breed studied by Lincoln (1976a).

The striking differences in short term prolactin secretion profiles have already been discussed. Seasonal differences in mean prolactin levels undoubtedly were caused by the difference in daily photoperiod (Pelletier, 1973; Barrell, 1976) which possibly exerts its influence by modifying activity of the pineal gland (Barrell, 1976).

CHAPTER IV

LH AND TESTOSTERONE SECRETORY RESPONSES FOLLOWING INTRAVENOUS INJECTION OR INFUSION OF GONADOTROPHIN RELEASING HORMONE (GnRH)

1. INTRODUCTION

Since Amoss and Guilleman (1969) demonstrated the release of LH in rams in response to injection of purified hypothalamic extracts of GnRH, the propensity of this hormone to elicit pituitary gonadotrophin release in a variety of circumstances in sheep has been widely documented (Convey, 1973; Pelletier, 1976). Prior to investigations described later in this thesis, in which GnRH was used extensively, it was considered necessary first to examine closely the activities of this hormone in the ram in view of limited studies which had been reported up to that time. Single intravenous injections and intravenous infusions of GnRH were studied to determine suitable doses and sampling routines, to evaluate hormonal interrelationships, and to provide base data for comparisons between the responses of adults and the immature rams used in later studies.

2. MATERIALS AND METHODS

(1) Animals and Management Procedures.

Animals, animal management, catheterization and sampling procedures have been described in Chapter 2. Four rams were used, and each was used in both experiments described in this chapter.

(2) Experimental Procedures.

(i) Experiment 2.1: Single Intravenous GnRH Injection. Four doses of GnRH, following logarithmic increments, were utilized: 3.1 µg, 12.5 µg, 50 µg and 200 µg. Each quantity was dissolved in 5.0 ml of acidified (0.01 M acetic acid) 0.9% saline and administered via a jugular catheter as a single rapid injection. Doses were administered at random to each ram as shown in Table 4.1.

Blood samples were withdrawn 10 and 20 minutes and immediately prior to injection of GnRH, and again 10, 15, 20, 25, 30, 40, 50, 60, 80, 100, 120, 150, 180 and 270 min following injection. After centrifugation, plasma was stored frozen until required for LH and testosterone assays.

TABLE 4.1

Allocation of GnRH doses (μg) in Experiments 2.1 and 2.2.

Date	Experiment 2.1				Date	Experiment 2.2			
	Ram Number					Ram Number			
	1	2	3	4		1	2	3	4
29.3.74	200	200	50		8.6.74	200	50	12.5	0
3.4.74	3.1	12.5	3.1	3.1	12.6.74	12.5	0	50	200
3.5.74			12.5	200	16.6.74	0	12.5	200	50
6.5.74	12.5	50	200	12.5	20.6.74	50	200	0	12.5
9.5.74	50	3.1		50					

(ii) Experiment 2.2: Intravenous Infusion of GnRH. Three doses of GnRH, or a control infusion of acidified saline, were infused via jugular catheter in the order shown in Table 4.1. Each dose was prepared in 8.0 ml of acidified saline as above, and infused at a constant rate of 1.0 ml/h for eight h using an infusion pump. Blood plasma samples were withdrawn from the opposite catheter 10 and 20 min and immediately prior to commencement of infusion, at 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0 h from the start of infusion, and 0.5, 1.0, 1.5, 2.0, 3.0, 4.0 and 7.0 h after cessation of the infusion. Plasma was frozen until assayed for LH and testosterone content.

To test the stability of the infused GnRH during the 8 h infusion period, a 200 µg aliquot was prepared as described above and held at room temperature overnight. Subsequently this hormone was injected intravenously to one ram and plasma samples collected at 0, 0.25, 0.5, 0.75, 1.0 and 1.5 h after administration and frozen until required for LH assay.

(3) Analysis of Data.

Total areas under LH and testosterone response curves were calculated as described in Chapter 2, and pre-injection hormone levels were calculated as the mean level recorded from pre-treatment samples. Two-way analyses of variance were performed on pre-injection and total hormone output data, and dose-response effects evaluated using standard orthogonal coefficients.

Analysis of variance of regression coefficients of total testosterone on total LH responses was performed to quantify the relationship between LH and testosterone output following GnRH administration.

Regression fits of mean LH and testosterone response curves for each dose of GnRH were calculated (see Chapter 2) and comparisons of regression coefficients between doses for each experiment were made by t tests based on standard orthogonal coefficients.

3. RESULTS

(1) Experiment 2.1

See Tables 4.2 and 4.3 and Figures 4.1 - 4.4.

TABLE 4.2

Mean pre-injection hormone levels and total responses to GnRH injection (Experiment 2.1) and a summary of analyses of variance of this data.

Dose of GnRH (μg)		LH		Testosterone	
		Pre-injection	Total	Pre-injection	Total
3.1		7.55	21.55	17.76	25.18
12.5		7.81	24.00	17.37	26.44
50		8.91	27.47	17.20	26.48
200		8.93	28.93	17.42	26.70
Source of variance	d.f.	Variance Ratios			
Dose	3	0.38	12.80**	0.02	1.45
Animal	3	1.01	12.20**	1.31	6.22*
Residual mean square	9	5.69	3.48	8.48	1.30

TABLE 4.3

Coefficients of regressions fitted to mean LH and testosterone response curves.

	Dose of GnRH (μg)	LH			Testosterone		
		linear	quadratic	cubic	linear	quadratic	cubic
Experiment 2.1							
	3.1	134.85	-852.18	2278.84	28.97	-77.83	150.50
	12.5	273.99	-1153.99	2776.25	35.70	-94.35	184.87
	50	614.61	-1878.99	3957.25	24.76	-55.52	97.75
	200	1104.08	-2028.16	2348.11	27.71	-72.71	145.28
Experiment 2.2							
	0	0.36	-0.14	0.05	-2.26	1.20	-0.56
	12.5	102.36	-53.41	24.36	6.04	-2.62	1.06
	50	154.30	-95.09	45.48	3.71	-2.15	0.94
	200	151.39	-113.20	56.21	5.14	-3.07	1.41

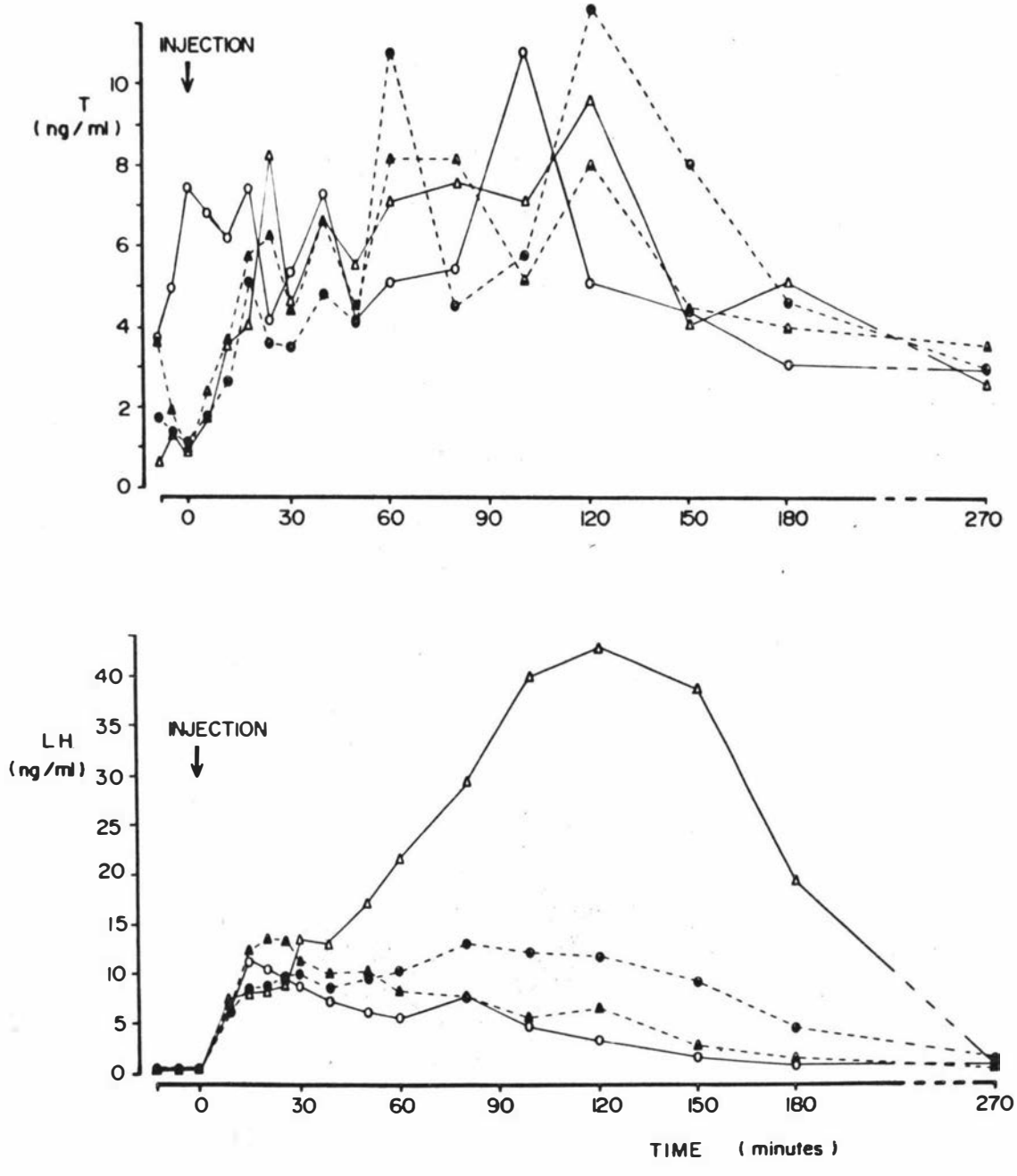


Figure 4.1: Ram 1. Plasma LH and testosterone concentrations following injection of GnRH. Doses were: O——O, 3.1 μg; ▲ - - ▲, 12.5 μg; ● - - ●, 50 μg; Δ——Δ, 200μg.

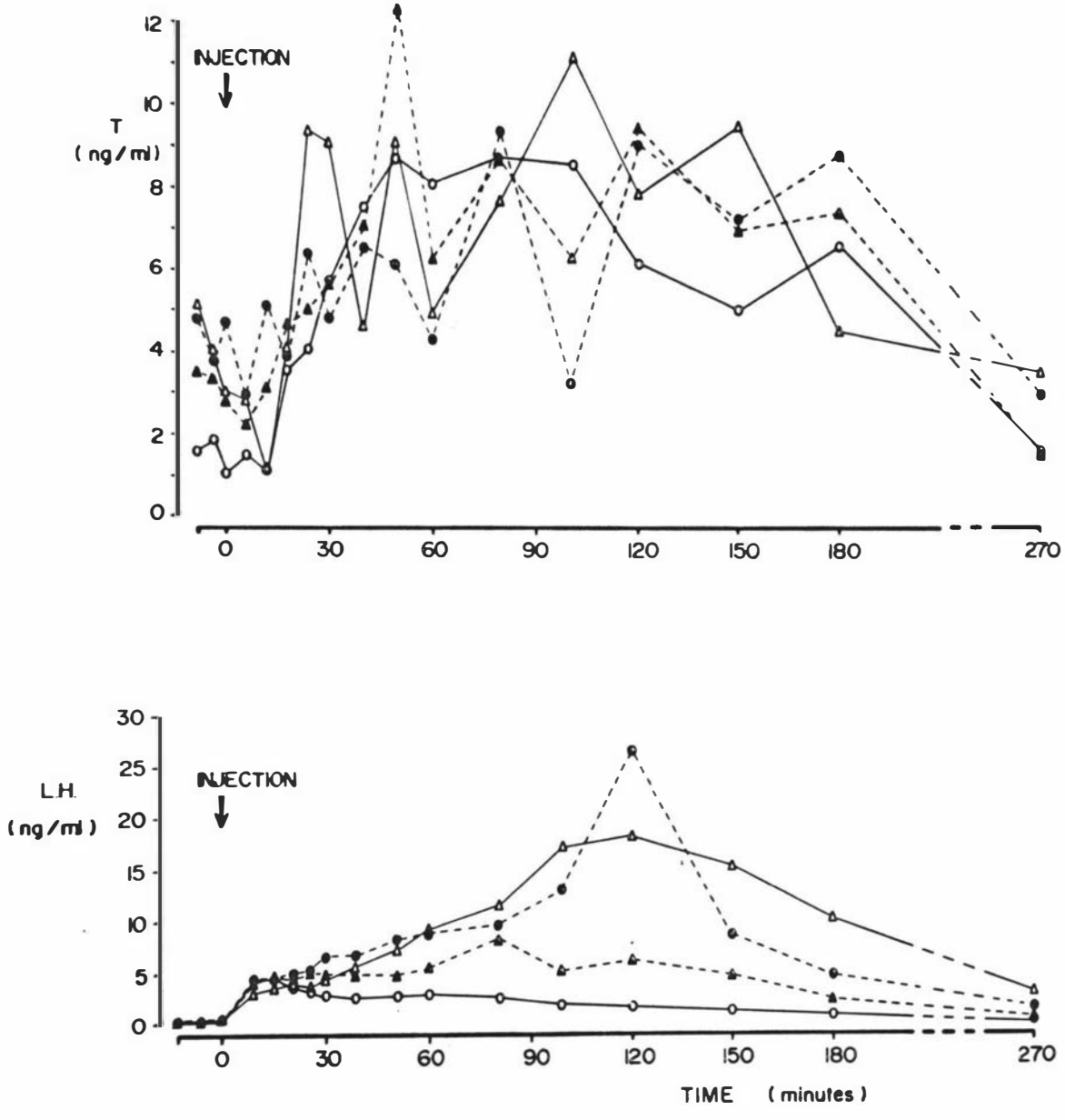


Figure 4.2: Ram 2. Plasma LH and testosterone concentrations following injection of GnRH. Doses were: 0———0, 3.1 μg; ▲ - - ▲, 12.5 μg; ● - - ●, 50 μg; Δ———Δ, 200 μg.

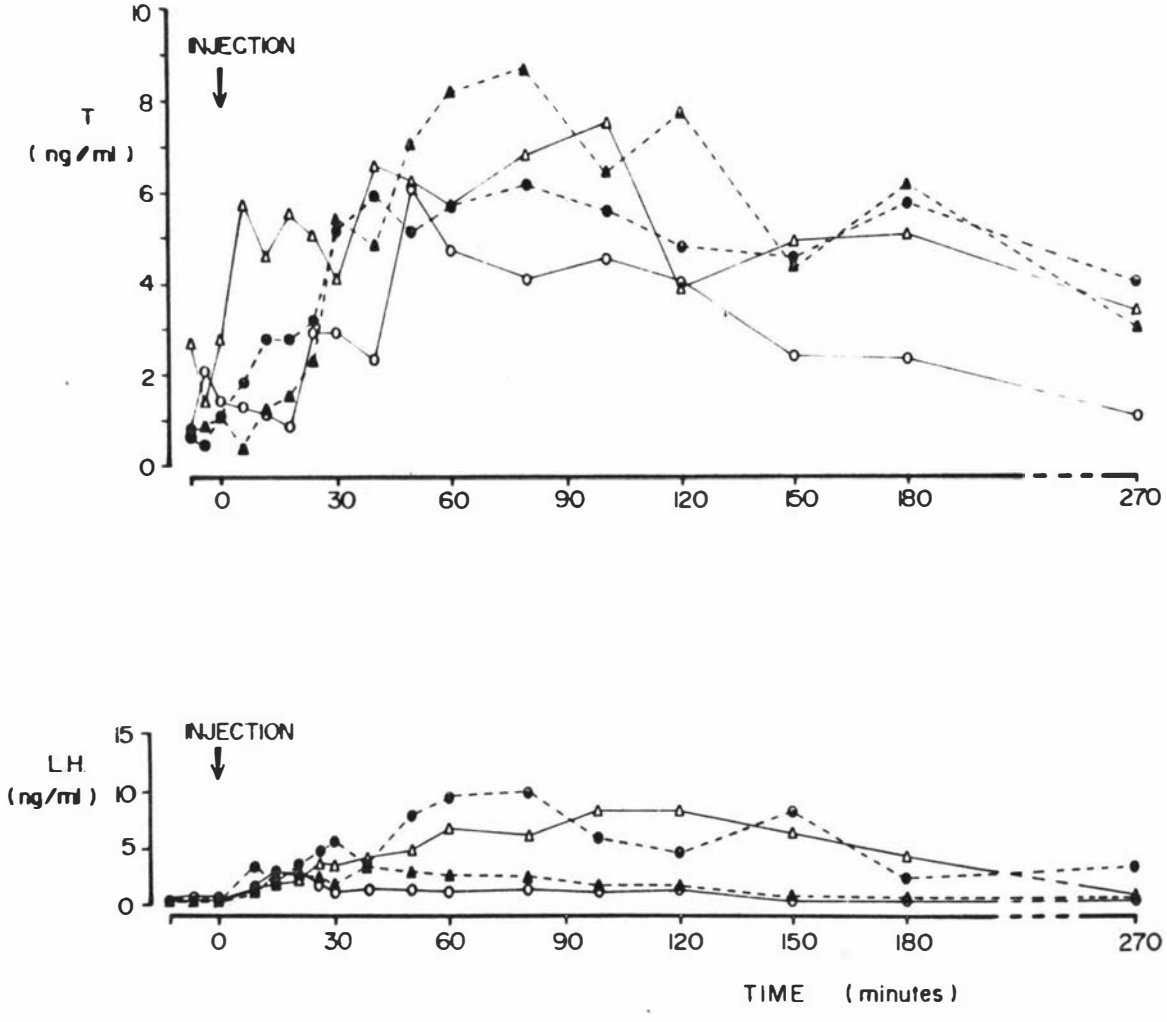


Figure 4.3: Ram 3. Plasma LH and testosterone concentrations following injection of GnRH. Doses were: 0——0, 3.1 μg; ▲ - - ▲, 12.5 μg; ● - - ●, 50 μg; Δ——Δ, 200 μg.

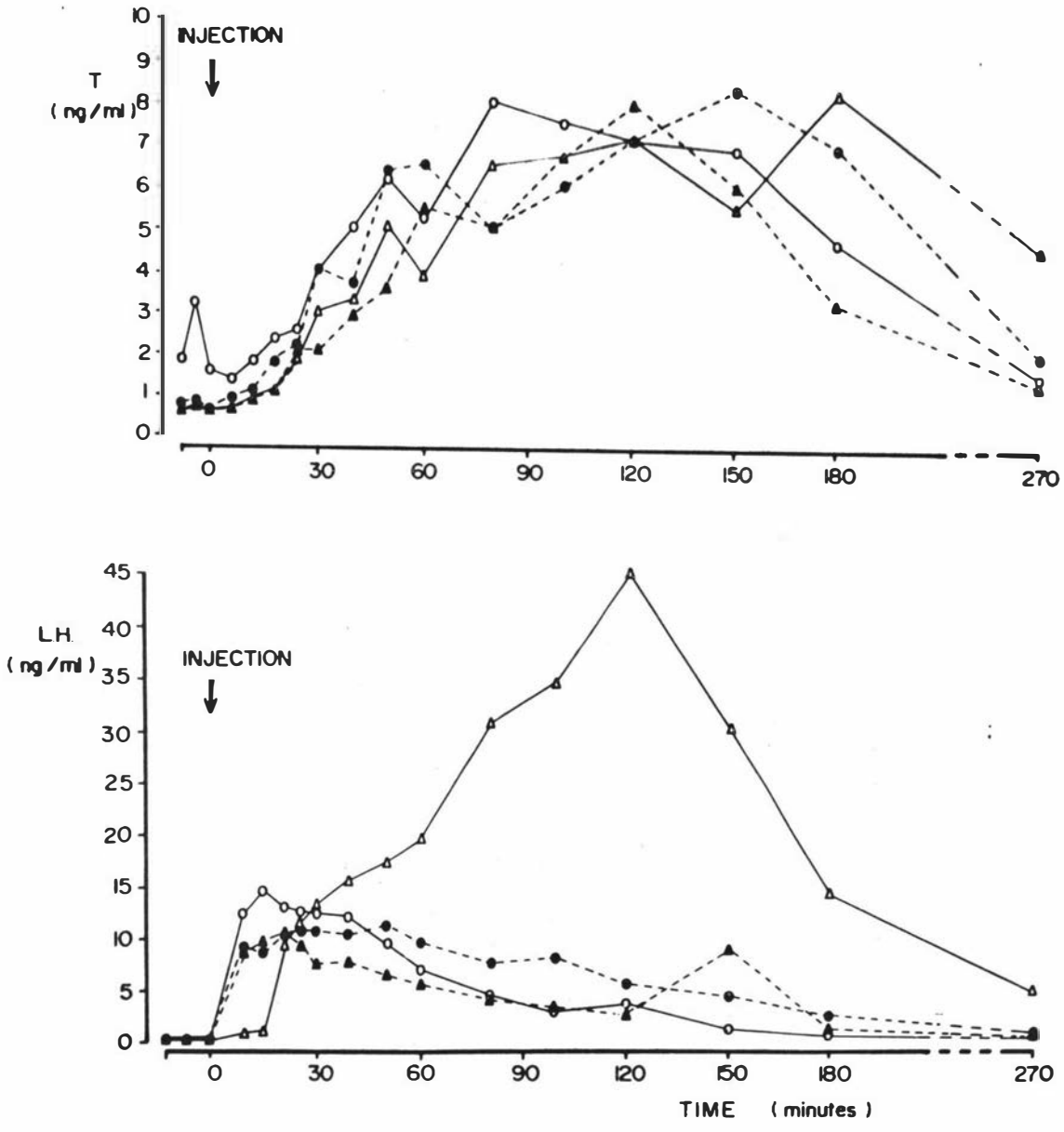


Figure 4.4: Ram 4. Plasma LH and testosterone concentrations following GnRH injection. Doses were: O——O, 3.1 μg; ▲ - - ▲, 12.5 μg; ● - - ●, 50 μg; Δ——Δ, 200 μg.

Prior to GnRH administration plasma LH levels ranged from undetectable (<0.05 ng/ml) to 4.41 ng/ml, and testosterone levels ranged from 0.39 to 7.41 ng/ml. All injections of GnRH resulted in elevations of LH concentrations within 10 min. Following injection of low doses (3.1 and 12.5 µg) plasma concentrations of LH rose rapidly to peak levels within 15 - 20 min then slowly declined during the subsequent 3 - 4 h. Higher doses of GnRH (50 and 200 µg) resulted in more prolonged elevations of plasma LH levels and generally a second peak was noted approximately 1.5 h after an initial elevation. Concentrations had returned to pre-injection levels within 4.5 h after injection in most subjects.

Elevation of testosterone concentrations occurred subsequent to those of LH. Testosterone levels fluctuated considerably and generally were elevated for longer than 4.5 h after GnRH injection.

Though the testosterone response to each GnRH injection did not differ significantly with dose, the LH output displayed a significant linear increase with increasing dose ($\underline{F}_{1,9} = 37.60$, $P < 0.001$). The regression of total testosterone secretion on total LH output was not significant.

Linear coefficients of regression fits of LH response curves (Table 4.3) showed a significant linear increase with GnRH dose increments ($\underline{t}_3 = 3.24$, $P<0.05$), but non-linear components of regression fits were not significantly different. No differences in shapes of testosterone response curves were detected.

(2) Experiment 2.2.

See Tables 4.3 and 4.4 and Figures 4.5 - 4.8.

Plasma levels of LH following injection of 200 µg of GnRH stored overnight as a check on GnRH stability in solution were:

Time after injection (h)	LH (ng/ml)
0	0.05
0.25	8.0
0.5	12.0
0.75	24.0
1.0	20.0
1.5	28.0

This response was similar to that observed following acute injection of equivalent quantities of freshly dissolved GnRH (Experiment 2.1).

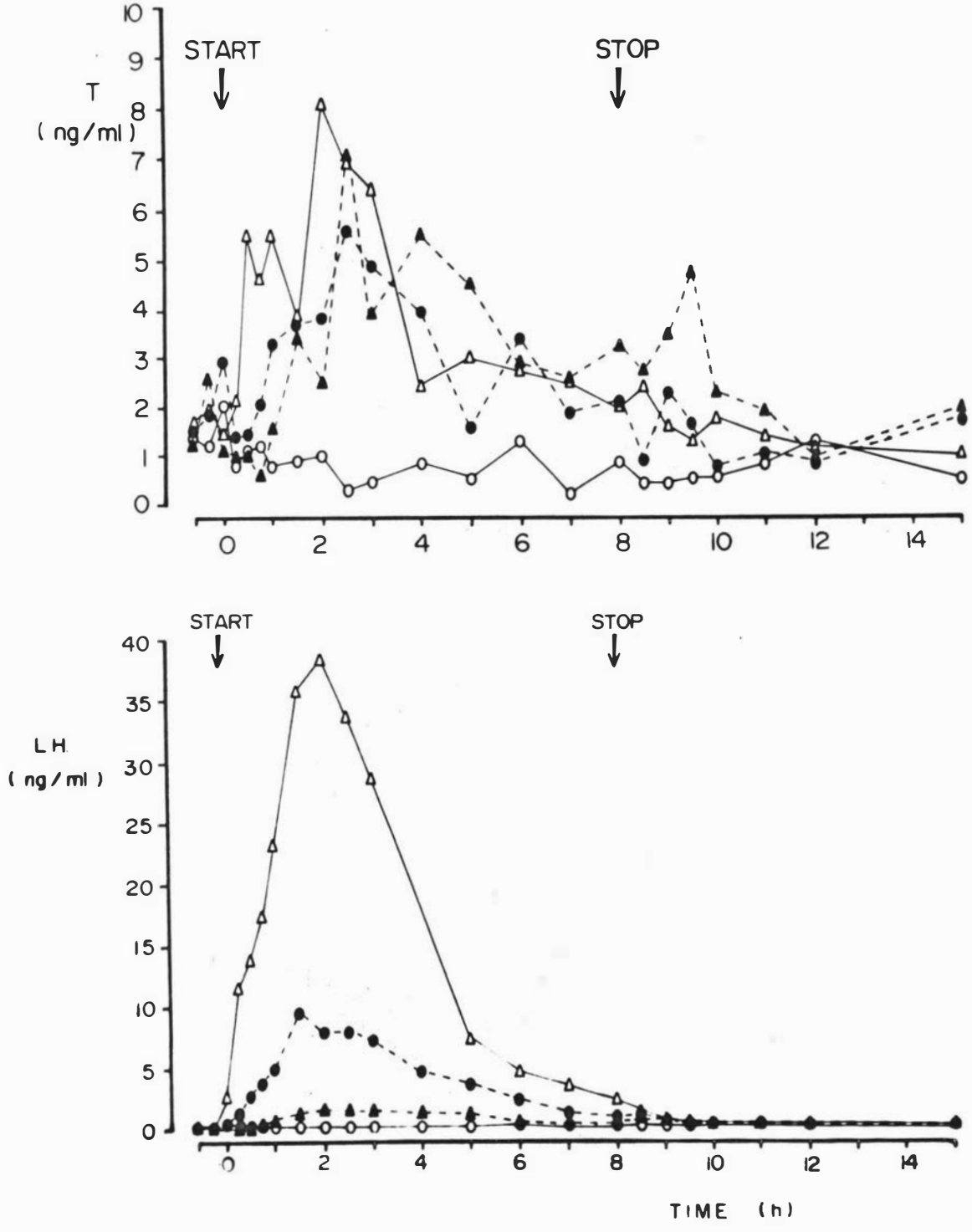


Figure 4.5: Ram 1. Plasma LH and testosterone concentrations during and after GnRH infusion. Total doses were: 0——0, 0 μg; ▲ - - ▲, 12.5 μg; ● - - ●, 50 μg; Δ——Δ, 200 μg.

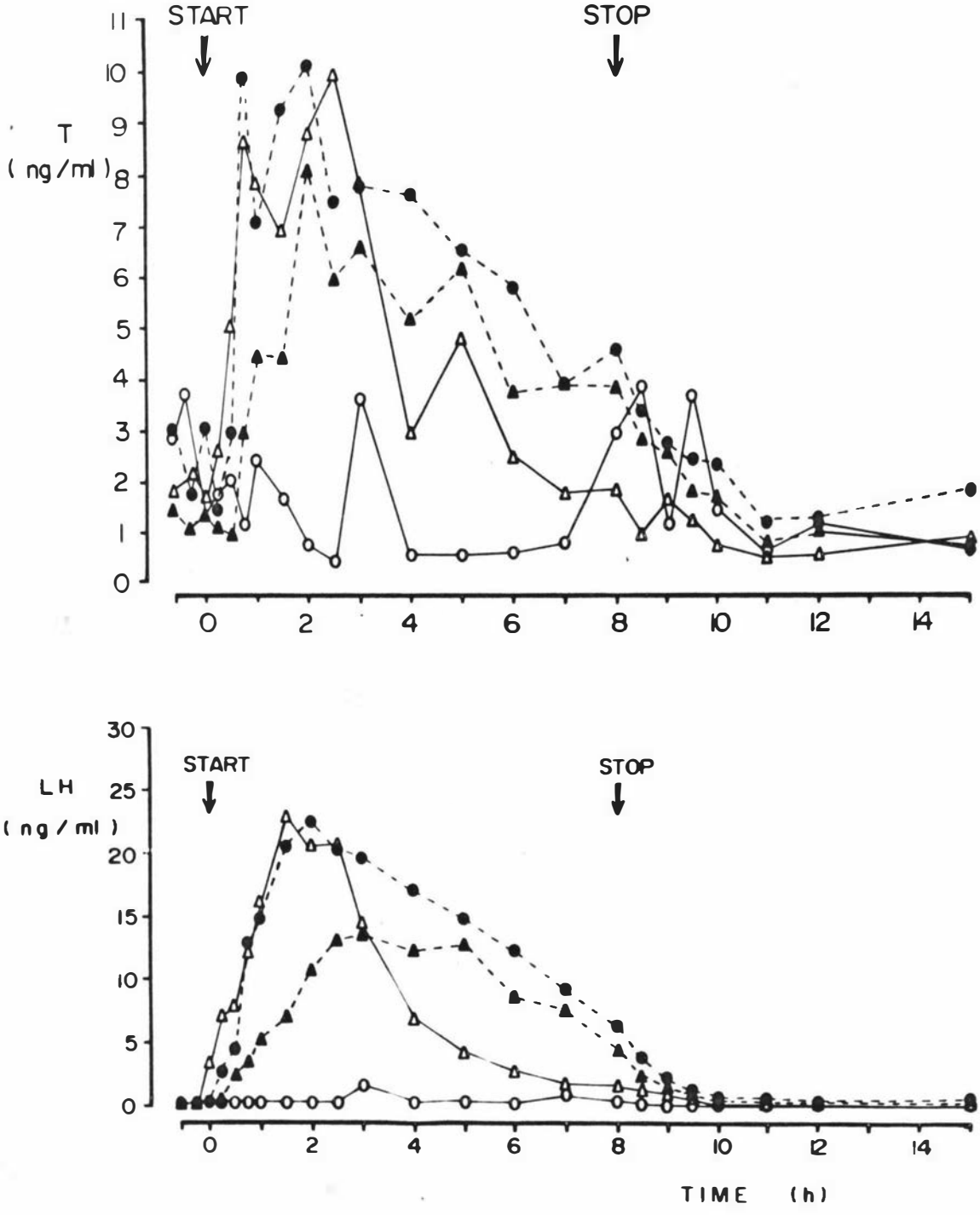


Figure 4.6: Ram 2. Plasma LH and testosterone concentrations during and after GnRH infusion. Total doses were; 0——0, 0 μg; ▲ - - ▲, 12.5 μg; ● - - ●, 50 μg; Δ——Δ, 200 μg.

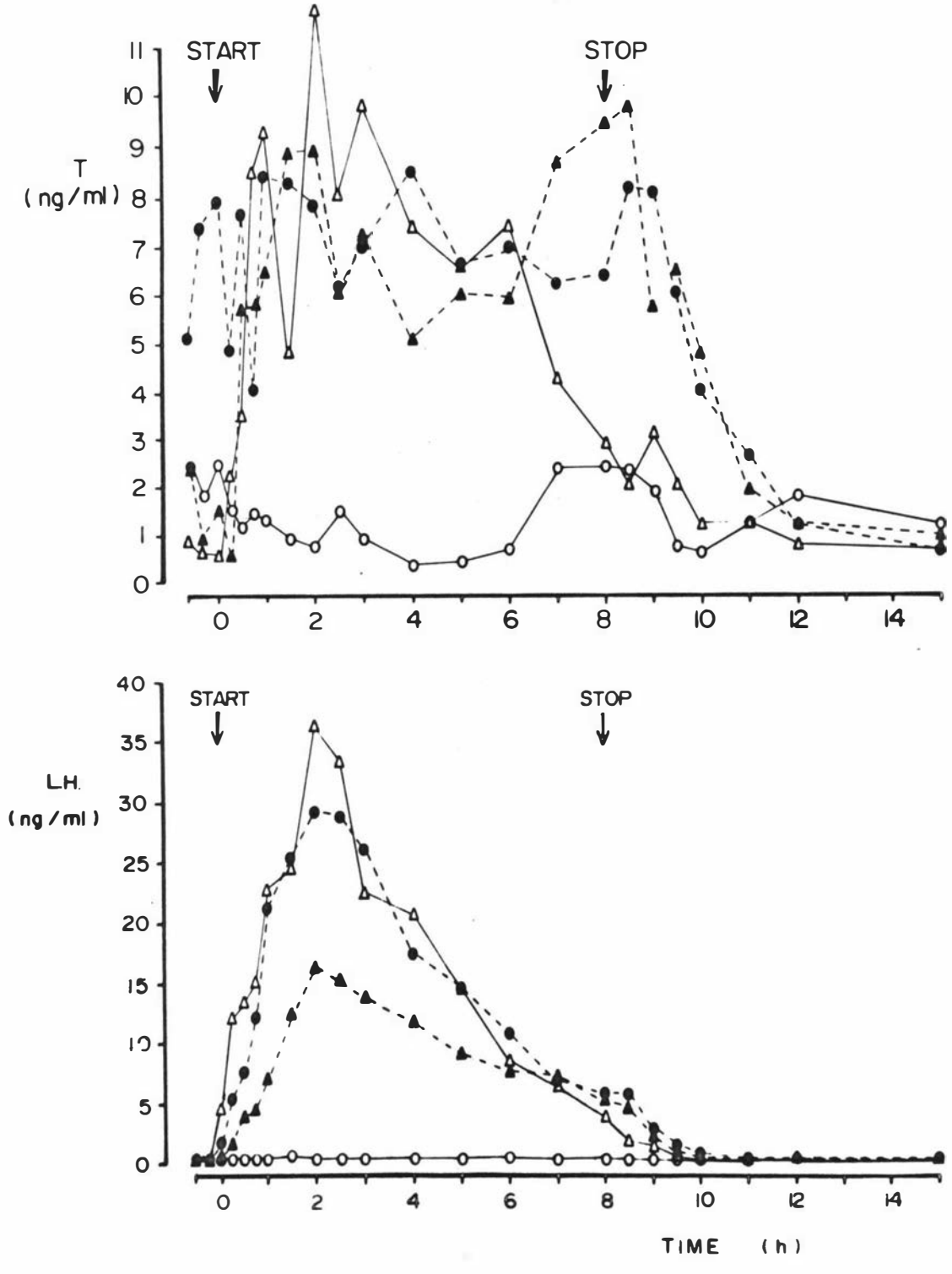


Figure 4.7: Ram 3. Plasma LH and testosterone concentrations during and after GnRH infusion. Total doses were: O——O, 0 μg; ▲ - - ▲, 12.5 μg; ● - - ●, 50 μg; Δ——Δ, 200 μg.

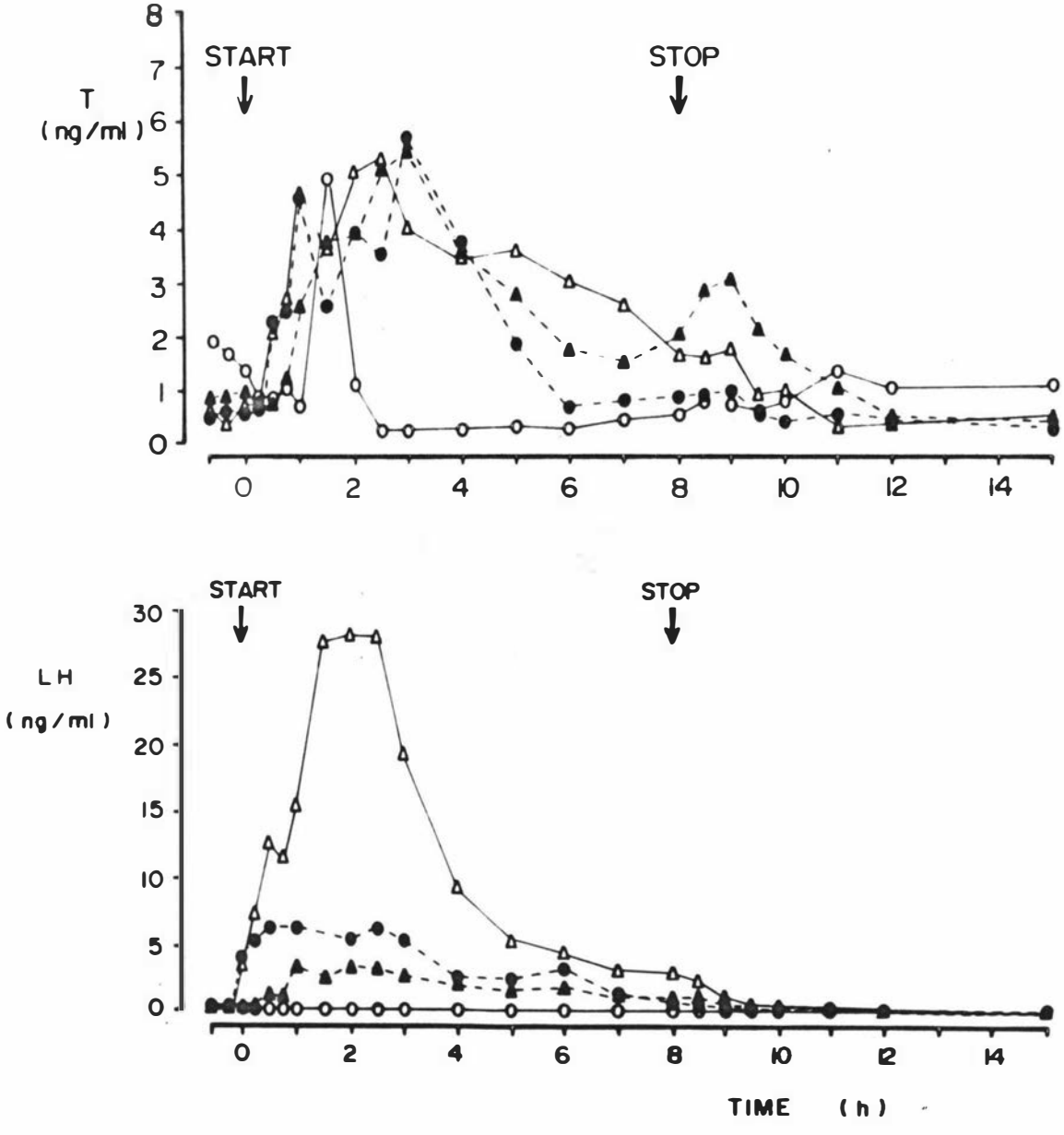


Figure 4.8: Ram 4. Plasma LH and testosterone concentrations during and after GnRH infusion. Total doses were: 0——0, 0 μg; ▲ - - ▲, 12.5 μg; ● - - ●, 50 μg; Δ——Δ , 200 μg.

TABLE 4.4

Mean pre-infusion hormone levels (ng/ml) and total responses to GnRH infusion (Experiment 2.2) and a summary of analyses of variance of this data.

Dose of GnRH (µg)	LH		Testosterone	
	Pre-infusion	Total	Pre-infusion	Total
0	0.18	14.99	2.04	35.59
12.5	0.12	37.65	1.31	45.24
50	0.61	44.50	3.04	45.35
200	1.26	47.70	1.23	44.85

Source of variance	d.f.	Variance Ratios			
Dose	1	7.87**	29.71***	1.81	21.51***
Animal	1	1.55	2.10	2.48	11.69**
Residual mean square	9	13.71	29.33	13.15	4.25

Prior to GnRH infusion plasma LH concentrations ranged from below 0.05 ng/ml to 4.87 ng/ml, and testosterone levels ranged from 0.38 to 7.99 ng/ml. LH levels rose steadily upon commencement of infusion of GnRH, reached peaks from 1 to 3 h later, then subsequently declined slowly during the remainder of the 8 h infusion period. After cessation of infusion of higher doses of GnRH, LH concentrations continued to be elevated above initial levels, but decreased to pre-infusion levels within two h. With lower doses of GnRH, LH concentrations often fell to pre-infusion levels before the infusion was completed. Testosterone levels increased subsequent to the LH rise and were highest during the first 4 h of GnRH infusion. Levels fell slightly thereafter, but generally were elevated above pre-infusion levels at the end of the infusion; the return to pre-infusion levels occurred 2 - 4 h later. Control infusions of saline resulted in no elevation of LH or testosterone plasma concentrations.

Estimates of total LH and testosterone responses to GnRH infusion (Table 4.4) were significantly greater than those following saline infusion ($\underline{F}_{1,9}$ = 81.9, $P< 0.001$, and $\underline{F}_{1,9}$ = 9.4, $P< 0.05$, respectively). Total LH response curves showed a significant linear increase with increasing GnRH dose ($\underline{F}_{1,9}$ = 6.94, $P< 0.05$), but there was no such increase in testosterone secretion following use of increasing doses of GnRH.

Analysis of variance of the regression coefficients of total of testosterone output on total of LH output showed a significant quantitative relationship between these two hormones ($P< 0.05$).

Linear, quadratic and cubic coefficients of regression fitted to LH and testosterone response curves following GnRH infusion (Table 4.3) were significantly greater in magnitude than those following saline infusion ($\underline{t}_3$ = 3.36, 4.60 and 4.29, $P< 0.05$) respectively for LH, and $\underline{t}_3$ = 4.86 ($P< 0.05$), 6.30 ($P< 0.01$) and 5.54 ($P< 0.05$) respectively for testosterone . No significant differences in regression coefficients following GnRH infusion indicated that the shapes of LH and testosterone following each dose of GnRH was essentially the same.

4. DISCUSSION

Results from this and other studies (Amos and Guilleman, 1969; Reeves et al., 1970; Arimura et al., 1972; Galloway et al., 1974;

Hopkinson et al., 1974; Galloway and Pelletier, 1974; Pelletier, 1976; Bremner et al., 1976) have shown conclusively that GnRH elicits anterior pituitary release of LH in adult rams; in turn, the release of LH results in an elevation of plasma testosterone concentrations.

The intravenous route of GnRH administration has been shown by others (Gonzalez-Barcena et al., 1973; Convey, 1973; Galloway and Pelletier, 1974; Hopkinson et al., 1974; Pelletier, 1976) to be efficient in inducing LH release and was adopted as the standard route for GnRH administration in experiments described in this thesis. Intra-arterial (Reeves et al., 1970; Amoss and Guilleman, 1969), intramuscular (Arimura et al., 1972), subcutaneous (Gonzalez-Barcena et al., 1973), intranasal (London et al., 1973), intrapituitary (Spies and Niswender, 1973) and intraventricular (Crammer and Barraclough, 1975) routes also have proven effective in stimulating LH output from the pituitary, even though the timing of responses have varied with the route of administration. In fact even when the dose has been held constant, wide variations in response to GnRH have followed the use of the same route of administration in the ram. Lincoln (1976a) suggested that pituitary responsiveness to GnRH varied in relation to lighting-induced seasonal breeding patterns. Use of different assays may have accounted for some of the differences in responses reported in the literature. A summary of responses recorded in some previous papers on the administration of GnRH to adult rams is presented in Table 4.5.

All doses of GnRH used in the present study (3.1 - 200 µg) elicited pituitary LH output and thereby caused testosterone release. Administration of saline did not elicit LH secretion in this experiment or those of others (Arimura et al., 1972; Hopkinson et al., 1974; Galloway and Pelletier, 1974). The present experiment showed a significant linear increase in LH output in response to logarithmic increments of injected or infused GnRH, together with a delay in attainment of peak values as dose increased; essentially similar results were recorded by Hopkinson et al. (1974).

The quantitative relationship between LH secretion and the subsequent testosterone output following GnRH administration is not clear; a positive relationship between LH and testosterone output was established after GnRH was given by infusion, but not following injection. Galloway and Pelletier (1974) showed a close linear

TABLE 4.5

Summary of responses recorded in previous research on the administration of GnRH to adult rams.

Authors	Breed	Number of rams	Route of administration	GnRH dose (µg)	Peak level (ng/ml)	Time to reach peak (mins)
Amoss and Guilleman (1969)	Whiteface	2	Intra-arterial	100	14.98 - 18.97	5
Reeves et al. (1970)	Whiteface	1	Intra-arterial	1	0	
				3	Approx.8	2.5 - 10
				9	"	"
				27	"	"
Arimura et al. (1973)	Whiteface	1	Intra-arterial	250	20	60
Hopkinson et al. (1974)	Unspecifeid	1	Intravenous	0.125	2.0 approx	5 approx
				0.25	4.0 "	20 "
				0.5	5.0 "	20 "
				1.25	10.0 "	20 "
				5.0	11.0 "	10 "
				10.0	13.5 "	60 "
				50.0	14.0 "	100 "
Galloway et al.(1974)	Ile de France	2	Intravenous	100	8.5 - 18.6	110 - 120
	"	2	"	800	30.5	140 - 160
Galloway and Pelletier (1975)	Ile de France	5	Intravenous	100	9.6 ± 3.4	120 ± 4
Lincoln (1976a)	Soay	7	Intravenous	5	4 - 50	10 - 45

relationship between the total secretion of these two hormones after injected GnRH, but these authors used only two widely divergent doses (100 and 800 μg) and two rams to establish this relationship. A consistent quantitative relationship between these hormones may have been obscured because of significant between-animal differences in output of LH and testosterone observed in this experiment.

Though for each ram the peak LH output following injection of high doses of GnRH was delayed, the initial LH rise was similar following each dose regardless of the occurrence of a second peak which was a characteristic response following higher GnRH doses. This constant initial response was interpreted as indicating a possible initial rapid saturation of gonadotroph receptors, regardless of dose, resulting in the immediate release of similar quantities of stored LH. Because of the short plasma half-life of GnRH (Crighton et al., 1973), a rapidly diminishing pattern of GnRH-receptor binding and LH release should follow administration of GnRH.

The reason for the second elevation of LH following high doses is not clear, but it has been suggested that high doses enhance synthesis of the gonadotrophin (Borgeat et al., 1972), or there may be two functional pools of LH in pituitary cells, differing in the period of exposure to GnRH required to elicit their release (Bremner and Paulsen, 1974). In either case biphasic responses to infused GnRH should have been expected, but none were observed in Experiment 2.2. However, Bremner and Paulsen (1974) did observe a biphasic response to GnRH infusion in men and following use of high doses of infused GnRH, biphasic output of LH has been shown in rams (Bremner et al., 1976).

The eventual fall in plasma LH levels was likely to have been due to plasma depletion of injected GnRH as well as receptor binding and metabolism of LH, but when GnRH was infused, LH levels fell despite continued administration. This probably was due to increasing steroid feedback inhibition of pituitary LH output (Galloway et al., 1974; Piper et al., 1975) or to refractoriness of the pituitary to prolonged exposure to GnRH (Bremner et al. 1976). Loss of biological activity of GnRH during infusion was an unlikely explanation since a separate small experiment demonstrated the stability of the decapeptide in solution. Also others have demonstrated the retention of biological activity by GnRH when incubated in saline (Virkkunen

et al., 1974). Exhaustion of pituitary LH supplies is unlikely to have occurred since it has been shown that considerable quantities of the gonadotrophin remain in sheep pituitaries following GnRH infusion, despite refractoriness to administration of larger doses of the decapeptide toward the end of the infusion period (Piper et al., 1975).

No episodic releases of LH were observed during either the GnRH or control saline infusions. A similar observation was made by Bremner and Paulsen (1974) who infused GnRH into men, but on the other hand, Piper et al., (1975) indicated that some pulsatile release of LH occurred during GnRH infusion into ewes, despite elevated plasma levels of LH. Bremner and Paulsen (1974) concluded that since exogenous GnRH administration inhibited episodic release of LH, episodic hormone release must have been initiated by centres other than the pituitary gland.

Exogenous testosterone administration to entire rams depressed the quantity of LH released from the pituitary in response to GnRH stimulation (Hopkinson et al., 1974). Similar effects were observed in vitro following high doses of testosterone, though low doses in vitro may have been stimulatory to LH output in response to GnRH (Kao and Weisz, 1974). Data published by Galloway et al. (1974) suggested that the levels of testosterone in plasma prior to GnRH injection may influence the pituitary response since LH output was lower when pre-injection levels of testosterone were high. By regression analysis, output of LH following GnRH administration was shown to be unaffected by testosterone levels prior to GnRH administration in this study. Investigation using one dose rate of GnRH and artificially varied plasma levels of testosterone in intact rams would be required to elucidate the possible short-term feedback activities of testosterone. In wethers (Galloway and Pelletier, 1975; Pelletier, 1976) testosterone treatment both lowered the peak response and delayed the onset of peak responses, following GnRH administration, indicating the complexity of the feedback activity of testosterone in modifying pituitary LH release.

CHAPTER V

ENDOCRINE AND ANATOMICAL PARAMETERS DURING SEXUAL MATURATION OF RAMS

1. INTRODUCTION

Many physiological aspects of puberty, including spermatogenesis, anatomical development and growth patterns have been studied in the ram (Drymundsson, 1973), but until recently, little has been published about the endocrine changes associated with puberty, or about the neuroendocrine mechanisms regulating the onset and progression of sexual maturation of rams. This study was undertaken to investigate hormonal factors which may be involved with puberty in the ram, to delineate pituitary and gonadal functional development and to relate the attainment of a reproductive capacity to corresponding hormonal events.

2. MATERIALS AND METHODS

(1) Experiment 3.1 : Pilot Study.

In spring 1973, four Dorset ram lambs were used in a pilot study of the changes in plasma hormone levels during sexual maturation, to provide data to assist in the design of Experiment 3.2, a major study of sexual development. From two weeks of age, single blood plasma samples were collected at approximately weekly intervals for 35 weeks and subsequently assayed for LH and testosterone concentrations.

Analysis of data was not performed because of the limited data available and the lack of a sound basis upon which to design a statistical analysis.

(2) Experiment 3.2: Basal Levels of Plasma LH, Testosterone and Prolactin.

(i) Control Rams. Thirty two lambs were selected at random from an initial flock of 40 rams and at each two-weekly sampling period from birth to 32 weeks of age, eight of these were selected for blood sampling. At birth a single blood plasma sample was collected, but from two weeks of age, four plasma samples were collected at 30 min intervals at each sampling period. Sampling commenced at 0900 h on each occasion. LH and prolactin estimations were performed on all

four samples, but testosterone levels were estimated only for the first sample collected.

(ii) GnRH Treated Rams. A further four ram lambs were given intramuscular injections of GnRH. The dose of GnRH was 1 µg/Kg given as a solution of 10 µg/ml in 0.9% saline each week from 1 to 31 weeks of age. Each two weeks, four blood plasma samples were collected as for (i) above, prior to injection of GnRH, and assayed for LH and testosterone levels.

(iii) Testosterone Treated Rams. The remaining four ram lambs were given intramuscular injections of testosterone cypionate ("Depo-testosterone", Upjohn Limited) each week at a dose rate of 2.5 mg/Kg, and plasma samples collected and assayed as for (ii) above.

(iv) Data Analysis. Data from the day of birth was not included in statistical analyses since one sample only was collected from each animal. Analyses of variance of testosterone and mean (within-animal) LH data were performed; age effects were partitioned using standard orthogonal coefficients (Fisher and Yates, 1963), while the effects of hormone treatments were determined using the coefficients shown below:

Contrast	Control	GnRH Treated	Testosterone Treated
1. Control vs GnRH Treated	+1	-1	0
2. Control vs Testosterone Treated	+1	0	-1

Mean prolactin levels (within-animals) were calculated and standard orthogonal coefficients used to partition the effects of age in a separate analysis of variance.

In order to examine the likelihood of a sampling-induced alteration of plasma LH or prolactin levels, a chi-square test was applied to test whether the number of animals displaying highest levels in each of the four samples collected from control animals departed significantly from expectations. This test was based on the expectation that highest levels should occur with equal frequency in each sample.

(3) Experiment 3.3: LH and Testosterone Output in Response to GnRH Administration.

(i) Control Rams. At 6, 10, 14, 18, 22, 26, 30 and 32 weeks of age, four of the lambs sampled at those ages in Experiment 3.2 (i) above were used to study pituitary LH and gonadal testosterone output following GnRH injection. Immediately after the fourth "basal level" sample was collected, a single jugular intravenous injection of GnRH (1 µg/Kg freshly dissolved in 0.9% saline at a concentration of 10 µg/ml) was given. Blood samples were collected at 15, 30, 45, 60, 90, 120 and 240 min after injection. The remaining four lambs sampled at the above ages in Experiment 3.2 (i) above were given control injections of saline and blood samples were collected 30 and 90 min later. Plasma was stored for LH and testosterone estimations.

For analyses of variance the mean plasma concentrations of LH and testosterone in the four pre-injection samples, and the total hormone output response curves following GnRH injection (see p 60) were used as parameters. Age effects were partitioned using standard orthogonal coefficients.

Regression fits of response curves (see p.60) were calculated and age effects partitioned using orthogonal coefficients after analyses of variance to determine differences in the timing of responses to GnRH injection.

(ii) GnRH and Testosterone Treated Rams. At 32 weeks of age, GnRH and testosterone treated animals were treated with GnRH then bled as for (3) (i) above. Analyses of variance of pre-injection levels and total hormone responses, and regression fits of response curves were performed. Between-group comparisons were made using contrasts shown in section (2) (iv) above.

(4) Testicular and Epididymal Histology and Epididymal Spermatozoal Reserves.

The four rams given GnRH injections at 6, 10, 14, 18, 22, 26, 30 and 32 weeks of age were utilized for a study of testicular and epididymal development and assessment of epididymal spermatozoal reserves. Immediately following the 240 min blood sampling after GnRH injection, animals were castrated, testes and epididymides removed and weighed, and sections collected for later histological examination (see p 41). Epididymides from lambs 18 weeks and over were stored frozen for later estimation of epididymal spermatozoal reserves, as outlined in Chapter 2.

Qualitative microscopic examination of testicular and epididymal sections was undertaken using the methods of cellular identification described by Sapsford (1962), Ortavant (1969) and Steffert (1971). Two sections of each organ were examined and seminiferous and epididymal tubule diameters measured (see p41).

Body weights, testicular and epididymal data all were subjected to analyses of variance in which age effects were partitioned using standard orthogonal coefficients. Analyses of regression and covariance of this data and hormone data collected from the animals at the ages of castration, were performed to determine relationships between various hormonal and anatomical parameters during sexual maturation.

Testicular and epididymal data including epididymal spermatozoal reserves of control and GnRH and testosterone treated groups sampled at 32 weeks, were compared by analyses of variance using the coefficients shown in (2) (iv) above.

Epididymal spermatozoal reserve data from 18 to 32 weeks was subject to analysis of variance and age effects determined using standard orthogonal coefficients. Regression and covariance analyses including testis and hormone data were performed to determine relationships between endocrine and anatomical parameters and the output of spermatozoa.

(5) Analysis of Body Weight Data.

Body weights from control and GnRH and testosterone treated rams were compared by analysis of variance. Growth curves of control, GnRH and testosterone treated animals reaching 32 weeks of age were calculated as the regression of $\log_{10}$ body weight against 1/age, and were compared by *t* tests based on coefficients shown in (2) (iv) above. These procedures were designed to examine body weight and growth rate differences attributable to GnRH or testosterone treatment.

3. RESULTS

(1) Experiment 3.1: Pilot Study.

See Figure 5.1.

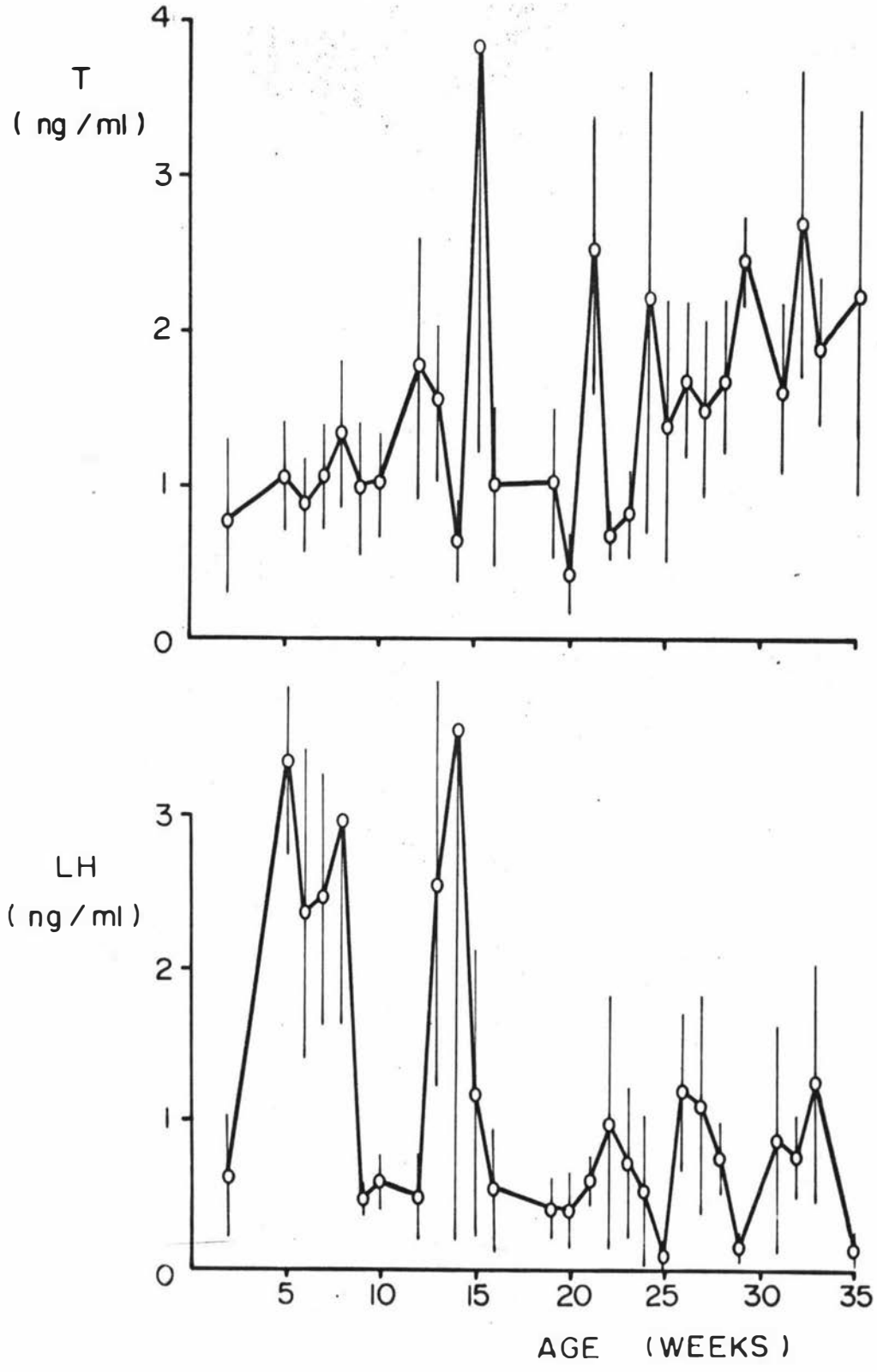


Figure 5.1: Plasma LH and testosterone concentrations (means ± SEM) recorded from Dorset ram lambs from birth to 35 weeks of age.

Plasma levels of LH were high (up to 3.54 ng/ml) between 5 and 8 weeks, but dropped to approximately 0.5 ng/ml between 9 - 12 weeks. Following an elevation at 13 and 14 weeks, concentrations fluctuated only slightly, and mean plasma content rarely exceeded 1.0 ng/ml. Mean plasma testosterone concentrations rose slowly from 0.75 ng/ml up to 1.73 ng/ml by 12 weeks, then during the following 12 weeks fluctuated between 0.40 and 3.84 ng/ml. From 25 to 35 weeks, levels increased steadily from 1.38 to 2.24 ng/ml.

(2) Experiment 3.2: Basal LH, Testosterone and Prolactin Plasma Levels.

See Figures 5.2, 5.3 and Table 5.1.

Peak plasma LH levels in control and GnRH treated lambs were reached at 6 weeks of age, and in testosterone treated animals at 12 weeks. Mean levels subsequently decreased until approximately 16 weeks then remained relatively stable at between 0.25 - 0.75 ng/ml. Analyses of variance (Table 5.1) showed that there were significant linear decreases in LH levels with age ($P < 0.001$) in both control and GnRH treated animals.

Mean plasma testosterone concentrations increased from birth and highest levels (approximately 2.7 ng/ml) were reached at between 26 and 30 weeks of age in control and GnRH and testosterone treated rams. The increase of testosterone concentration was shown to be linear in each group ($P < 0.001$, Table 5.1).

Mean plasma content of prolactin was low at birth (14.7 ng/ml), increased rapidly to approximately 55 ng/ml at 6 weeks and remained at approximately that level until 18 weeks, whereupon levels declined gradually to 13.4 ng/ml at 32 weeks. This pattern of prolactin secretion was the reason for the significant ($P < 0.001$) quadratic component in the analysis of variance of the age effects for the prolactin data.

Analyses of variance testing the effects of weekly GnRH and testosterone treatments (Table 5.1) showed no effect of treatment on LH levels, but testosterone levels measured in plasma from testosterone treated rams were significantly lower than those from control rams ($P < 0.001$).

A chi-square analysis was performed to determine whether

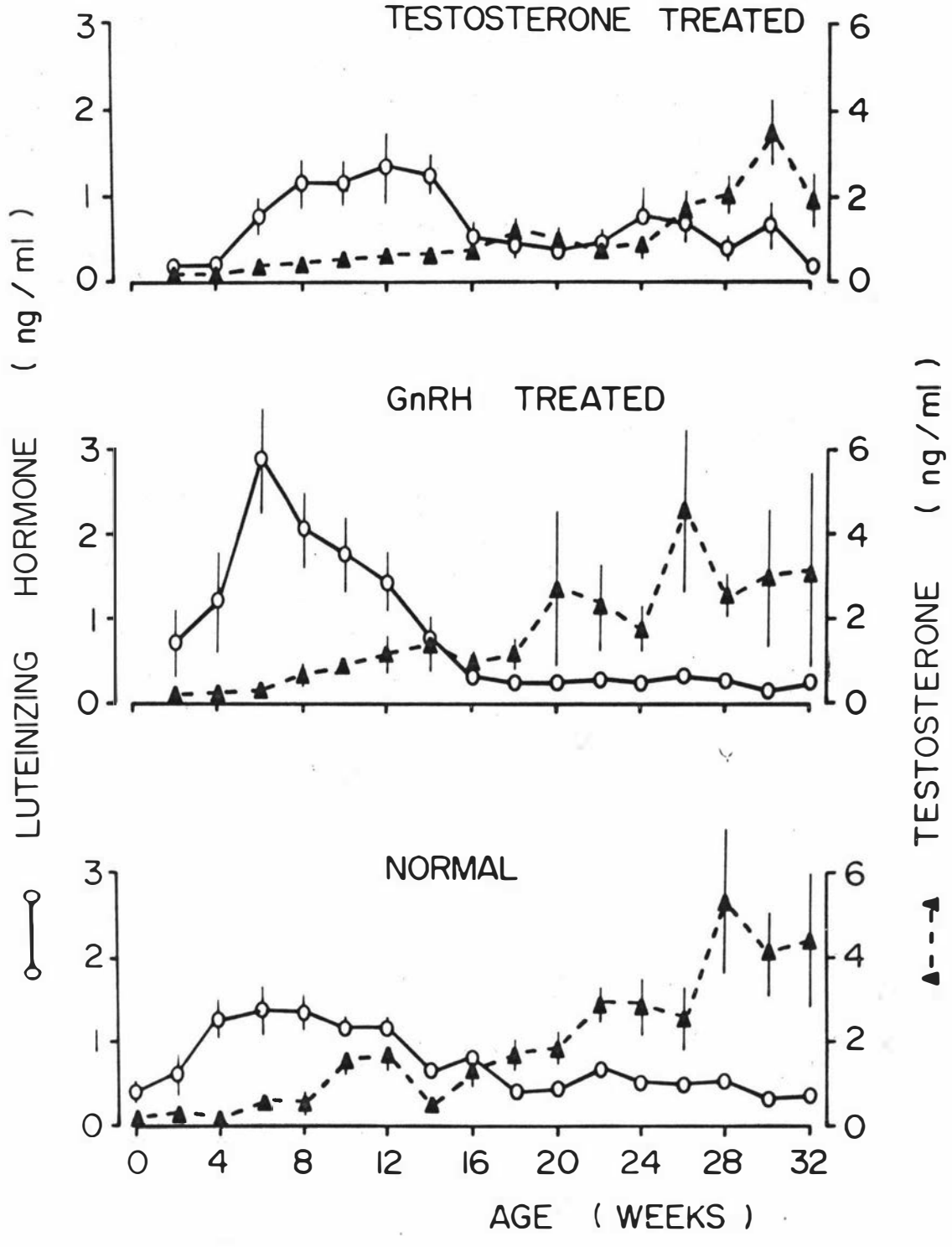


Figure 5.2: Plasma LH and testosterone concentrations (means ± SEM) recorded from control, GnRH treated and testosterone treated rams from birth to 32 weeks of age.

Figure 5.3: Plasma concentrations of prolactin (means ± SEM) recorded from control ram lambs from birth to 32 weeks of age.

TABLE 5.1

Summary of analyses of variance of basal LH, testosterone and prolactin data for control and GnRH and testosterone treated rams.

Source of Variation	d.f.	Variance Ratios		
		control	GnRH treated	testosterone treated
1. AGE				
LH				
linear	1	14.53***	42.49***	0.56
quadratic	1	4.15*	0.00	7.89**
cubic	1	9.43**	17.76***	3.84*
Residual mean square	235	161.5	161.5	161.5
TESTOSTERONE				
linear	1	129.88***	37.82***	31.99***
quadratic	1	0.25	2.24	0.39
cubic	1	0.39	0.39	0.19
Residual mean square	235	331.9	331.9	331.9
PROLACTIN				
linear	1	3.18		
quadratic	1	16.09***		
cubic	1	0.41		
Residual mean square	109	303.5		

2. HORMONAL TREATMENT		LH	Testosterone
Control vs GnRH Treated	1	0.04	1.44
Control vs Testosterone Treated	1	2.53	17.22***
Residual mean square	235	161.5	331.9

sampling-induced elevations of LH and prolactin concentration occurred in untreated ram lambs. Data for these analyses and chi-square values are shown below:

Hormone	Number of rams with highest Basal Level in Samples:				Expected Number	χ^2_3
Sample No.	1	2	3	4		
LH	94	16	9	4	30.75	170.29***
Prolactin	50	31	29	13	30.75	22.39***

Results indicated that significantly greater numbers of animals than expected showed higher levels of both LH and prolactin in the first of the four samples collected ($P < 0.001$).

(3) Experiment 3.3: LH and Testosterone Output in Response to GnRH Administration.

See Figures 5.4 - 5.8 and Tables 5.2 and 5.3.

Each GnRH injection was followed by an elevation of LH concentrations within 15 min. The mean peak LH level was highest (64 ng/ml) at the first sampling at 6 weeks of age, then decreased progressively to 20.5 ng/ml at 32 weeks. Analysis of variance (Table 5.2) showed this change to be a significant linear decrease with age ($P < 0.001$). Peak responses occurred within 15 min of injection at 6, 10, 14 and 18 weeks of age, at 45 min at 22 weeks, 30 min at 26 weeks, and 60 min at 30 and 32 weeks.

The principal significant finding from the analyses of variance of regression fits (Table 5.3) was that the linear ($P < 0.01$), negative quadratic ($P < 0.001$) and cubic ($P < 0.001$) regression fits of LH response curves decreased linearly with age, indicating a progressive delay in the peak LH response as sexual maturation progressed.

Conversely, mean peak testosterone levels were low (3.6 ng/ml) at 6 weeks, increased slightly at 10, 14 and 18 weeks, and reached progressively higher levels thereafter, being maximal (9.86 ng/ml) at 32 weeks of age. In the analysis of variance (Table 5.2) this change was shown by the significant linear increase with age in the testosterone response ($P < 0.001$).

Peak testosterone content in plasma at 6 weeks of age occurred 120 min after GnRH injection, and in the subsequent experiments at

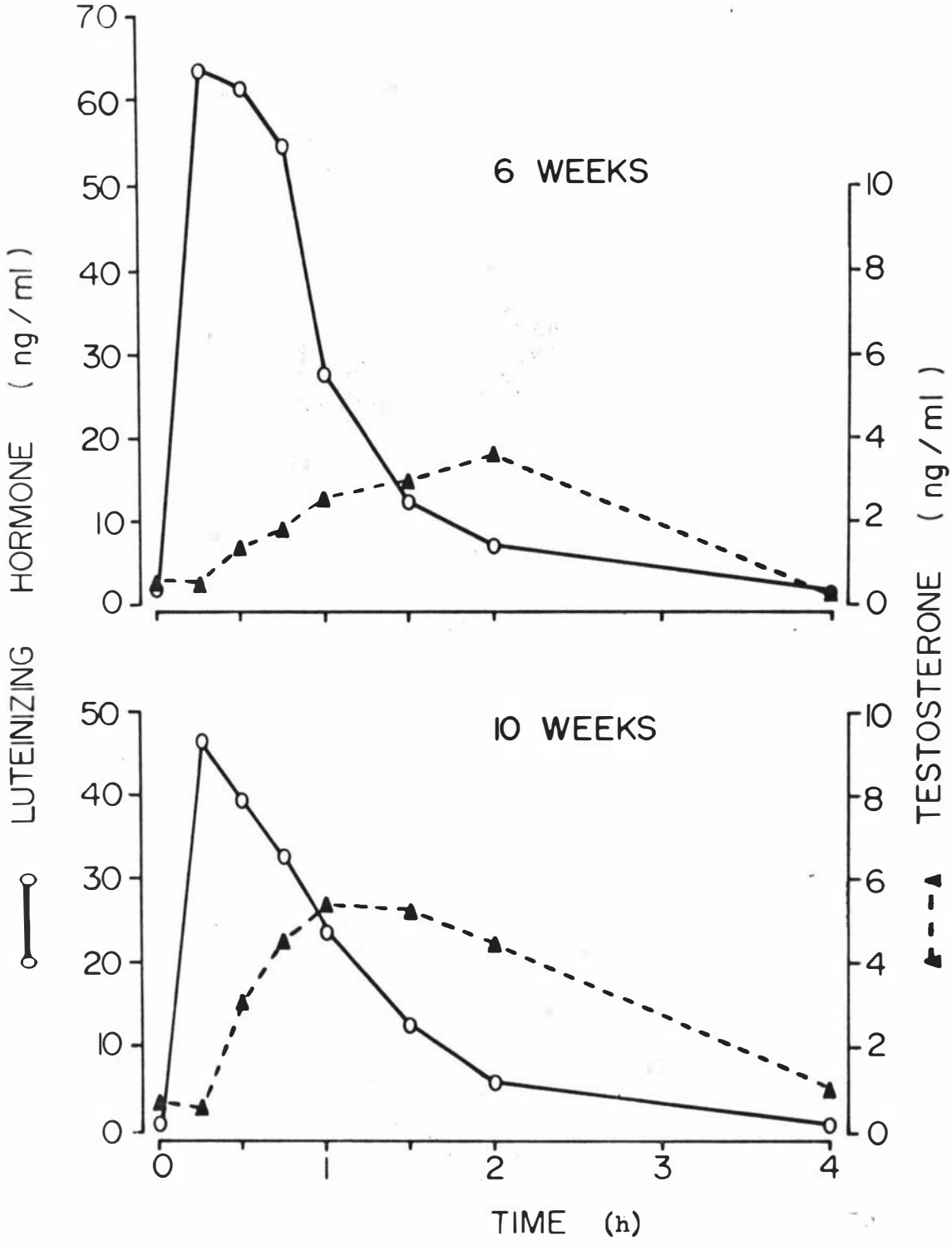


Figure 5.4: Mean LH and testosterone responses following injection of GnRH (immediately after the zero h sample) at 6 and 10 weeks of age.

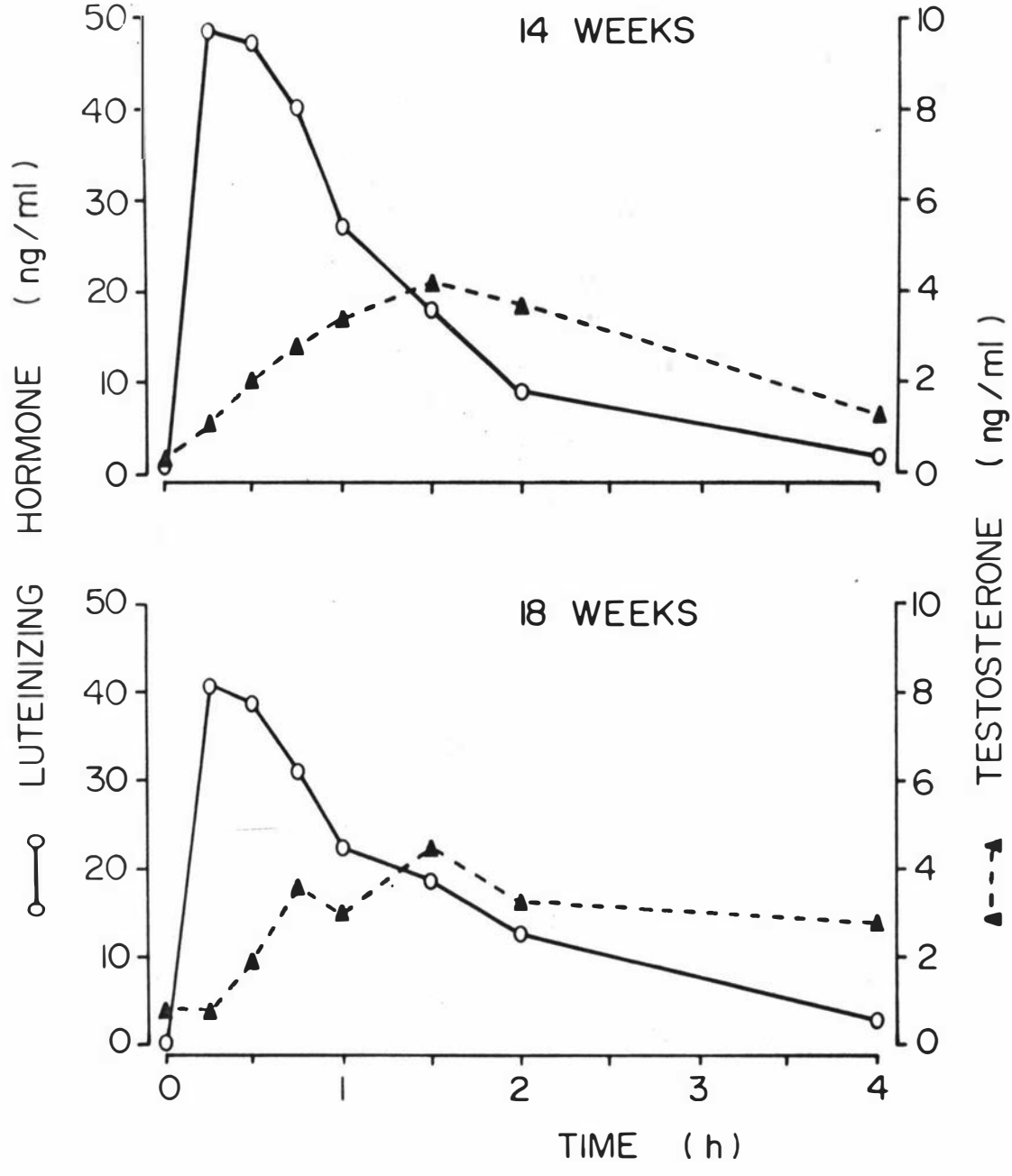


Figure 5.5: Mean LH and testosterone responses following injection of GnRH (immediately after the zero h sample) at 14 and 18 weeks of age.

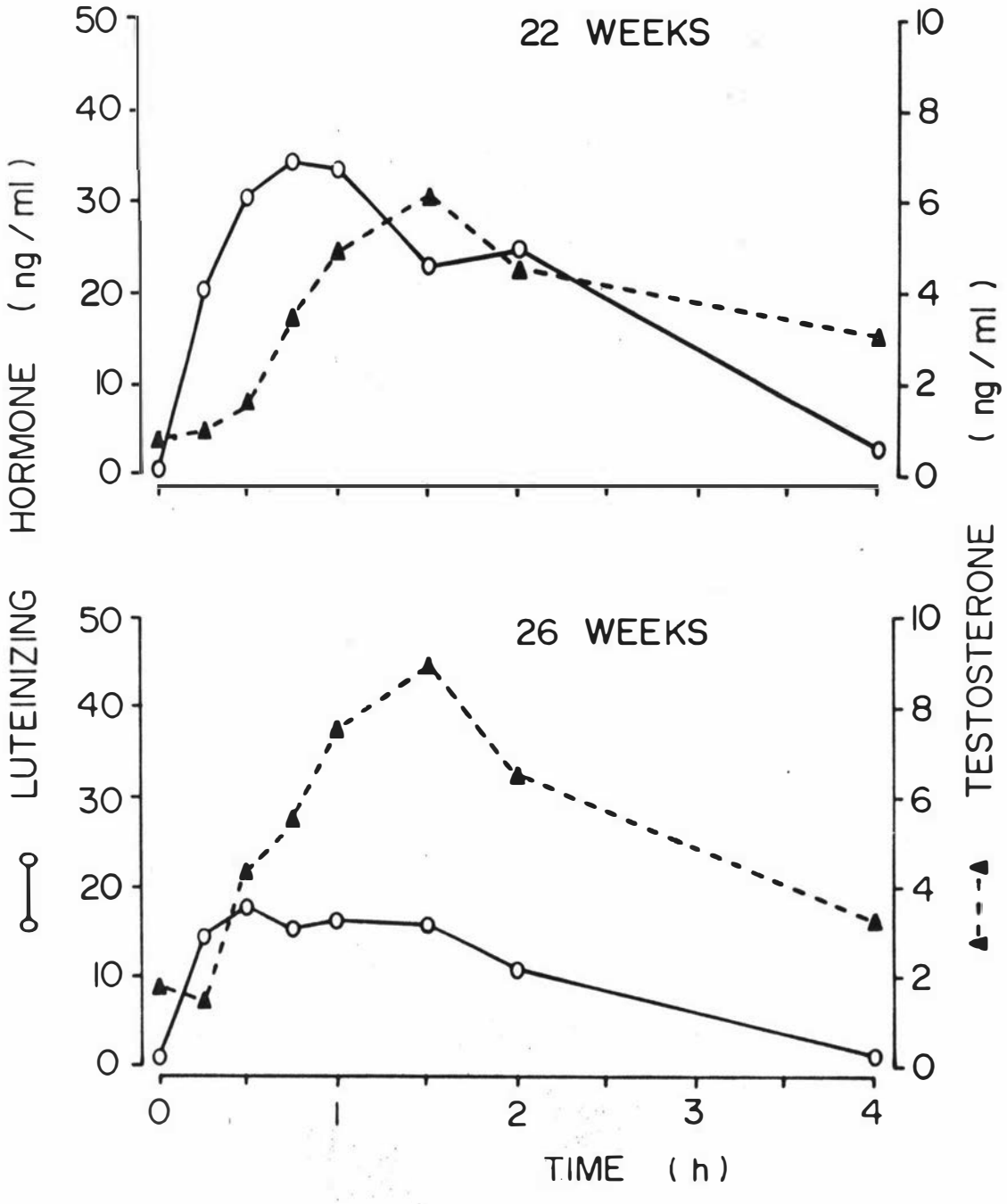


Figure 5.6: Mean LH and testosterone responses following injection of GnRH (immediately after the zero h sample) at 22 and 26 weeks of age.

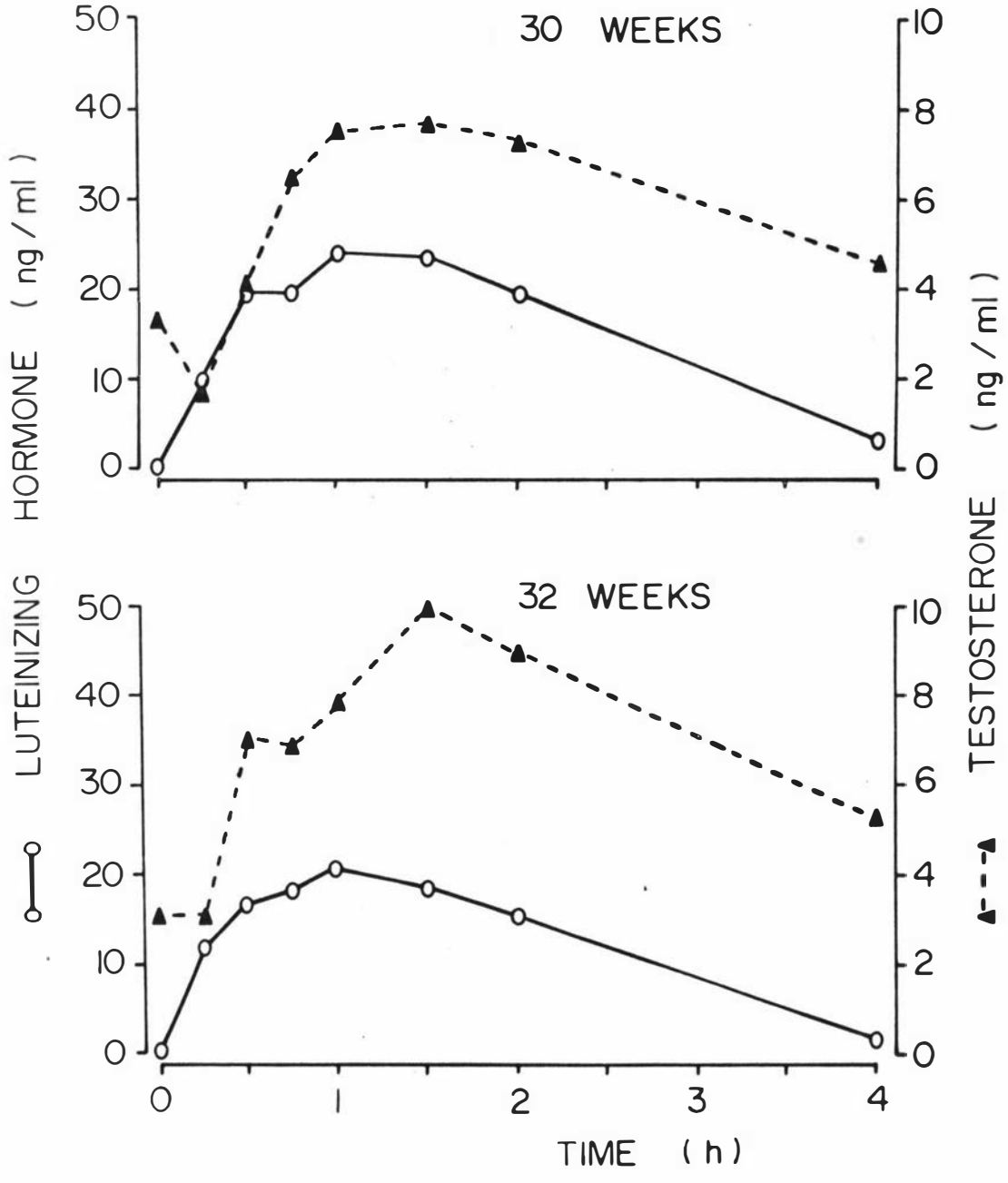


Figure 5.7: Mean LH and testosterone responses following injection of GnRH (immediately after the zero h sample) at 30 and 32 weeks of age.

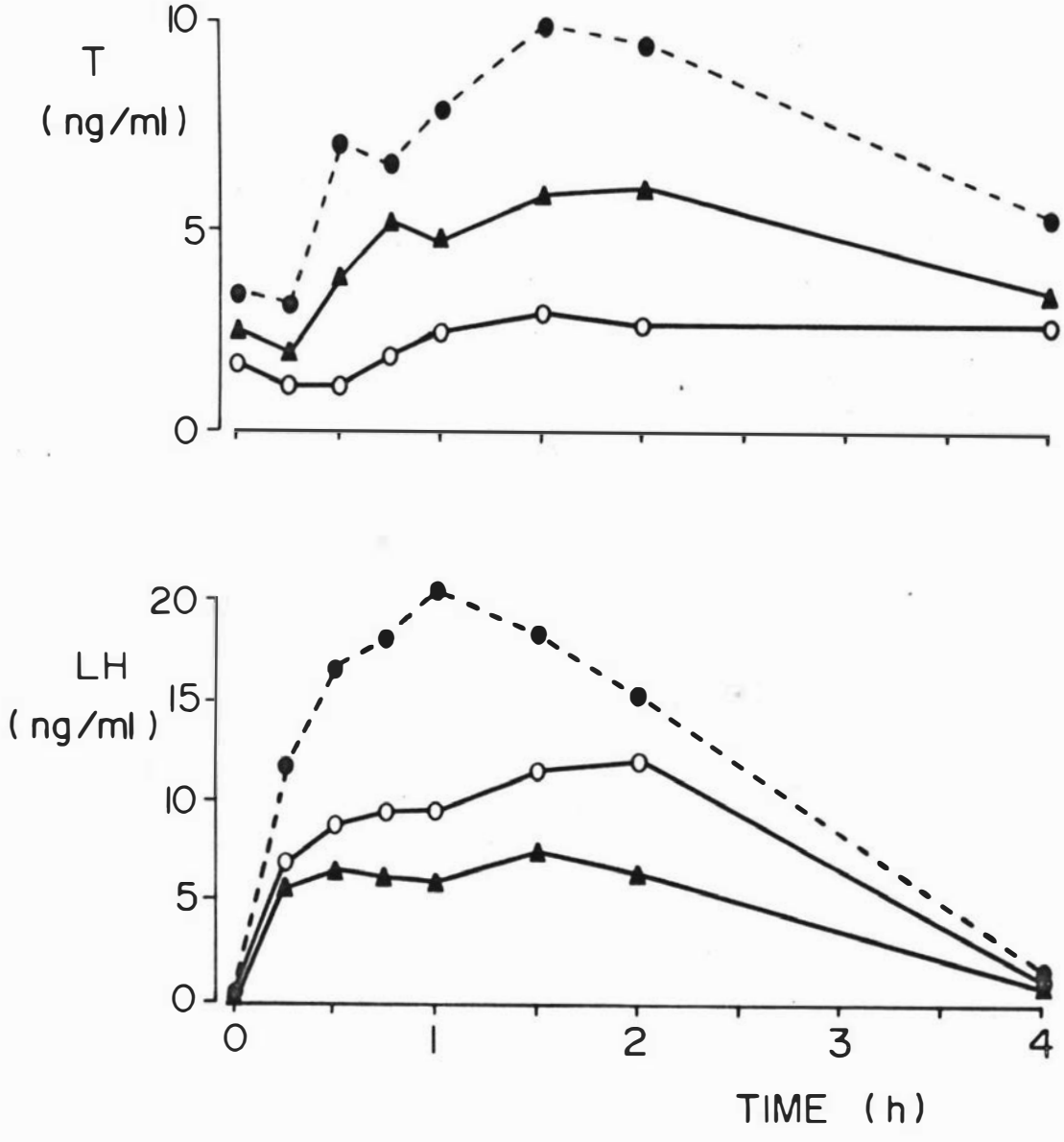


Figure 5. 8: Mean plasma LH and testosterone responses of control (● - - ●), GnRH treated (▲——▲) and testosterone treated (○——○) ram lambs following GnRH injection (immediately after the zero h sample) at 32 weeks of age.

TABLE 5.2

Experiment 3.3: Mean pre-injection levels (ng/ml) and total LH and testosterone output responses to GnRH, and summary of analyses of variance of this data.

Group	Age (weeks)	LH		Testosterone	
		Pre-injection	Total	Pre-injection	Total
CONTROL					
	6	1.01	17.43	0.31	9.86
	10	0.62	16.75	0.62	11.73
	14	0.48	17.46	0.19	11.05
	18	0.26	17.02	0.80	11.33
	22	0.25	17.28	0.71	11.80
	26	0.40	15.64	1.89	13.17
	30	0.16	16.24	3.32	13.06
	32	0.27	15.95	3.03	14.18
GnRH TREATED					
	32	0.08	13.36	0.21	12.75
TESTOSTERONE TREATED					
	32	0.08	13.58	0.17	10.21

Source of Variation	d.f.	Variance Ratios			
1. AGE					
linear	1	11.45**	15.93***	49.16***	57.60***
quadratic	1	3.76	0.82	3.35	0.70
cubic	1	0.74	0.53	0.15	0.14
Residual mean square	24	0.02	0.98	0.01	1.58
2. HORMONAL TREATMENT					
Control vs GnRH Treated	1	0.37	3.53	0.06	1.05
Control vs Testosterone Treated	1	0.50	2.95	0.98	8.85*
Residual mean square	9	0.002	3.80	0.053	3.86

TABLE 5.3

Summary of analyses of variance of regression fits of LH and testosterone response curves.

		Variance Ratios					
		LH			Testosterone		
Source of Variation	d.f.	linear	quadratic	cubic	linear	quadratic	cubic
1. AGE							
linear	1	13.76**	48.00***	89.12***	1.33	1.17	0.12
quadratic	1	1.73	0.03	0.13	1.19	1.07	1.94
cubic	1	3.96	3.77	8.85**	3.75	8.34**	5.97*
Residual mean square	25	420628	685443	467.67	402184	188365	365.91
2. HORMONAL TREATMENT							
Control vs GnRH Treated	1	4.50	0.13	0.23	1.03	1.12	0.99
Control vs Testosterone Treated	1	4.47	0.41	0.78	7.24*	1.48	1.53
Residual mean square	10	606890	694421	496.03	564310	502394	414.03

60 - 90 min post-injection. There were no major differences between the regression fits of testosterone response curves from 6 to 32 weeks of age, indicating the shapes of the response curves were essentially the same.

The only significant difference observed between treated and control groups was that the total testosterone output from testosterone treated rams was significantly less than that from control rams ($P < 0.05$, Table 5.2).

(4) Body Weight, Testis, Epididymal and Hormonal Data.

(i) Body Weight. See Figure 5.9 and Table 5.4.

Analysis of variance (Table 5.4) showed that both GnRH and testosterone treated animals had significantly greater body weights than untreated animals ($P < 0.001$). t test comparisons of growth curve regression coefficients showed no significant differences, indicating that while body weights were significantly different, growth rates of animals in each group were similar. Thus body weight differences were likely to have been a reflection of differing body weights at birth: GnRH treated and testosterone treated groups were respectively 0.80 and 0.68 Kg heavier than controls at birth.

Table 5:4. Summary of Analysis of Variance of GnRH and Testosterone Treatment Effects on Body Weight.

Source of Variation	d.f.	Variance Ratio
1. Normal vs GnRH Treated	1	59.38***
2. Normal vs Testosterone Treated	1	29.88***
Residual Mean Square	385	0.0063

(ii) Body Weights, Testis and Epididymal Growth, Epididymal Spermatozoal Reserves and Hormone Data at Ages of Castration.

See Tables 5.5 - 5.8 and Figures 5.10 and 5.11.

LH and testosterone data relevant to this section have been presented in sections (2) and (3) above (see Table 5.2).

Body weights, testis weights, epididymal weights, seminiferous and epididymal tubule diameters (Table 5.5) increased linearly with age ($P < 0.001$) from 6 to 32 weeks. Epididymal spermatozoal reserves increased linearly from 18 to 32 weeks ($P < 0.001$); prior to 18 weeks

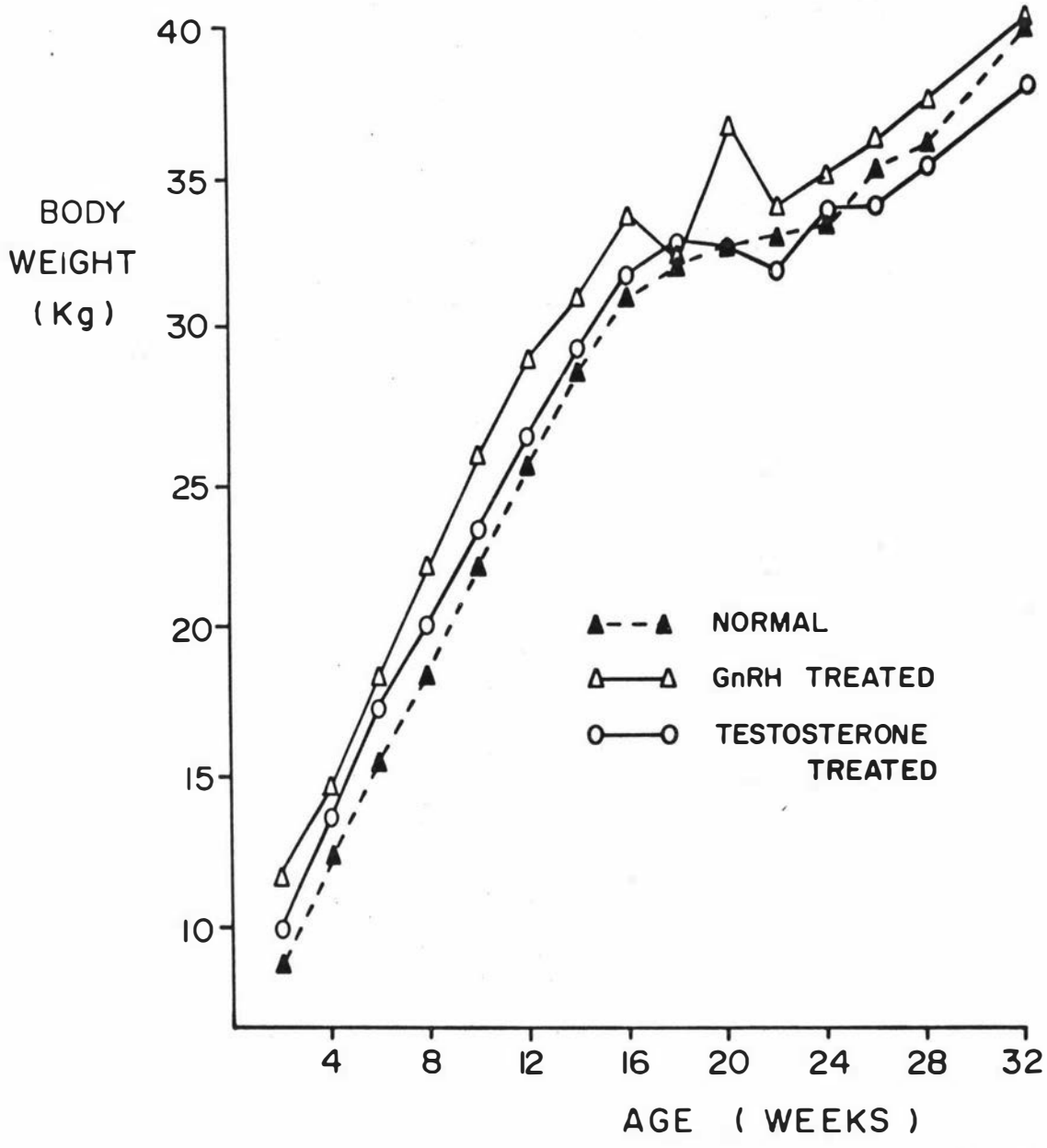


Figure 5.9: Mean body weights of control, GnRH treated and testosterone treated rams from birth to 32 weeks of age.

TABLE 5.5

Body weights, testis, epididymal and hormone data (means ± SEM) from rams castrated from 6 to 32 weeks of age. (n=4)

Age (weeks)	Body Weight (Kg)	Testis Weight (g)	Epididymal Weight (g)	Seminiferous Tubule Diameter (μ)	Epididymal Tubule Diameter (μ)	Epididymal Spermatozoal Reserves ($x10^9$)	Basal Prolactin (ng/ml)
6	17.27 ±1.37	4.61 ±0.40	2.62 ±0.37	51.70 ±1.25	142.4 ±13.50		53.93 ±2.16
10	24.75 ±2.07	17.05 ±1.82	5.80 ±0.47	88.45 ±4.90	222.71 ±9.97		53.95 ±2.11
14	24.75 ±2.59	22.87 ±2.82	5.97 ±0.52	99.36 ±3.75	201.53 ±4.09		50.40 ±1.66
18	27.40 ±1.59	54.08 ±7.24	8.40 ±0.92	131.85 ±5.48	229.24 ±10.56	0.00 ±0.00	60.90 ±0.93
22	33.82 ±3.11	64.94 ±6.72	10.68 ±1.17	146.95 ±3.90	153.73 ±5.30	6.47 ±3.76	46.95 ±11.73
26	33.50 ±2.82	101.55 ±11.06	15.08 ±1.22	131. 71 ±9.59	268.13 ±9.91	19.58 ±8.78	24.72 ±1.92
30	36.25 ±3.11	119.45 ±14.19	18.13 ±1.63	174.98 ±8.36	306.55 ±13.40	25.95 ±1.29	36.55 ±1.24
32	40.20 ±2.90	143.15 ±7.98	22.15 ±1.43	196.71 ±2.84	347.72 ±9.42	59.04 ±9.51	13.40 ±4.53

TABLE 5.6

Summary of analyses of variance of data presented in Table 5.5.

Source of variation	d.f.	Variance Ratios						
		Body Weight	Testis Weight (1)	Epididymal Weight (1)	Seminiferous Tubule Diameter (2)	Epididymal Tubule Diameter (2)	Epididymal Spermatozoal Reserves (4)	Basal Prolactin
AGE								
linear	1	80.50***	274.33*** (131.11***)	182.41*** (67.50***)	217.38*** (5.44*)	124.73*** (6.28*)	40.59***	31.49***
quadratic	1	3.63	21.61*** (11.86**)	0.84 (0.00)	23.80*** (0.00)	0.03 (0.00)	0.97	8.56**
cubic	1	0.47	1.91 (0.74)	1.65 (0.47)	2.40 (0.30)	6.17* (3.28)	0.42	0.03
Residual mean square	25	0.0055	0.0236	0.0149	0.0042	0.0028	1610	399.51

Figures in parentheses represent variance ratios of means corrected for:(1) Body Weight;(2) Testis Weight; (3) Epididymal Weight.

(4) Error degrees of freedom = 16 (data from 22 to 30 weeks only)

TABLE 5.7

Variance ratios of regressions of data presented in Tables 5.2 and 5.4.

Dependent Variable	Independent Variable: Body Weight	Testis Weight	Epididymal Weight	Seminiferous tubule Diameter	Epididymal Tubule Diameter	Total LH	Total Testosterone	Basal LH	Basal Testosterone	Basal Prolactin
Testis Weight	16.20***	----	----	----	----	0.44	8.46**	0.36++	0.38	4.63*
Epididymal Weight	18.92***	62.25***	----	----	----	1.26	4.77*	0.00	1.09	1.80
Seminiferous Tubule Diameter	3.86	46.66***	----	----	----	0.22	3.37	0.06	0.38	3.35
Epididymal Tubule Diameter	----	----	18.56***	11.27**	----	0.41	0.94	0.05	0.01++	1.66
Epididymal (1) Spermatozoal Reserves	----	7.96*	8.62*	5.21*++	1.02	0.84	7.88*	5.43*	3.78++	4.99*++

(1) d.f. = 15; all other regressions have 24 d.f.

++indicates negative regression.

TABLE 5.8

Body weight, testis and epididymal data from GnRH and testosterone treated groups at 32 weeks of age (Means ± SEM), and analyses of variance of this data. (Data for control rams is presented in table 5.5).

Group	Body Weight (Kg)	Testis Weight (g)	Epididymal Weight (g)	Seminiferous Tubule Diameter (μ)	Epididymal Tubule Diameter (μ)	Epididymal Spermatozoal Reserves($\times 10^9$)
GnRH Treated	40.75 ±2.56	125.67 ±12.11	21.46 ±2.44	172.25 ±4.73	333.61 ±15.25	49.67 ±10.99
Testosterone Treated	38.00 ±1.47	37.22 ±7.51	11.73 ±1.85	124.62 ±16.16	231.40 ±5.60	1.15 ±0.54

Source of Variation	d.f.	Variance Ratios				
HORMONAL TREATMENT						
Control vs GnRH Treated	1	0.06	0.11(1) (0.20)	0.23(1) (0.04)	0.40(2) (0.04)	0.07(3) (0.07)	0.43
Control vs Testosterone Treated	1	0.25	12.30** (11.41**)	8.11* (6.79*)	8.22* (0.28)	16.11** (2.02)	122.90***
Residual mean square	10	0.003	0.093	0.033	0.019	0.003	367.59

Figures in parentheses represent variance ratios for means corrected for: (1) Body weight; (2) Testis weight; (3)Epididymal weight.

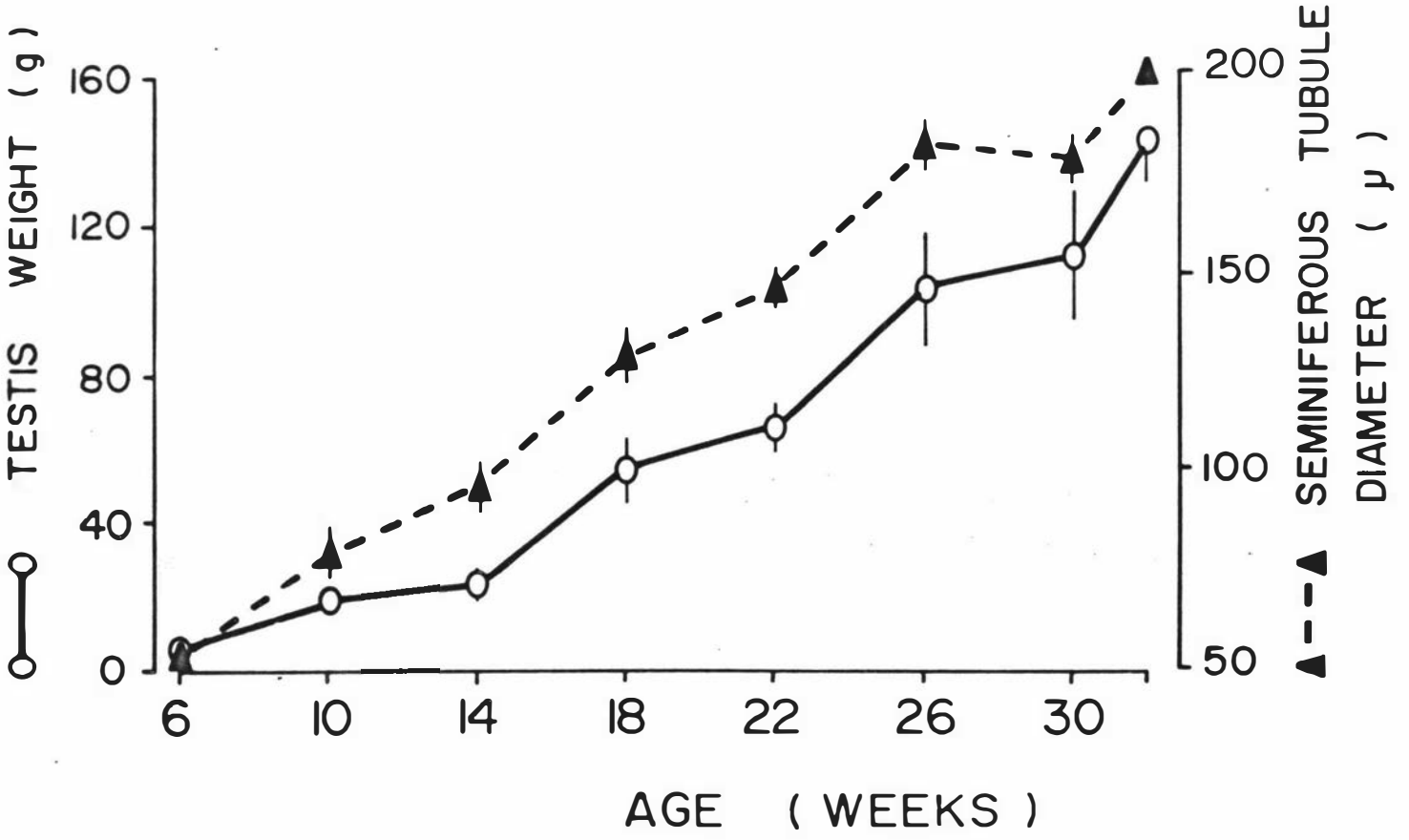


Figure 5. 10: Testis weights and seminiferous tubule diameters (means ± SEM) of control ram lambs from 6 to 32 weeks of age.

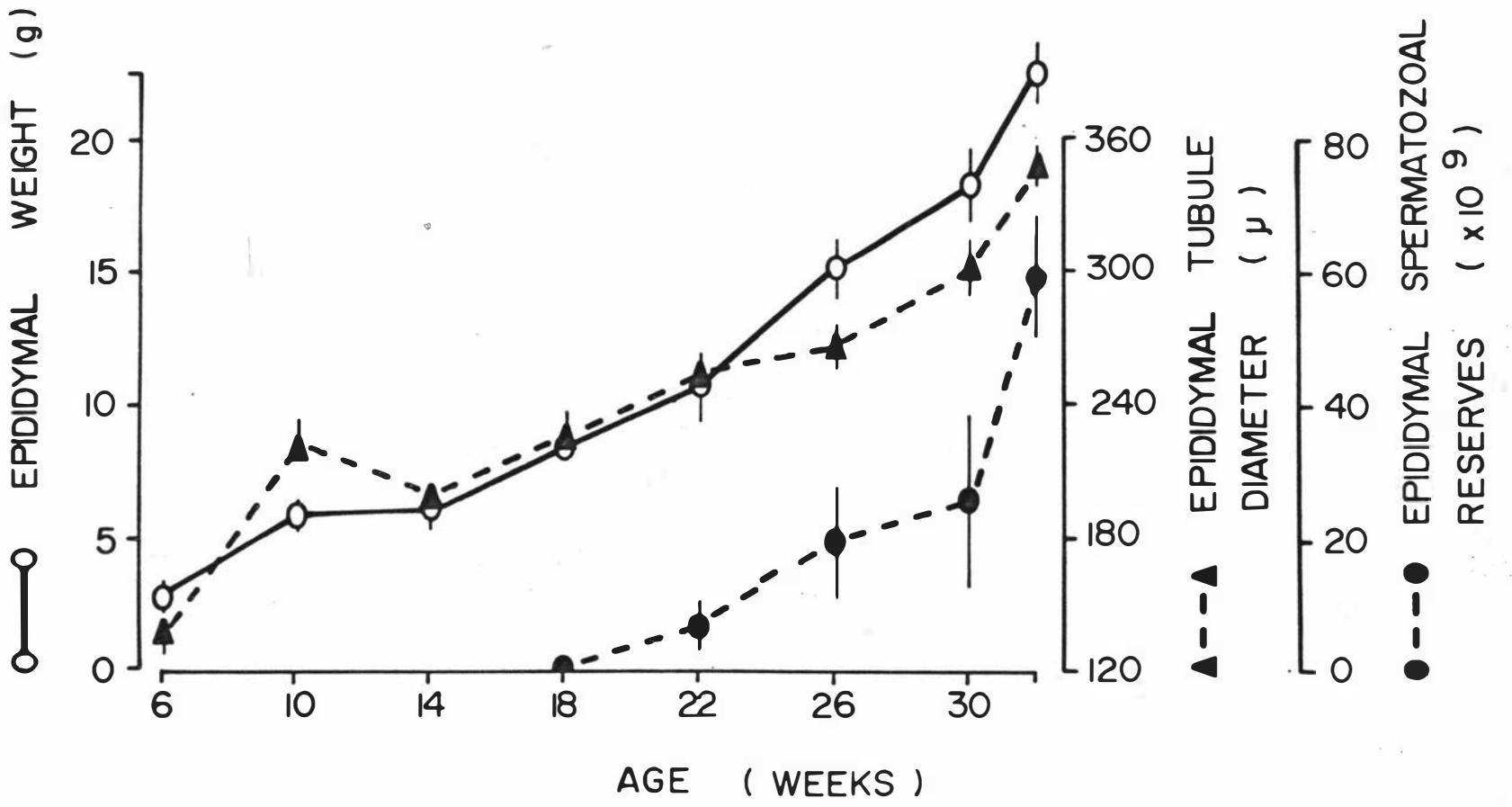


Figure 5.11: Epididymal weights and epididymal tubule diameters from 6 to 32 weeks of age, and epididymal spermatozoal reserves from their first appearance at 22 weeks of age (means ± SEM).

of age no epididymal spermatozoa were present.

When body weight was used as a covariate, testis and epididymal weights increased significantly ($P < 0.001$) indicating that the relative growth rate of these organs was greater than that of body weights. Use of testis weight and epididymal weight as covariates showed seminiferous tubule and epididymal tubule diameters to increase relatively more rapidly than the respective organ weights ($P < 0.05$).

Analyses of variance of regression coefficients showed the following important significant relationships (Table 5.7):

(a) Testis growth was related to body growth and to the increasing total testosterone responses to GnRH during the period of study.

(b) Epididymal weights increased with increasing body weights, testis weights and total testosterone responses to GnRH injection.

(c) Epididymal spermatozoal reserves increased as testis weights, epididymal weights, seminiferous tubule diameters and total testosterone responses to GnRH increased. Though epididymal spermatozoal reserves were related to basal LH levels, LH levels did not change significantly from 18 - 32 weeks and consequently the correlation of this relationship was small($r^2 = 0.25$).

Analyses of variance of data from control and hormonally treated rams at 32 weeks of age (Table 5.8) showed that testis weights, epididymal weights, seminiferous tubule and epididymal tubule diameters and epididymal spermatozoal reserves, all were lower in testosterone treated rams than in untreated rams. GnRH treatment had no significant effect on any of the parameters measured.

(5) Testicular Histology.

See Figures 5.12 - 5.23. A summary of histological observations is presented in Table 5.9.

(i) Seminiferous Tubular Histology During Sexual Maturation

(a) Control Rams.

6 Weeks: Seminiferous tubules consisted of solid sex cords (Figure 5.12) within which were dense populations of supporting cells lining the basement membrane, while numerous gonocytes and some

TABLE 5.9

Number of rams displaying each testicular feature during sexual maturation.
(Four animals were examined at each age)

AGE (weeks)	Seminiferous Tubule Lumina	Gonocytes	Prospermatogonia	Spermatogonia	Primary Spermatocytes		Spermatids		Spermatozoa	
					Young	Old	Round	Elongated	Within Seminiferous Tubule	Within Epididymal Tubule
Control Rams										
6	0	4	4	-	-	-	-	-	-	-
10	3	2	4	2	2	-	-	-	-	-
14	4	-	-	4	4	3	-	-	-	-
18	4	-	-	4	4	4	2	2	2	-
22	4	-	-	4	4	4	4	4	4	2
26	4	-	-	4	4	4	4	4	4	4
30	4	-	-	4	4	4	4	4	4	4
32	4	-	-	4	4	4	4	4	4	4
GnRH Treated Rams										
32	4	-	-	4	4	4	4	4	4	4
Testosterone Treated Rams										
32	3	1	1	3	3	3	3	3	3	3

actively dividing prospermatogonia were present in both central and eccentric locations.

10 Weeks: Most tubules contained well defined lumina, particularly those nearer the rete testis. Though many tubules contained eccentrically placed prospermatogonia, the majority of these germ cells were located close to the basement membrane (Figure 5.13). A high degree of mitotic activity was evident in these cells, and many had assumed the appearance of spermatogonia. In a few tubules, single lines of primary spermatocytes were observed. Supporting cells had begun to assume mature Sertoli cell morphology and were interspersed between germ cells in most tubules.

14 Weeks: The testes of one ram castrated at this age were approximately twice the weight of those of the other three; seminiferous tubules of the testes of this animal all contained lumina, large numbers of early spermatocytes were present and many of these were actively dividing (Figure 5.15). In sections of testes from the remaining rams some seminiferous tubules lacked lumina, though germ cells were all in their peripheral positions (Figure 5.14) and a small number of tubules contained primary spermatocytes.

18 Weeks: In sections from two animals, both early (young) and late (old) primary spermatocytes (Figure 5.17) were abundant. Sections from the other two rams showed a much more mature appearance with round and elongated spermatids present in many tubules, and spermatozoa present in a few (Figure 5.15). The latter cells usually were scattered throughout the lumen, but often displayed characteristic streaming toward Sertoli cells. Sertoli cells were relatively less abundant on the basement membrane than in younger testes and had assumed the characteristics of mature Sertoli cells with pale staining, often flattened or triangular shaped nuclei.

22 Weeks: All sections possessed tubules with spermatozoa present. As observed in the adult, not all tubules contained all cell types, indicating that the spermatogenic cycle had been implemented even at this early stage of spermatogenesis.

26 - 32 Weeks: The histological appearance of all sections from rams 26 weeks and older were similar. All elements of the spermatogenic cycle were present (Figures 5.17 and 5.18) and the histological appearance was that of the adult shown in Figure 5.20.

(b) GnRH and Testosterone Treated Rams. Testes from rams treated with GnRH then castrated at week 32 were indistinguishable histologically from those of control animals.

Three of the animals treated with testosterone also showed testicular histological features similar to those of untreated rams, but a fourth ram possessed testes which resembled the histological structure of 6 week old lambs (Figure 5.21). No tubular lumina were present and supporting cells were immature in appearance. Gonocytes and prospermatogonia were present both centrally and peripherally, but appeared to be present in larger numbers than in testes of untreated six week old rams (Figure 5.12).

(ii) Interstitial Tissues. Animals at all ages possessed Leydig cells which often were characterised by an eosinophilic granular cytoplasm (Figure 5.12). Though interstitial tissue became relatively less abundant as seminiferous tubules increased in size, the morphology of the Leydig cells, interstitial fibrous connective tissue and blood vascular tissue remained constant.

Neither GnRH nor testosterone treatment produced any morphological effects on interstitial tissue histology.

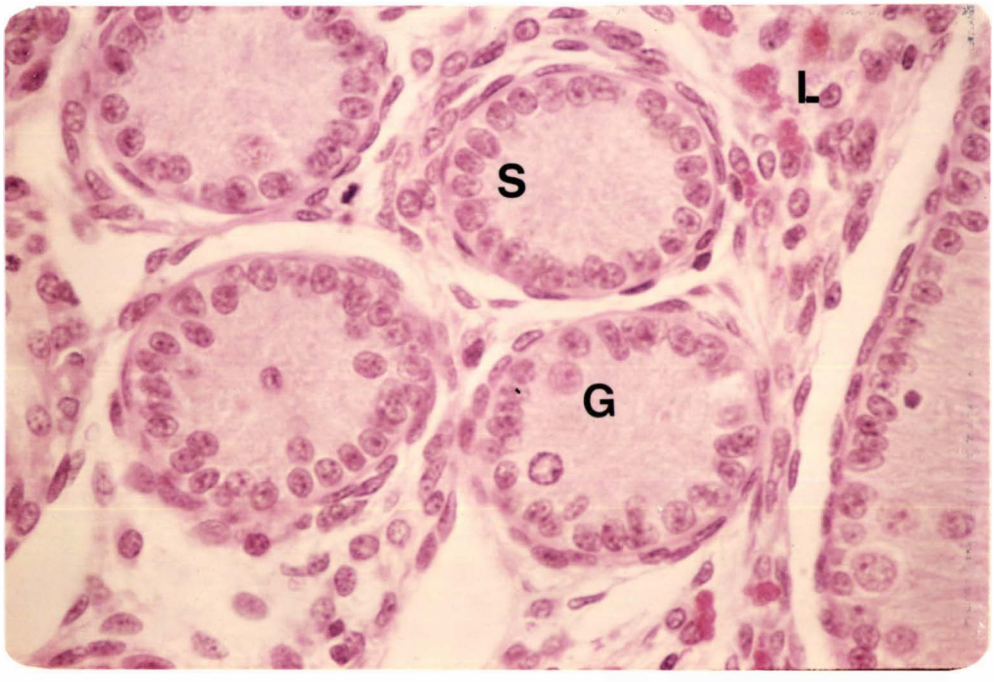
(6) Epididymal Histology.

(i) Control Rams. At six weeks of age, the epididymal tubules possessed distinct lumina (Figure 5.22). The epithelium was columnar and ciliated. Epithelial basal cells were evident in all sections from animals 10 weeks and older, but only in sections from one animal at 6 weeks. From 10 weeks, the histological appearance of the epididymal tubule lining did not change apart from an increase in height of the epithelial lining. At 18 weeks, despite testicular histological examination indicating that two rams were producing spermatozoa, no spermatozoa were evident in the body of the epididymis. The epididymal tubules of two rams at 22 weeks, and of all older animals contained spermatozoa (Figure 5.23).

(ii) GnRH and Testosterone Treated Rams. The epididymides of the GnRH treated animals were indistinguishable in structure from those of control animals, as were those of three androgen treated rams. The fourth androgen treated ram, the ram with a prepubertal testicular histological appearance, possessed no epididymal spermatozoa, but abundant basal epithelial cells were present in the epithelial lining.

Figure 5.12: 6 Weeks (x400). Solid sex cords containing gonocytes (G) and supporting cells (S). Interstitial tissue contains numerous Leydig cells (L), many of which possess cytoplasmic eosinophilic granules.

Figure 5.13: 10 Weeks (x400). Sex cords containing dividing prospermatogonia (D) and type-A spermatogonia (S).


L
S
G

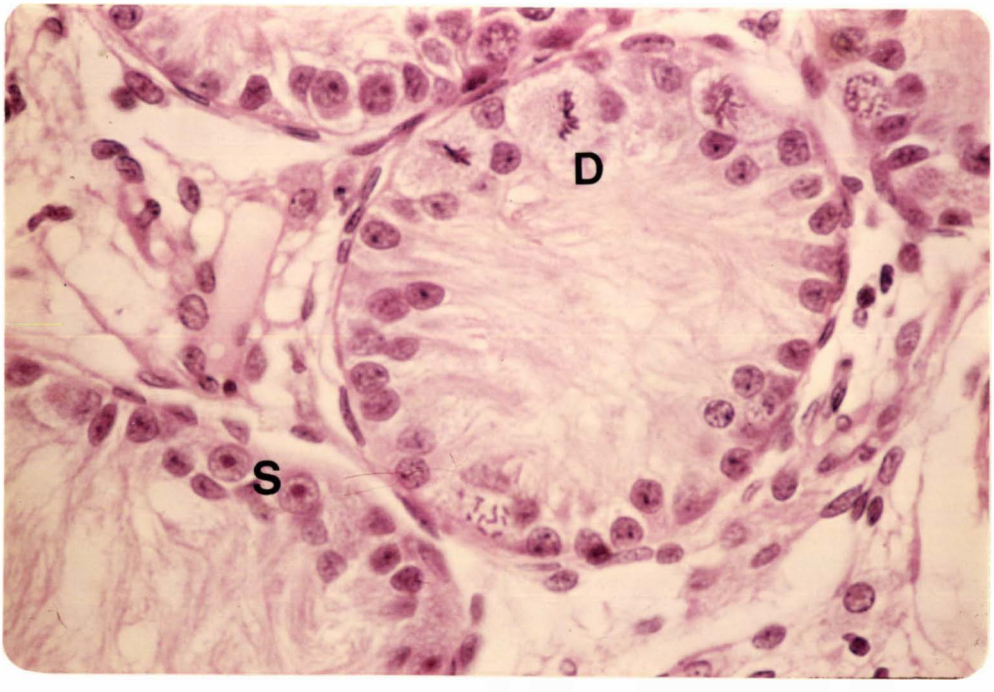
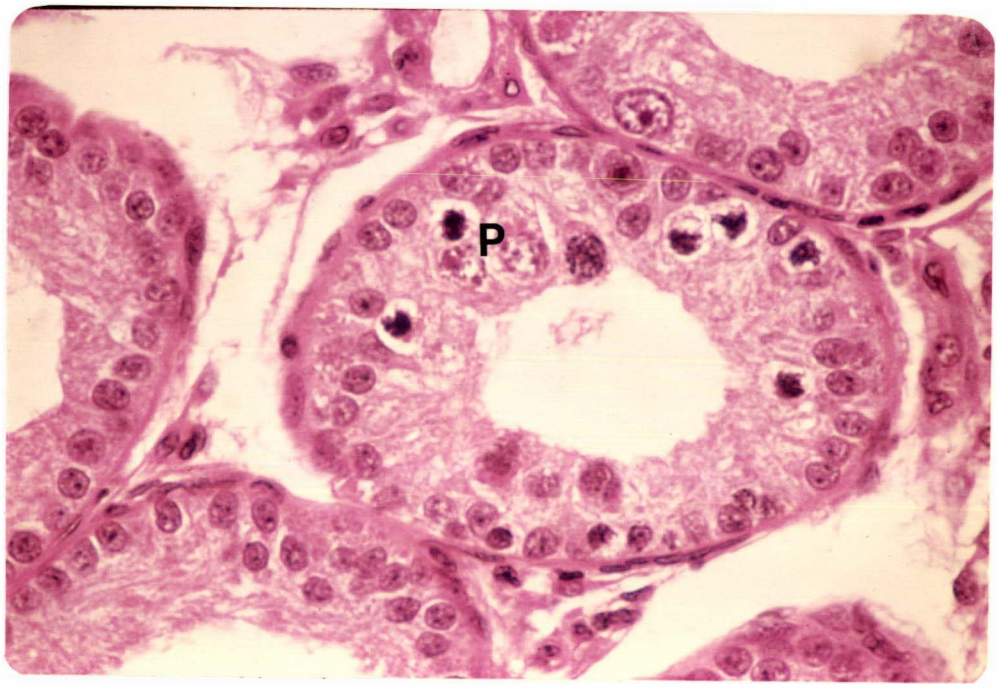

D
S

Figure 5.14: 14 Weeks (x400). Seminiferous tubule showing lumina, spermatogonia interspersed between supporting cells, and primary spermatocytes (P).

Figure 5.15: 18 Weeks (x400). Seminiferous tubules showing mature appearance; Sertoli cells (S), and dividing spermatocytes (D).


P

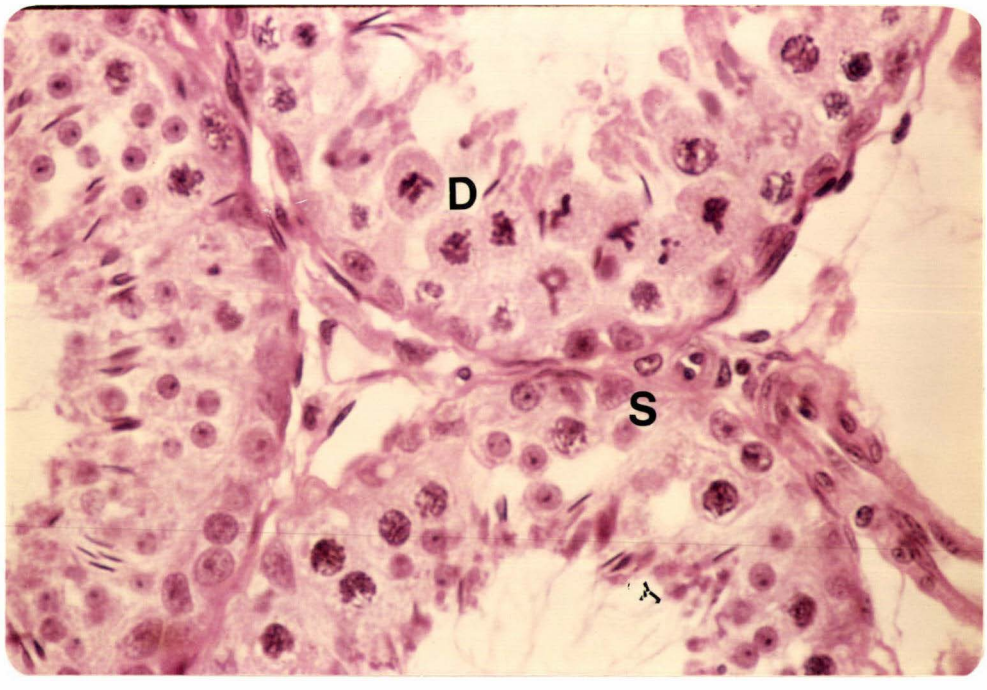
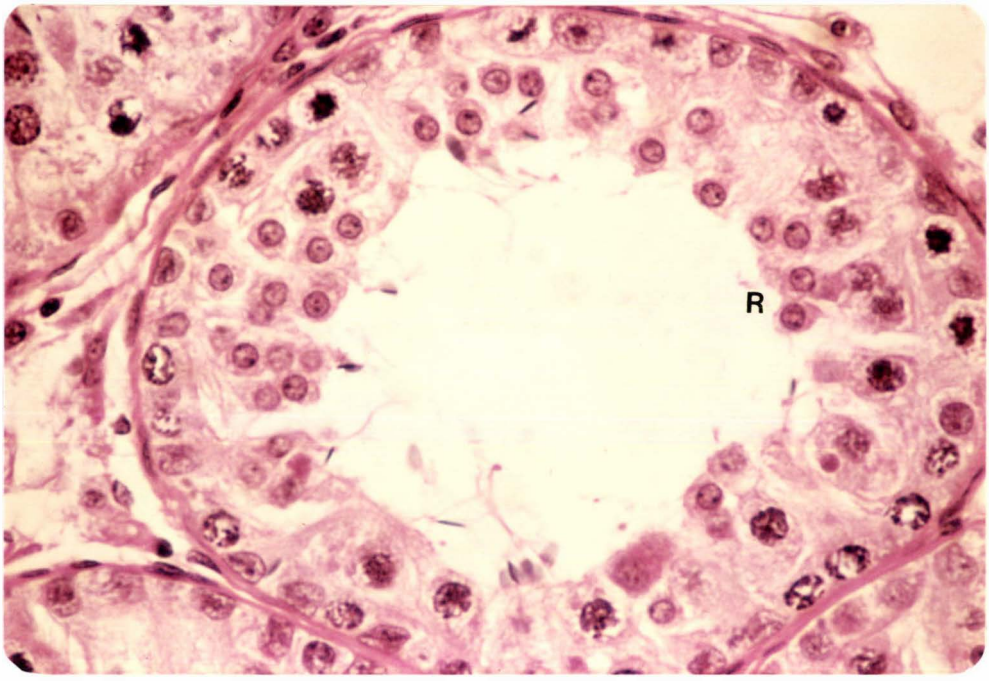

D
S

Figure 5.16: 22 Weeks (x400). Mature seminiferous tubule showing numerous spermatogonia, primary spermatocytes, round spermatids (R) and spermatozoa.

Figure 5.17: 26 Weeks (x1000). Type-A spermatogonia (A), type-B spermatogonia (B), Sertoli cells (S), young primary spermatocytes (Y), old primary spermatocytes (O) and Leydig cells (L).


R

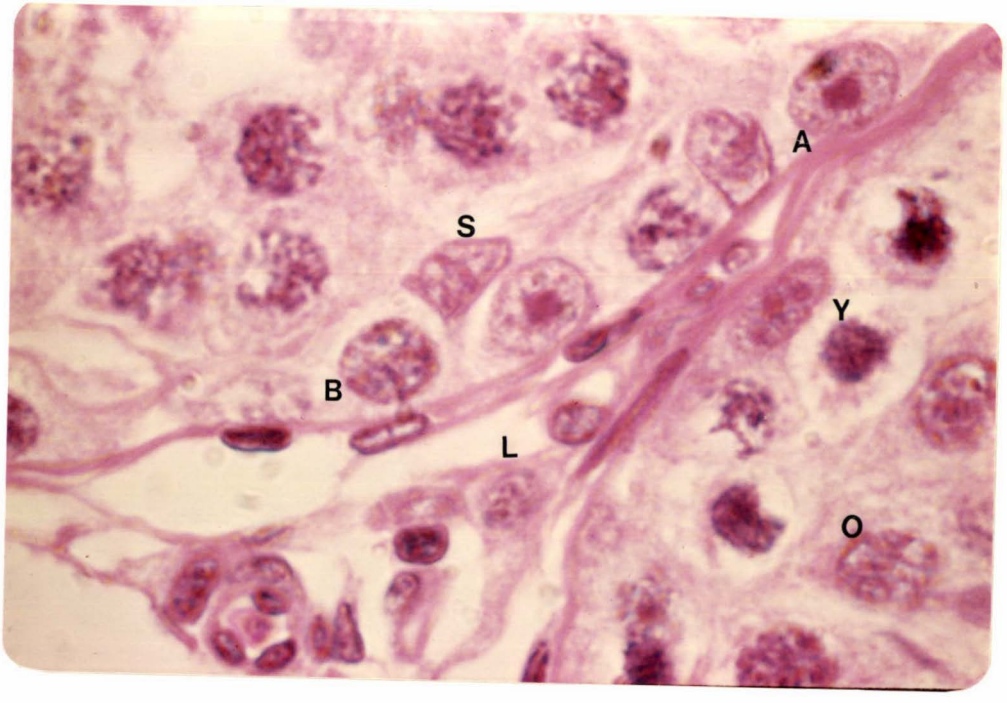
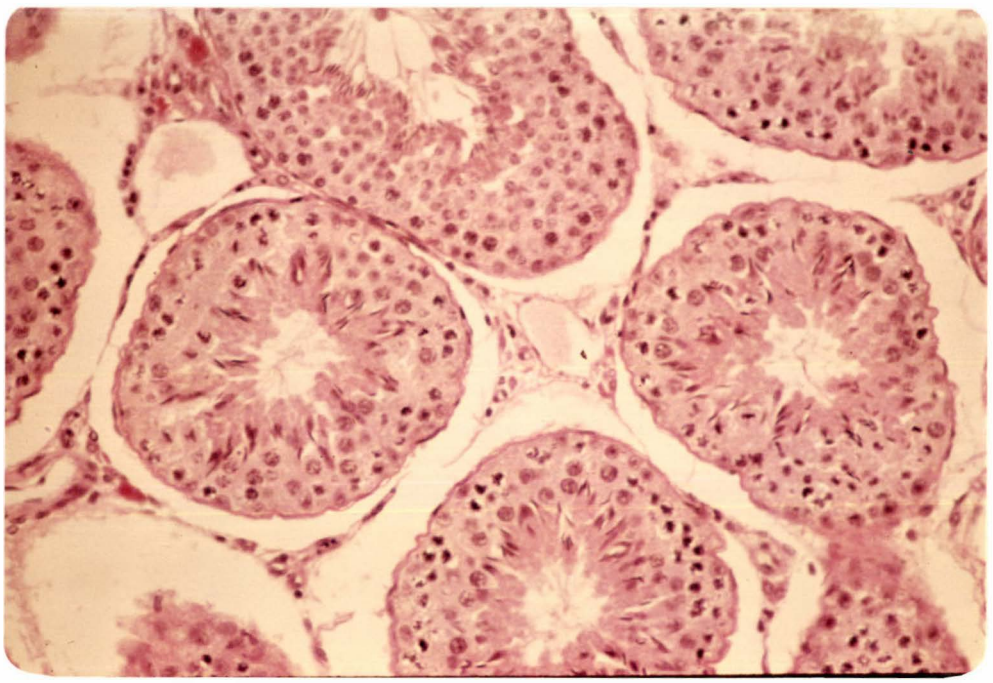

A
S
Y
B
L
O

Figure 5.18: 30 Weeks (x400). Mature seminiferous tubule with spermatozoa (S).

Figure 5.19: 32 Weeks (x160). Mature testis. Note: Leydig cells containing eosinophilic cytoplasmic granules; seminiferous tubule diameter similar to that of the adult (Figure 5.20).



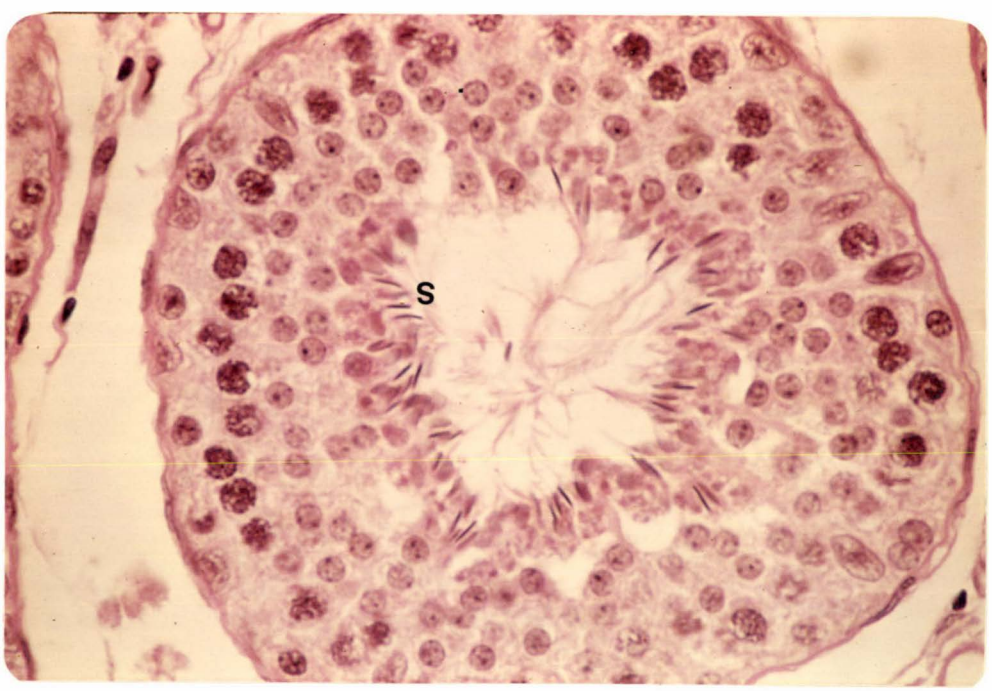
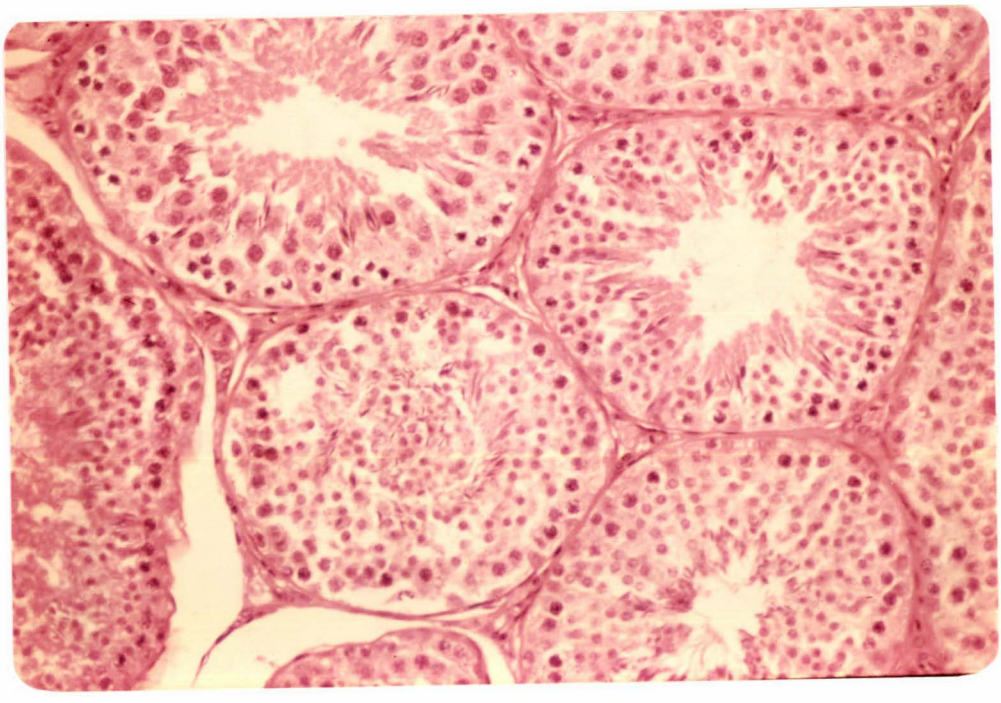

S

Figure 5.20: Mature adult testis (x160).

Figure 5.21: 32 Weeks (x400). Testosterone treated ram showing immature testicular histological appearance of sex cords, supporting cells and gonocytes. Note similarity with testis of a six week old ram (Figure 5.12).



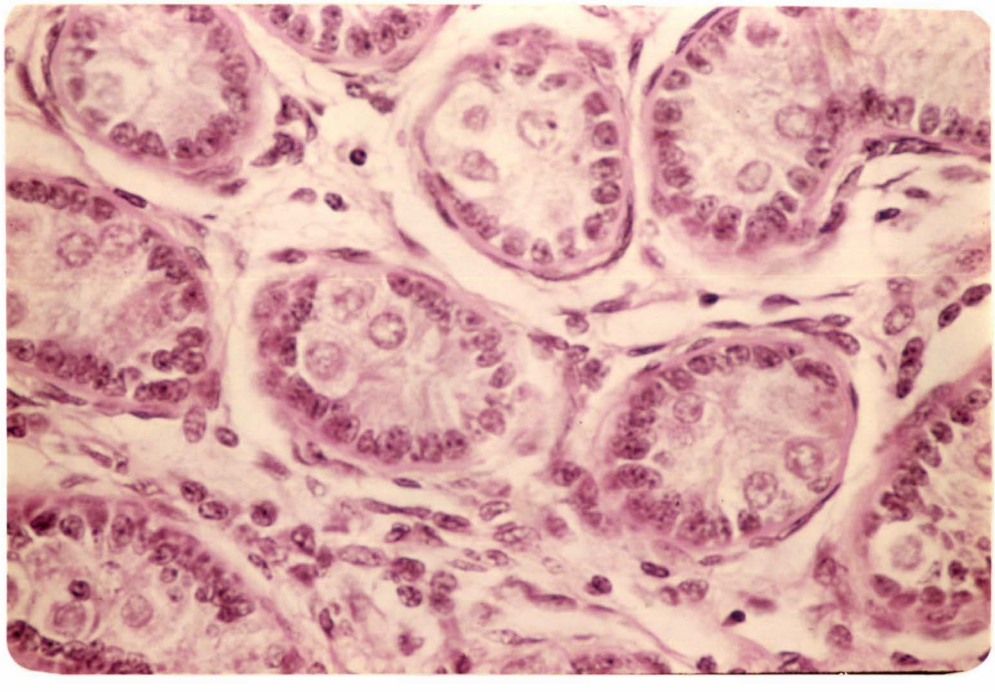
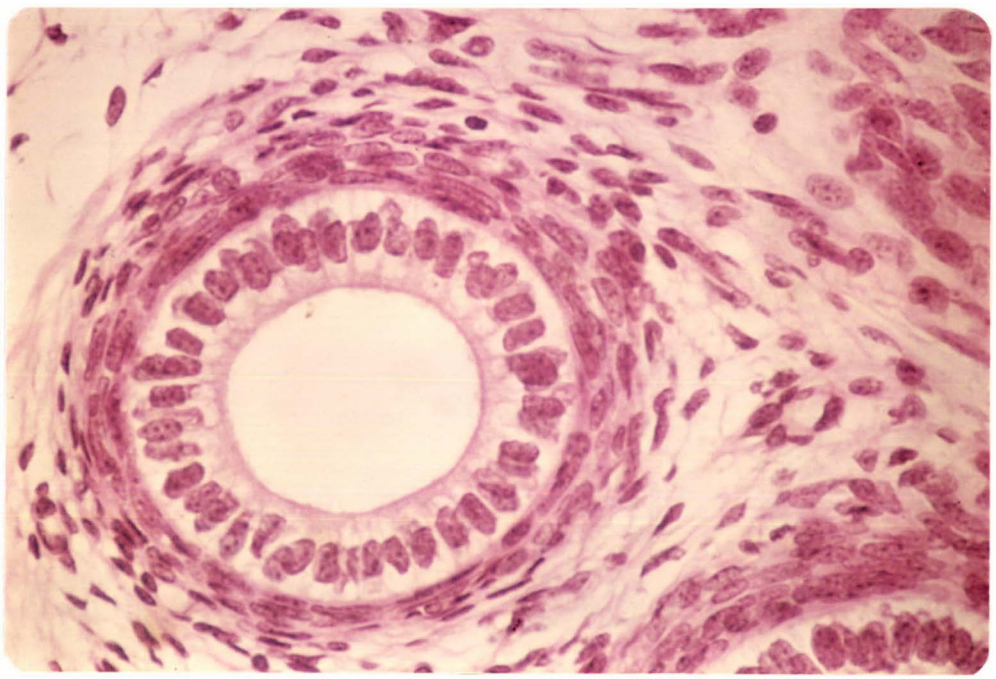
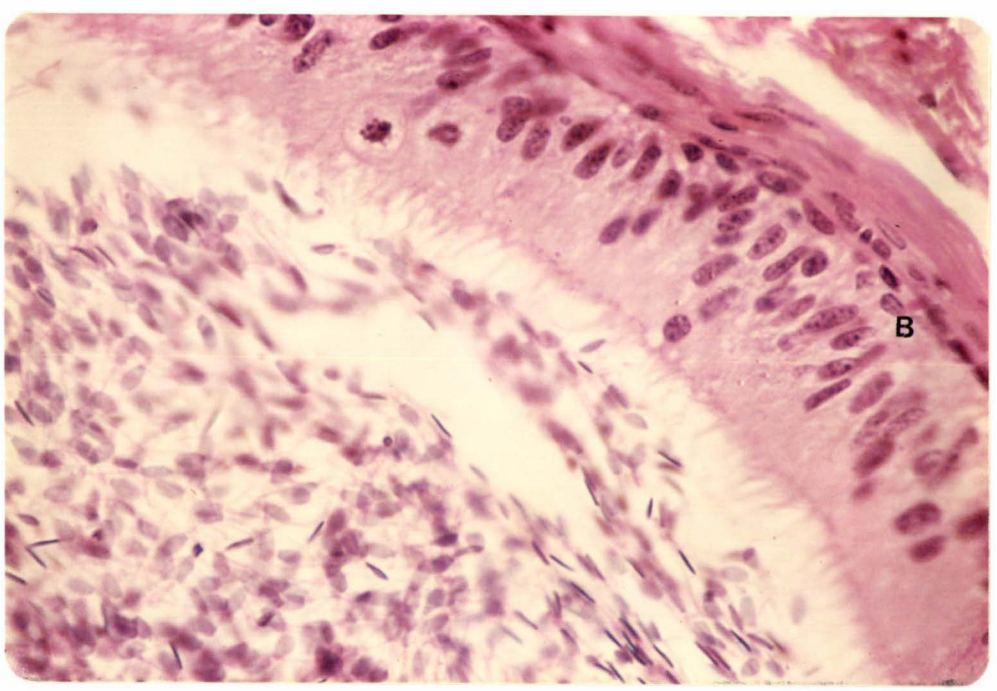


Figure 5.22: 6 Weeks (x400). Epididymal tubule showing low columnar epithelium.

Figure 5.23: 32 Weeks (x400). Epididymal tubule containing spermatozoa. Pseudostratified columnar epithelium containing basal cells (B).




B

4. DISCUSSION

(1) Experiments 3.1 and 3.2

(i) Basal LH and Testosterone Levels.

(a) Control Rams. Profiles of plasma LH concentrations in Dorset and Romney rams during puberty were similar. Concentrations were highest during the first 15 weeks of life, although Romneys did show one major elevation of long duration while Dorsets produced a biphasic elevation with peaks at 5 - 8 and 13 - 14 weeks. Beyond 15 weeks, levels were lower and relatively stable. Mean peak levels in Dorsets (3.33 and 3.58 ng/ml) were considerably higher than the mean peak level (1.36 ng/ml) recorded from the Romneys. This was suggestive of a breed difference in plasma LH concentration similar to that reported by Carr and Land (1975). On the other hand Barrell (1976) did not detect any difference in levels of this hormone between adults of these breeds. Since the samples from lambs of these two breeds were collected in separate experiments, statistical comparison of results was not attempted.

Neonatal increases in plasma LH concentrations correlated with previous observations of rising pituitary LH content and concentrations in rams up to approximately 10 weeks of age (Skinner et al., 1968; Courot et al., 1975). Since pituitary LH contents remained high thereafter, the decrease in plasma LH concentrations was likely to have been due to the increasing testosterone levels inhibiting LH release, as demonstrated by Crim and Geschwind (1972b) to occur in ram lambs, rather than to decreased pituitary synthesis or storage of the hormone.

Changes in plasma concentrations of LH observed in this study were similar to those reported by Courot et al. (1975) to occur in Ile de France ram lambs, regardless of the season of birth. In Grade-Targhee rams, LH levels in plasma were highest at 90 days of age (Crim and Geschwind, 1972b). Sanford (1974) noted higher LH levels in 2 - 3 month old rams than in 6 - 7 month old or adult rams. Experiment 3.2 showed prepubertal and pubertal plasma LH levels to be higher than levels in adults (Experiment 1), a finding also in accord with those of Courot et al. (1975). Lee et al. (1976a) observed highest levels in Merino-Corriedale crossbreds at 5 weeks of age, but also observed peaks at 11 and 33 weeks.

On the other hand, plasma testosterone levels showed a linear increase from birth to 32 weeks of age in Romneys, and similar but

not so markedly increasing levels were observed in Dorsets; concentrations in Dorsets were higher during the early phase of development but had decreased relatively to only half the concentration in Romneys by 32 weeks of age. Breed or managemental factors may have contributed to these differences. A similar pattern of increasing testosterone levels also has been observed in Ile de France ram lambs (Courot, 1974; Cotta et al., 1975) and in Merino-Corriedale crossbreds (Lee et al., 1976a). Skinner et al. (1968) reported a rise in total testicular content of testosterone related to testis weight in sexually developing Suffolk rams. Cotta et al. (1975) showed a significant correlation between LH and testosterone levels during the prepubertal phase up to 70 days, but thereafter, testosterone levels continued to rise while those of LH remained stable.

(b) GnRH and Testosterone Treated Rams. Treatment of rams with GnRH or testosterone from week 1 had no significant influence on overall mean plasma LH concentrations. However, mean LH concentrations in GnRH treated animals were considerably higher during the first 12 weeks than those recorded from control animals. This may have been an indication of greater synthesis and subsequent release of LH at a time when endogenous production of testosterone was low and consequent negative feedback influences were minimal. On the other hand, testosterone administration depressed LH levels in plasma from 2 - 6 weeks of age, a period when endogenous production was low.

Exogenous testosterone significantly depressed plasma testosterone levels measured 1 week after each injection, which suggested that the 2.5 mg/Kg dose was cleared from the body within 1 week. Larger depots of testosterone cypionate (200 mg/animal) given to ewes (Experiment 5) resulted in measurable quantities being detectable in plasma for up to three weeks. That testosterone treatment suppressed testosterone levels in the absence of depressed LH levels possibly was due to active suppression of LH levels following injection, in the short-term (less than 7 days), inhibiting long-term development of the gonadal steroidogenic capacity.

(ii) Basal Prolactin Levels. Plasma prolactin levels were low at birth, rose steadily to a plateau from 6 to 16 weeks of age, then by 32 weeks had gradually declined to levels equivalent to those recorded on the first day of life. Large standard errors which were noted during periods in which mean levels rose and fell suggested that the frequency

and/or magnitude of pulastile output may have been greater during these periods, or that differences between individuals occurred in the timing of such changes in levels of prolactin secretion.

In autumn born rams, Ravault and Courot,(1975) observed an increase in plasma prolactin concentrations in three subjects at 1 - 2 weeks, yet six others showed no such increase. All nine rams produced dramatic but short-lived peaks of plasma prolactin concentrations at between 10 and 12 weeks; this age corresponds to that at which testicular growth accelerates as spermatogenesis is initiated. Courot (1974) showed that mean prolactin levels were slightly elevated at 14 days and a marked peak occurred at 84 days of age. Since this pattern was not observed in spring born lambs, and since prolactin levels of adult rams showed marked seasonal, photoperiod-related changes (Pelletier, 1973; Barrell, 1976), it was probable that the pattern of prolactin levels during puberty in spring born lambs was influenced more by changes in photoperiod than stage of sexual development. The early elevation of levels in spring born lambs preceded the gonadal growth spurt in this study. Thus the use of autumn born rams has clearly implicated the involvement of prolactin in the establishment of puberty.

Barrell (1976) observed that seasonal fluctuations in plasma prolactin levels preceded similar but successively later changes in plasma LH and testosterone levels and in seminal fructose levels. Photoperiod induction of changes in prolactin content of plasma may augment the activity of LH in stimulating testosterone output (as suggested by Bartke (1971a,b) and Hafiez et al. (1972)), thus enhancing the onset of spermatogenesis. The observation by Skinner and Rowson (1968) that reproductive development was more advanced in spring rather than autumn born rams may have been related to such an effect occurring due to greater rates of secretion of prolactin during the spring and summer periods.

Though no direct relationships were found between prolactin and LH or testosterone levels in this study or that of Courot (1974), the potential role of prolactin during puberty in the ram cannot be discounted. Studies using specific prolactin inhibiting factors (e.g. Bromergocryptine) or constant photoperiods would advance the understanding of the role of prolactin during puberty.

(iii) Sampling Induced Elevation of Hormone Levels. Chi-square tests confirmed that a significantly disproportionate number of rams had their highest levels of LH and prolactin in the first samples collected (74% and 39% respectively). It is generally accepted that prolactin levels in sheep can be elevated by "stressful" procedures (Davis, 1972), but it has not previously been shown that handling and venepuncture can influence the output of LH in rams. Data from Experiment 4.1 also supported the concept that blood sampling elevated plasma concentrations of LH, particularly in younger animals. This conclusion is in direct conflict with that of Roche et al. (1970) who claimed that blood sampling method did not influence plasma levels of LH in sheep. The fact that physical procedures may influence LH levels should be borne in mind when planning future studies.

Recently, Sitarz et al. (1977) reported that handling and sampling procedures induced LH elevations in young bulls, and they suggested that animals accustomed to handling and sampling may not show this phenomenon. These authors were unable to show any elevation of prolactin levels following venepuncture sample collection in the bovine.

(2) Experiment 3.3: LH and Testosterone Output in Response to GnRH Administration.

The linear decrease in total LH output in response to GnRH administration corresponded with the linear decrease in basal LH levels. As reported by Galloway and Pelletier (1974) for Ile de France ram lambs there was a progressive delay in the timing of the LH peak as the lambs increased in age up to 32 weeks. In wethers, testosterone has been shown to delay the timing of peak LH concentrations in response to GnRH stimulation (Pelletier, 1976) and also to depress peak levels. It is likely that the decreased and delayed response was induced by the generally increasing plasma content of testosterone.

Contrary to the findings in this study, Galloway and Pelletier (1974) observed an increase, from 1 to 20 weeks, in the total pituitary LH output in response to GnRH stimulation. These authors showed significantly increased LH output in one group from 7 to 40 days and in the second group, from 20 to 60 days. Using a more appropriate test, an analysis of variance of data presented, based on the model used in this thesis, a significant linear increase in LH output with age ($P < 0.001$) was shown.

The different age patterns of LH response to GnRH observed in this study and by Galloway and Pelletier (1974) were likely to have been due to different doses of GnRH used; this study employed a dose rate of 1 μg/Kg but Galloway and Pelletier used 4 μg/Kg. Higher doses of GnRH produced a biphasic release of LH (Experiment 2.1); a pattern also illustrated in older lambs by Galloway and Pelletier (1974), but not evident in their younger rams. Ideally, such experiments should employ wide ranges of doses of GnRH to determine both the "sensitivity" and the "capacity" of the pituitary at different ages.

Intra-arterial infusion of GnRH to ram lambs also resulted in marked elevations in plasma LH and testosterone concentrations (Lee et al., 1976b). Though the data presented by Lee et al. was difficult to interpret and doses varied, these authors claimed that the highest LH levels were achieved at 3 - 4 months, even though inspection of their results suggested that the maximal response was at day 1.

Testosterone output following GnRH induced LH elevations increased with age, from 6 to 32 weeks, as did basal testosterone levels. This increased androgen output was in accord with reports of increased numbers of Leydig cells observed (Sapsford, 1962) and an increased gonadal testosterone content during puberty (Skinner and Rowson, 1968).

(3) Testicular and Endocrine Parameters.

The onset of spermatogenesis in the animals used in Experiment 3.2 closely followed the patterns described by others (e.g. Courot, 1962; Sapsford, 1962; Steffert, 1971). Primary spermatocytes appeared at approximately 70 days and spermatogenesis was established in all animals by 22 weeks, when body weights were between 27 and 33 Kg. Drymundsson (1973) noted that there were great differences in body weights at the attainment of puberty in different breeds. It was observed in this study that there were close relationships between body weights, testis weights and the appearance of spermatozoa in histological sections and in epididymides.

The growth pattern of ram testes has been well documented (Skinner et al., 1968; Steffert,1971; Drymundsson, 1973). The quadratic component of the testicular growth curve indicated accelerated testicular growth during puberty; at this stage testicular growth was proportionately more rapid than body weight increases. The tubular component of the testes increased from 50%

to 80% of the testicular volume during puberty (Drymundsson, 1973), this increase being caused by increased tubular length and diameter. Covariance analysis in the present study confirmed relatively greater tubular growth. Tubular diameters corresponded closely with data recorded from Suffolk rams by Skinner et al. (1968). Though testis growth has been considered to be responsible for rising testosterone concentrations in plasma and consequently the significant relationship between testis weight and testosterone output in response to GnRH administration, it has been suggested that testis growth itself is partly dependent on testicular testosterone secretion (Courot, 1967).

Epididymal weights followed testis weights closely, as observed by Watson et al. (1956) and Skinner et al. (1968), and also were correlated with the testosterone output in response to GnRH administration. From 18 weeks of age, epididymal spermatozoal reserves increased in proportion with increasing testicular weights and by 32 weeks had reached values corresponding closely to those recorded previously from adult Romney rams (Barrell, 1976). The relationship between testis weight and spermatozoal production is well established (Drymundsson, 1973).

While weekly GnRH treatment had no significant effect on any of the parameters measured, testosterone treatment did depress many of these parameters (Table 5.8). Courot (1967) presented evidence suggesting that LH, FSH and testosterone were all essential for the initiation of spermatogenesis, though it was known that testosterone alone could maintain spermatogenesis in mature hypophysectomised animals (Steinberger, 1971). It is possible that the exogenous testosterone suppressed FSH secretion in the short term, as discussed earlier in relation to basal LH levels (p 140), and that this weekly suppression delayed the development of the germinal epithelium and hence depressed testis weights, epididymal weights, seminiferous and epididymal tubule diameters and epididymal spermatozoal reserves. A depression of FSH output may also have inhibited Leydig cell receptor formation (Odell and Swerdloff, 1976) and therefore lowered endogenous testosterone output. However, the role of testosterone in suppressing FSH output in sheep is still unclear (Pelletier et al., 1977). An extreme result was recorded from one ram in which testicular development was totally inhibited by weekly exogenous testosterone. In this animal LH and testosterone levels always were low, and responses to GnRH were exceptionally low.

Since GnRH disappeared from plasma of lambs very rapidly (Courot et al., 1975), it was unlikely that weekly injections could have had long term effects on either hormone levels or on physiological development. The brevity of overall effect would have been compounded by the short half-lives of both LH and testosterone. However, Schanbacher and Lunstra (1977) recently demonstrated improved mating activity and significantly greater testis weights and spermatozoal motility in adult rams given twice-daily injections of GnRH during the non-breeding season. In the future, longer acting implants or depot forms of GnRH may prove useful tools in experiments on advancing the maturation of the reproductive system.

CHAPTER VI

LUTEINIZING HORMONE, TESTOSTERONE AND PROLACTIN SECRETION PROFILES, AND RESPONSES TO REPEATED GnRH INJECTIONS, IN PREPUBERTAL, PUBERTAL AND EARLY POSTPUBERTAL RAMS

1. INTRODUCTION

The patterns of LH, testosterone and prolactin secretion, and pituitary responses to GnRH injection throughout sexual maturation in Romney rams, have been described in Chapter 5. However, little has been reported on the short term secretion profiles of these hormones prior to and during puberty, or on the capacity of the pituitary gland of young rams to respond to repeated doses of GnRH.

In the experiments described in this chapter Romney ram lambs were utilized in an investigation of short term hormone output patterns, and of responses to repeated administration of GnRH, at selected phases (prepubertal, pubertal and early postpubertal) of sexual development, as an aid to elucidation of the maturational changes occurring in the hypothalamo-pituitary-gonadal axis.

2. MATERIALS AND METHODS

(1) Animals.

16 rams lambs were identified at birth and divided into two groups: 8 born in the first week of lambing were allocated to Experiment 4.1, and 8 born in the second week to Experiment 4.2. This selection method obviated practical difficulties associated with sampling mixed-age groups. These experiments were carried out in September and November of 1975 and January of 1976.

(2) Experiment 4.1 : LH, Testosterone and Prolactin Secretion Profiles.

Blood samples were collected each twenty min for 8 h on each of the 6th, 14th and 22nd weeks of life. Sampling commenced at 09.00 h on each occasion. All plasma samples were assayed for LH and prolactin in individual assays, while for testosterone estimations all samples from individual animals were measured in the same assay.

(3) Experiment 4.2 : LH and Testosterone Responses of Young Rams to Repeated Administration of GnRH.

The second group of 8 ram lambs was subjected to four repeated injections of GnRH at ages 6, 14 and 22 weeks. Blood samples were collected 20 min and immediately prior to the first GnRH injection, then at further 20 min intervals. Jugular intravenous injections of GnRH at a dose rate of 1 µg/Kg (constituted in 0.9% saline at 10 µg/ml immediately prior to injection) were given at hourly intervals immediately after collection of the blood sample scheduled for that time. Following the fourth GnRH injection, blood samples were collected 20, 40, 60, 90 and 150 min later. All plasma samples were assayed for LH in a single assay, while for testosterone estimations all samples from individual animals were measured in the same assay.

(4) Data Analysis

(i) Experiment 4.1. Cumulative regression coefficients were calculated for individual LH, testosterone and prolactin profiles at each age. Because hormone levels tended to be higher in the first samples collected, suggesting a sampling-induced elevation, regression coefficients were calculated firstly for cumulative regressions when all samples were included (complete profiles), and secondly when the first three samples from each profile were discarded (reduced profiles). Paired t tests were then performed on differences between the complete and reduced profile cumulative regression coefficients for each hormone to determine the influence of the higher levels recorded in the first samples collected. The appropriate regression coefficients (see p159) were subjected to analyses of variance and age effects determined by use of standard orthogonal coefficients.

An analysis of regression was performed to examine the degree to which the quantity of testosterone released episodically at each age studied was determined by the height of the preceding (causative) LH peaks; LH and testosterone peaks have been defined in Chapter 3 (p 64). To examine differences in peak levels of LH and testosterone peaks observed from the three sampling periods, analysis of variance of peak levels was performed and age effects partitioned using standard orthogonal coefficients.

(ii) Experiment 4.2. Total LH and testosterone responses were calculated as described in Chapter 2 (p 60). The pre-injection

hormone level used was the mean of the two pre-injection levels. Analyses of variance of pre-injection and total hormone responses was performed and age effects partitioned using standard orthogonal coefficients.

An analysis of variance of regression coefficients of total testosterone response on total LH response was performed to determine the degree to which testosterone output was determined by the quantity of LH released following GnRH injection.

3. RESULTS

(1) Experiment 4.1

See Tables 6.1 and 6.2, and Figures 6.1 - 6.8.

Generally LH levels were low (0.05 - 0.50 ng/ml) at each of the three ages studied, but secretory pulses were observed. At each age, some animals produced no pulses of LH while others exhibited up to three spontaneous peaks during the 8 hour sampling period.

Plasma testosterone levels fluctuated but generally were low (0.05 - 0.50 ng/ml). At 14 and 22 weeks, testosterone concentrations rose subsequent to each LH spike (Table 6.1), but at 6 weeks, only six of the 11 LH peaks were followed by noticeable androgen secretory responses.

Plasma prolactin concentrations fluctuated more markedly than did LH or testosterone levels, and generally were higher in the first samples collected at each sampling period than in subsequent samples. Prolactin secretion patterns were similar at each sampling period.

Analyses of variance of LH and testosterone peak levels (Table 6.1) showed LH peak levels at each age to be similar, while peak testosterone levels showed a significant linear increase with age ($P < 0.001$).

The regression of testosterone peak levels on LH peak levels at 6 weeks of age was not significant, but at both 14 and 22 weeks this regression was significant ($r^2 = 0.48$ and $r^2 = 0.31$, $P < 0.05$, respectively), indicating that the quantitative relationship between LH and testosterone peaks was not evident in prepubertal rams.

At 6 weeks of age, it was observed that seven of the eight animals sampled displayed LH elevations in the first three samples, an occurrence observed also in four animals at 14 and 22 weeks.

TABLE 6.1

Total numbers of LH and subsequent testosterone peaks, ranges of peak LH, testosterone and prolactin levels observed during each 8 hour sampling period, and a summary of anlyses of variance of LH and testosterone data.

Age (weeks)	LH		Testosterone		Prolactin
	Total Number of Peaks	Range of Peak Levels (ng/ml)	Total Number of Peaks	Range of Peak Levels (ng/ml)	Range of Levels
6	11	1.49 - 8.92	6	0.05 - 1.24	5.0 - 94.7
14	7	0.55 - 5.44	7	0.36 - 2.10	13.2 - 129.3
22	11	0.59 - 6.46	11	0.60 - 4.39	11.4 - 130.3

Source of variation	d.f.	Variance Ratios	
		LH Peak	Testosterone Peak
AGE			
linear	1	0.24	18.96***
quadratic	1	0.30	1.62
Residual mean square	26	0.10	0.13

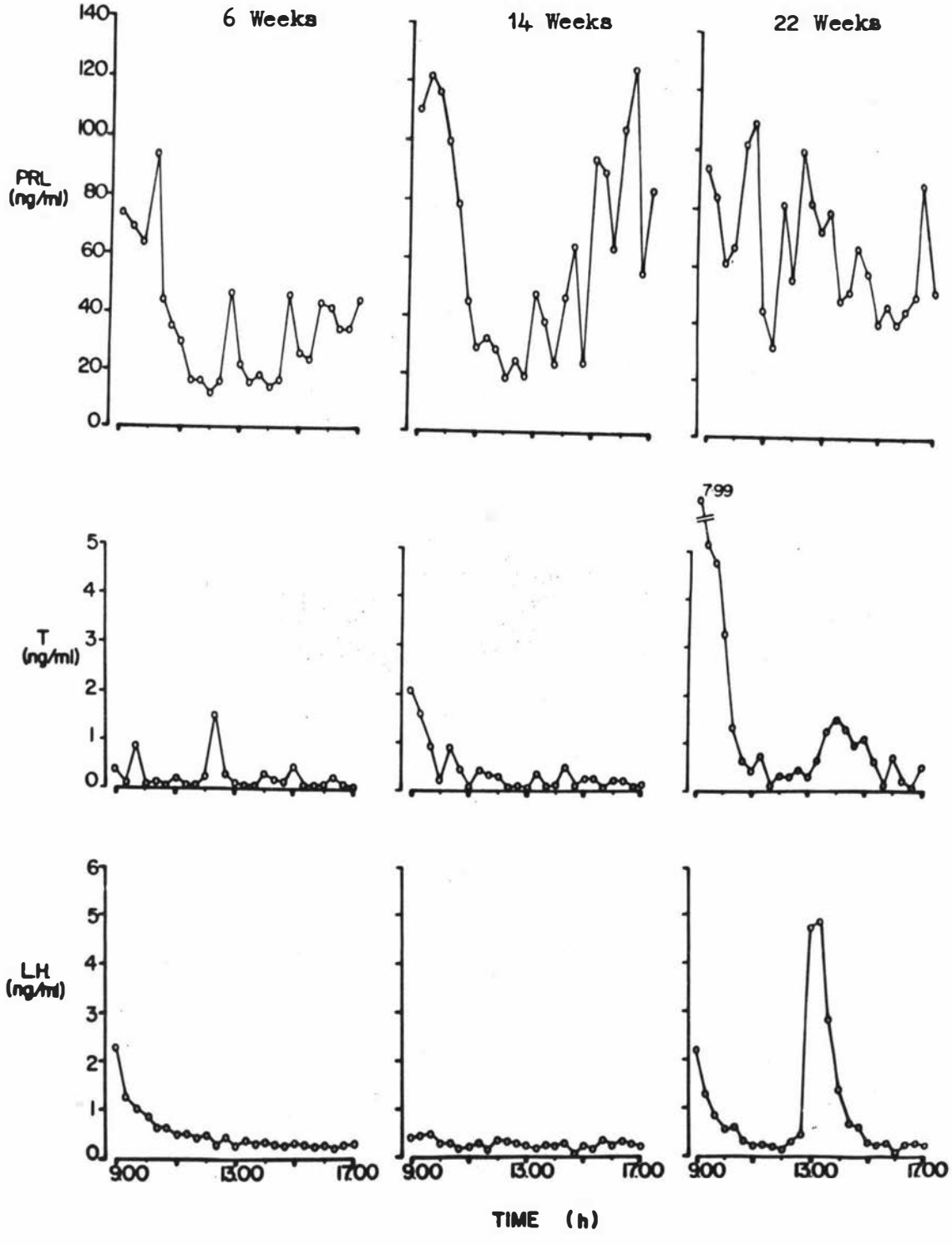


Figure 6.1: Ram 103. Eight hour LH, testosterone and prolactin secretion profiles at 6, 14 and 22 weeks of age.

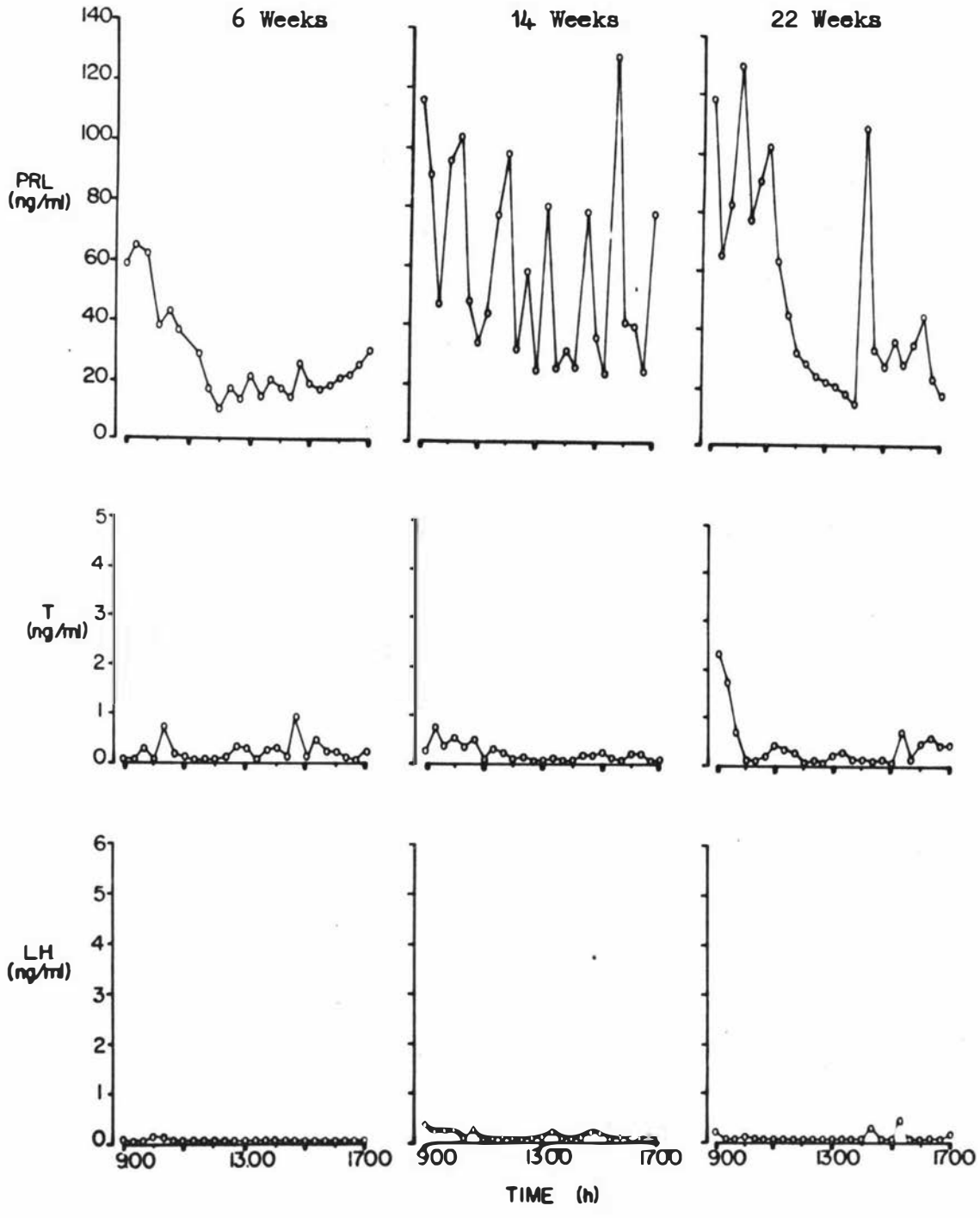


Figure 6.2: Ram 105. Eight hour LH, testosterone and prolactin secretion profiles at 6, 14 and 22 weeks of age.

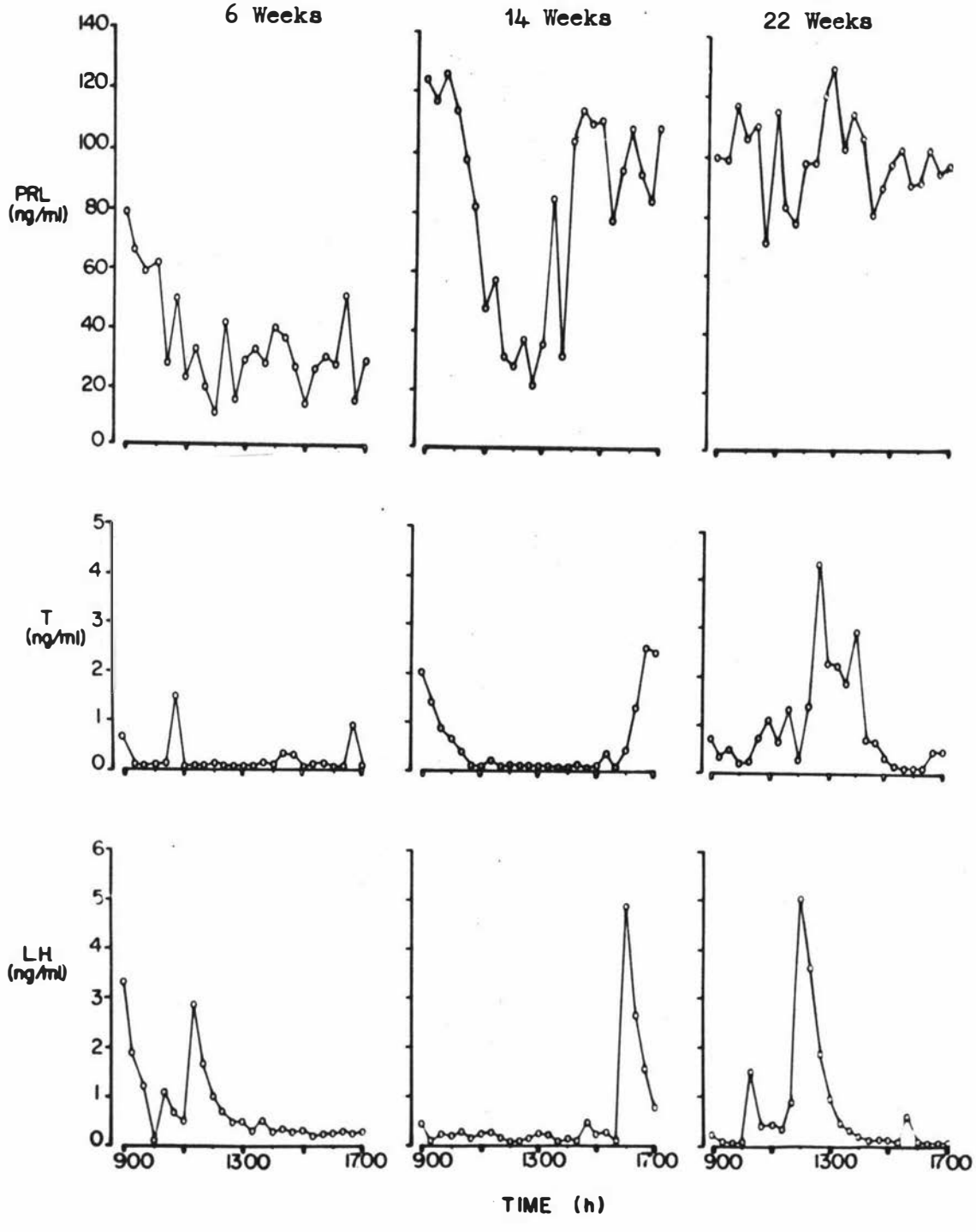


Figure 6.3: Ram 106. Eight hour LH, testosterone and prolactin secretion profiles at 6, 14 and 22 weeks of age.

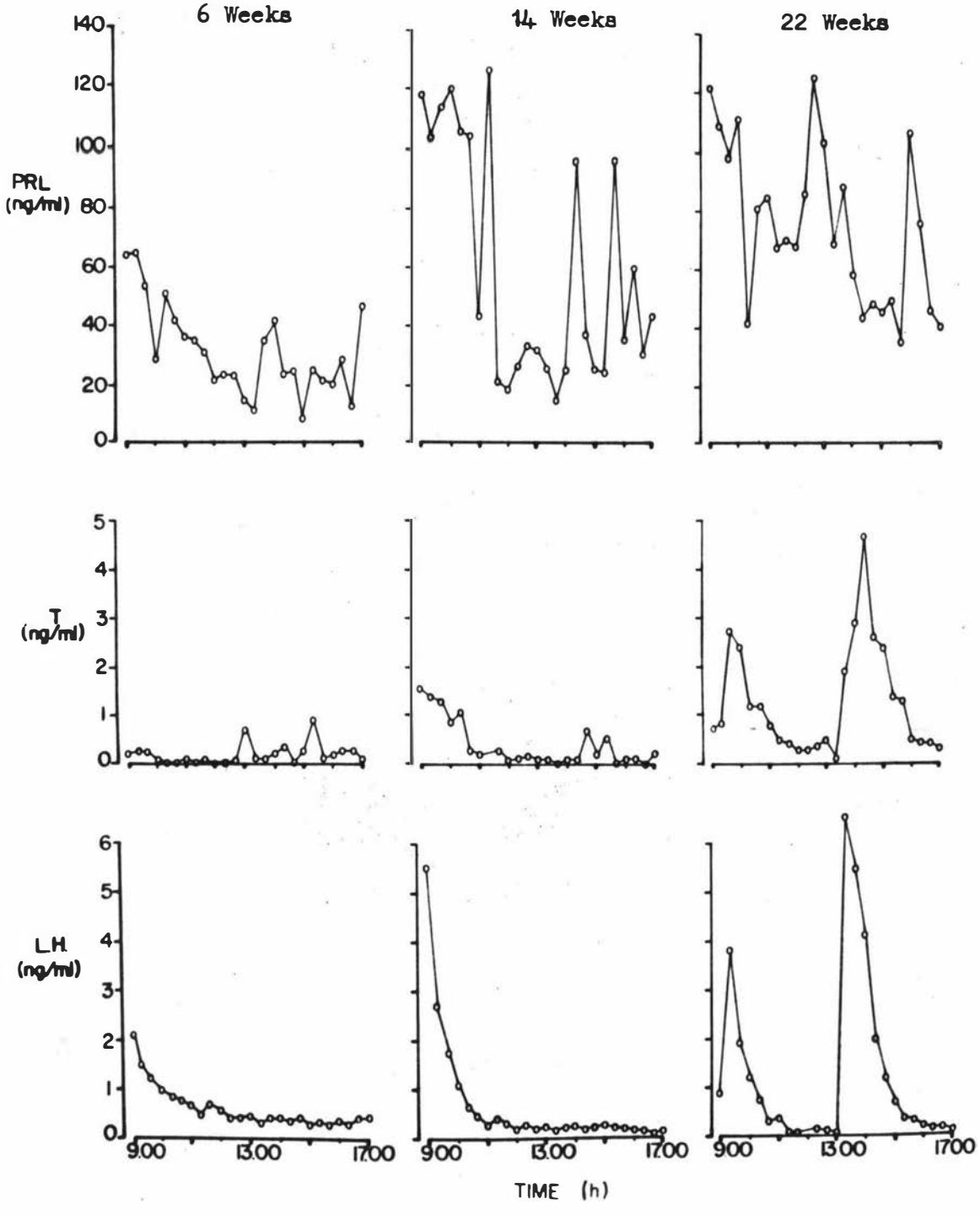


Figure 6.4: Ram 107. Eight hour LH, testosterone and prolactin secretion profiles at 6, 14 and 22 weeks of age.

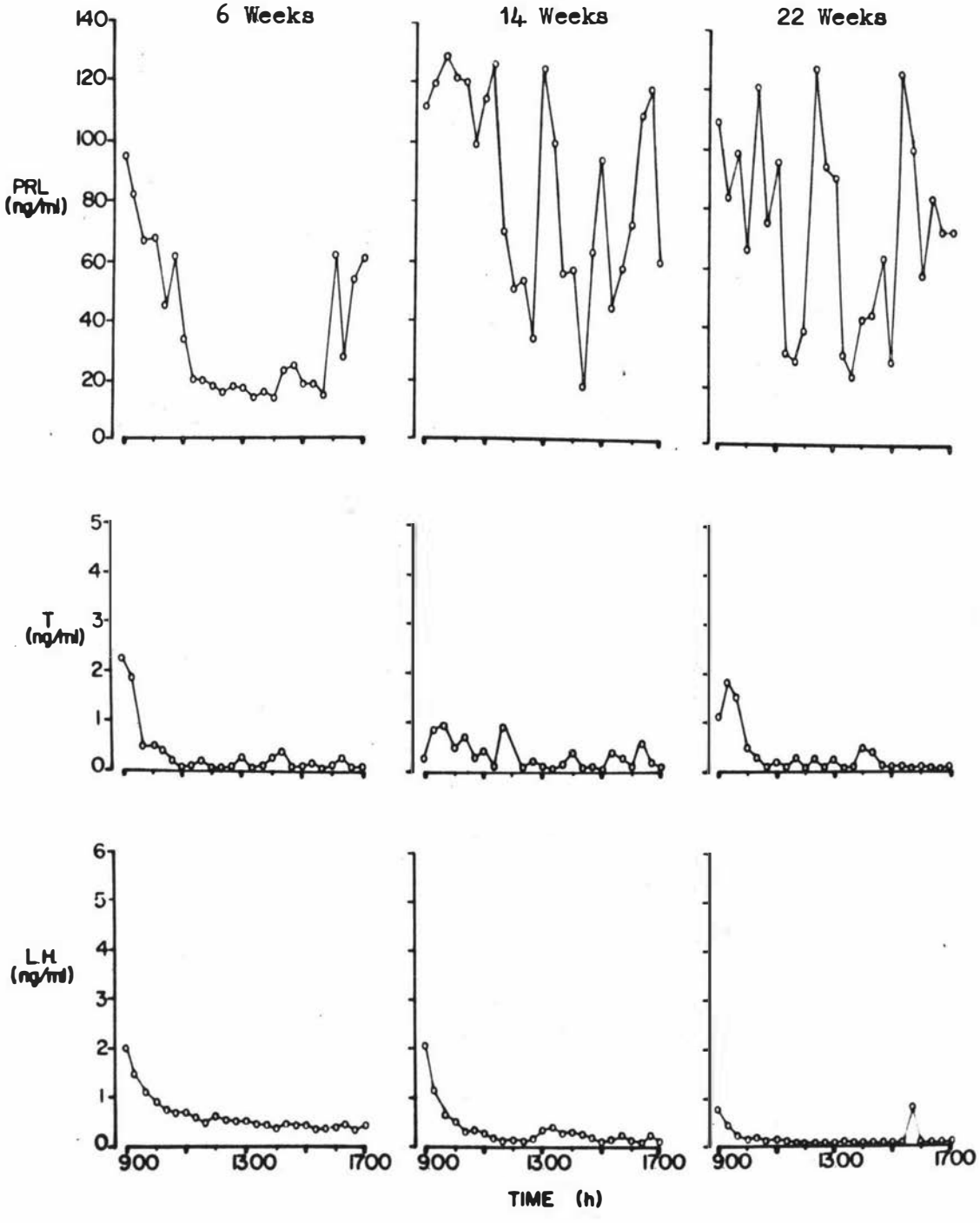


Figure 6.5: Ram 108. Eight hour LH, testosterone and prolactin secretion profiles at 6, 14 and 22 weeks of age.

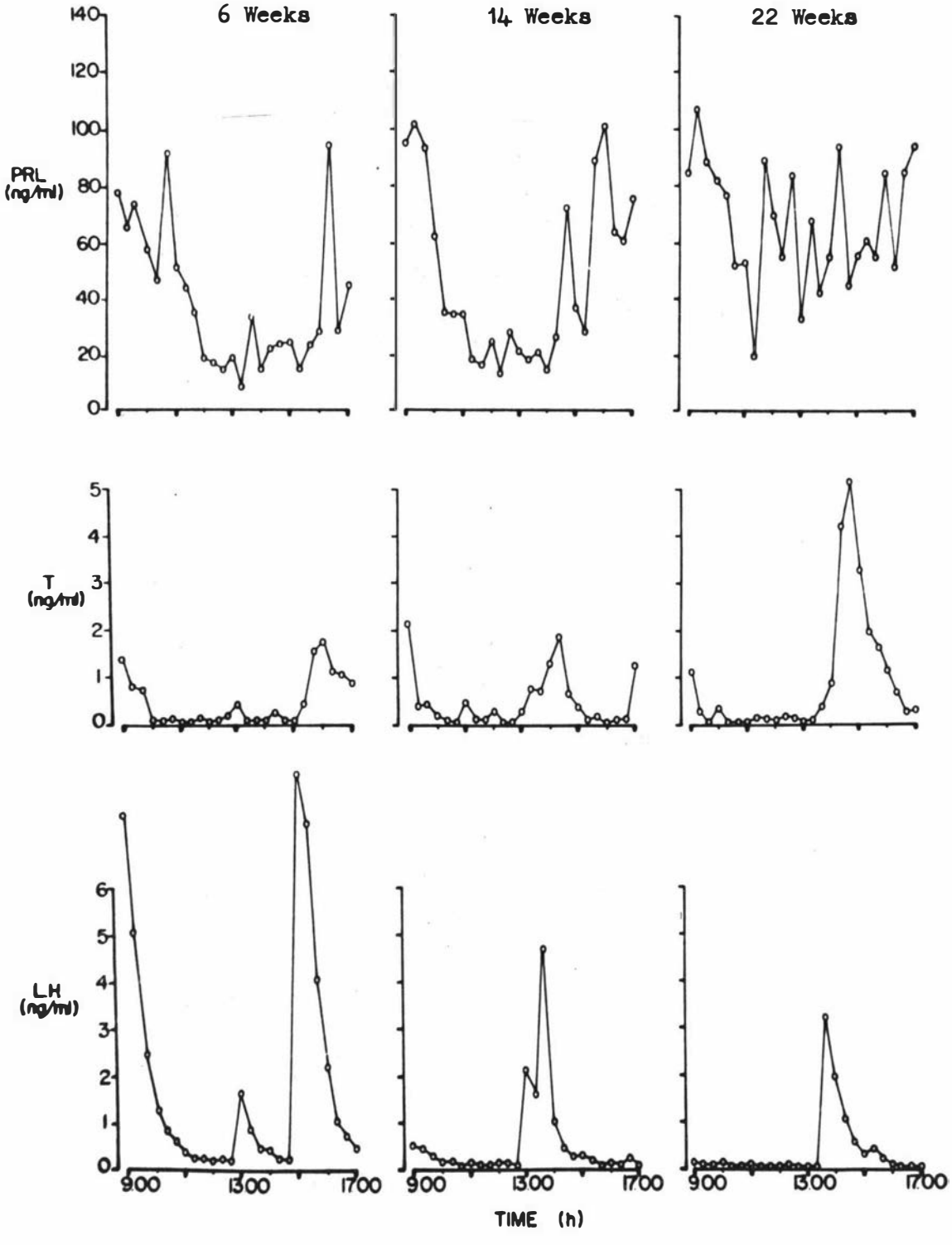


Figure 6.6: Ram 109. Eight hour LH, testosterone and prolactin secretion profiles at 6, 14 and 22 weeks of age.

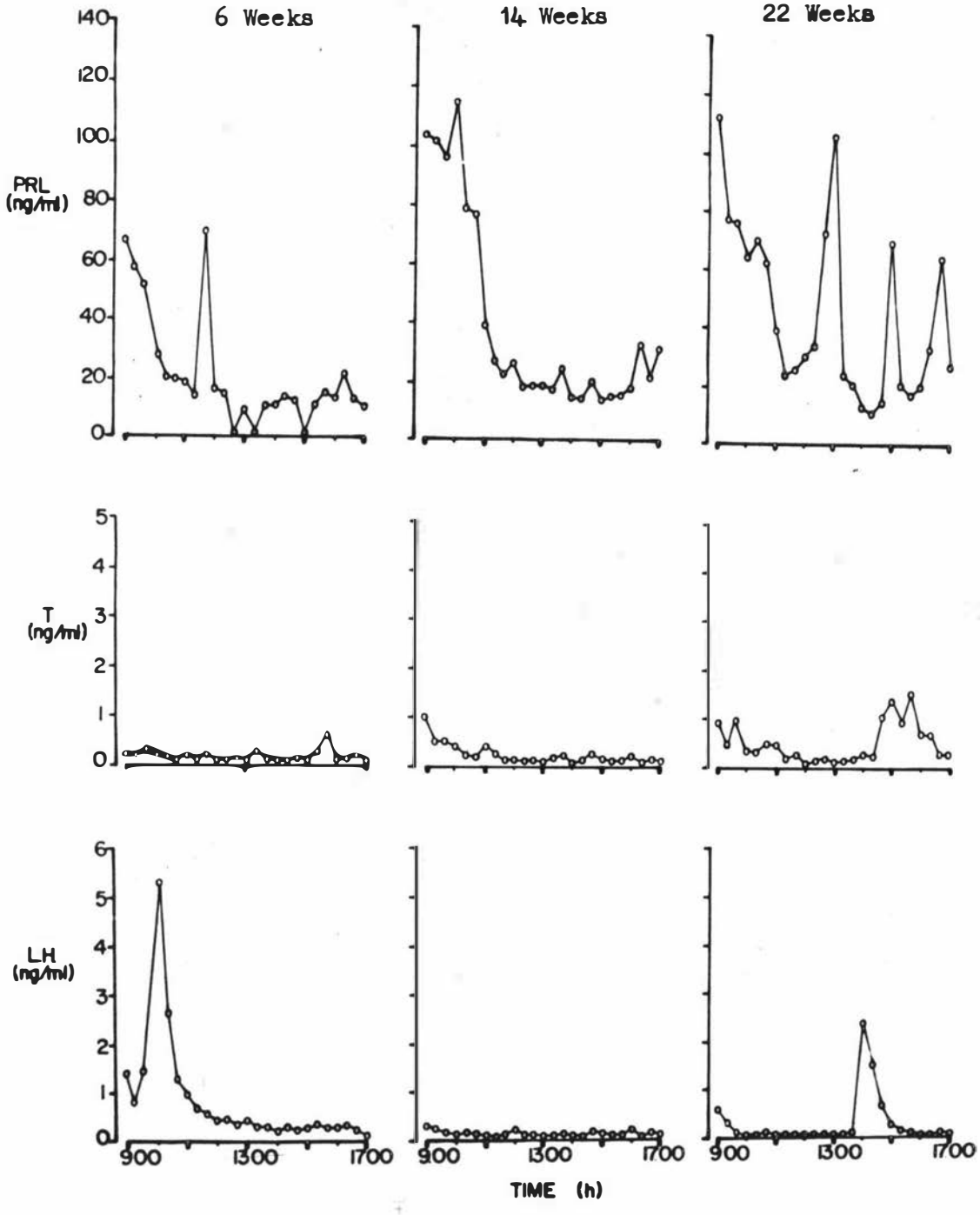


Figure 6.7: Ram 110. Eight hour LH, testosterone and prolactin secretion profiles at 6, 14 and 22 weeks of age.

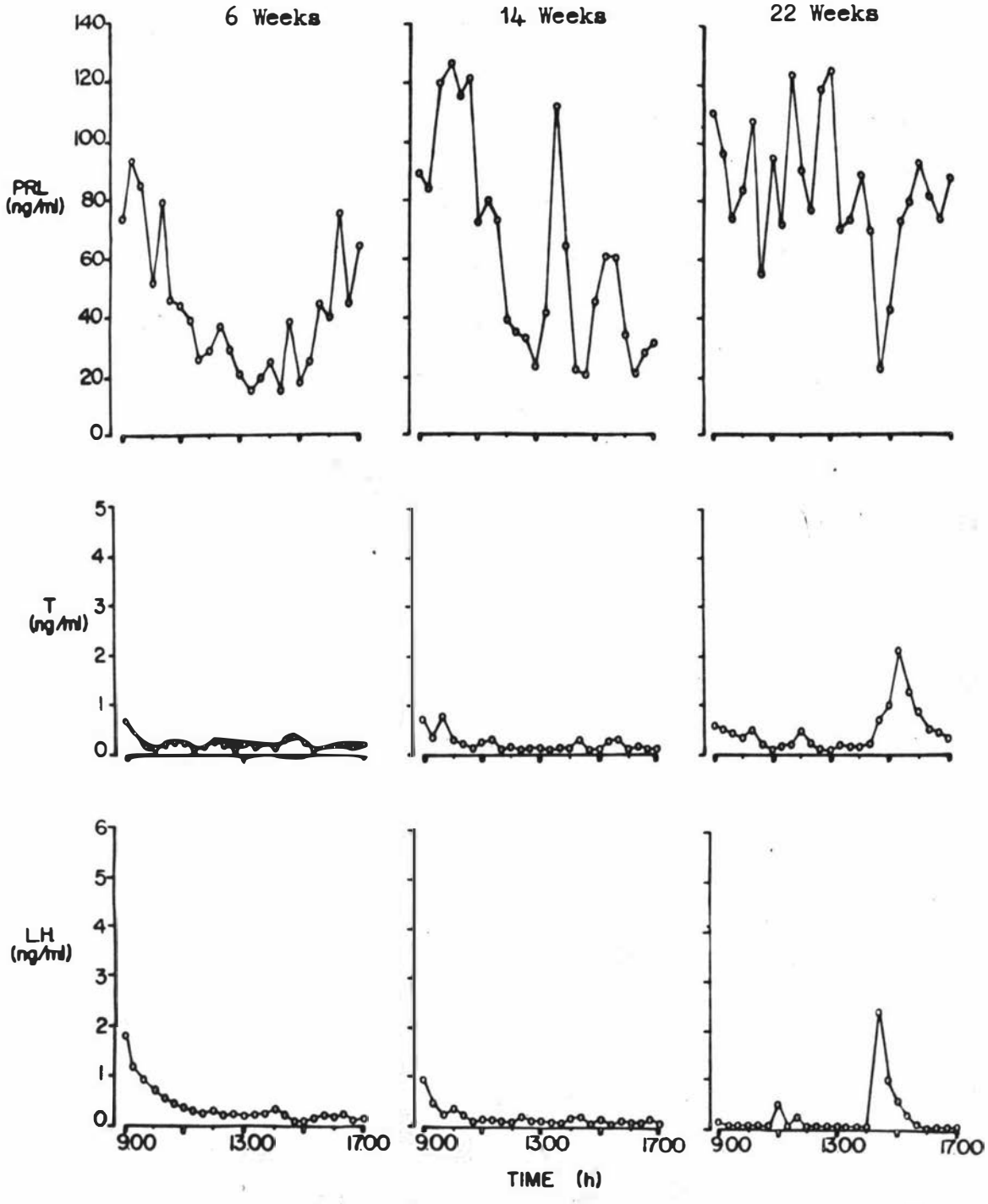


Figure 6.8: Ram 111. Eight hour LH, testosterone and prolactin secretion profiles at 6, 14 and 22 weeks of age.

TABLE 6.2

Mean cumulative regression coefficients for 8-hour secretion profiles and a summary of analyses of variance of this data.

Age	LH			Testosterone			Prolactin++		
	Linear	Quadratic (x10)	Cubic ($x10^3$)	Linear	Quadratic (x10)	Cubic ($x10^3$)	Linear	Quadratic (x10)	Cubic ($x10^3$)
6	0.0729	-0.0365	0.0707	0.0590	-0.0116	0.0242	0.0959	-0.0401	0.0695
14	0.0424	-0.0160	0.0295	0.0591	-0.0303	0.0622	0.0963	-0.0394	0.0676
22	0.0228	-0.0024	-0.0205	0.0470	-0.0206	0.0705	0.1022	-0.404	0.0654

Source of variation	d.f.	Variance Ratios								
AGE										
linear	1	17.92***	17.73***	17.90***	0.17	0.00	10.73**	0.00	0.00	0.64
quadratic	1	0.14	0.15	0.05	0.05	0.33	1.47	0.21	0.00	0.00
Residual mean square	21	0.00056	0.00034	0.00186	0.00236	0.00034	0.00080	0.00005	0.00002	0.00010

++ Coefficients calculated using reduced profile data.

Other peaks occurred randomly throughout the sampling period. Paired t tests comparing cumulative regression coefficients of complete and reduced secretion profiles showed that the high levels noted in early plasma samples had no significant effects on LH or testosterone cumulative regression coefficients. Consequently regression coefficients derived from complete profiles of these two hormones were utilized for the analyses of variance. Conversely, the high levels of prolactin in early samples significantly altered cumulative regression coefficients for this hormone, thus cumulative regression coefficients derived from reduced prolactin profiles were used in the analysis of variance (Table 6.2).

Cumulative regression coefficients derived from complete LH and testosterone profiles were essentially linear, indicating a relatively uniform secretion of both hormones throughout each 8 hour sampling period.

Analyses of variance of cumulative regression coefficients (Table 6.2) showed major changes in LH secretion profiles: linear, quadratic and cubic components decreased significantly with age ($P < 0.001$) indicating a diminished LH output (linear component), and less variability of LH levels (quadratic and cubic components) as animals progressed toward sexual maturity. The cubic component of testosterone output increased ($P < 0.001$) indicating greater variation in plasma levels with age. No significant changes in prolactin secretion profiles were observed.

(2) Experiment 4.2 .

See Table 6.3 and Figures 6.9 - 6.16.

The first injection of GnRH was followed by an increase of LH concentrations similar to those observed earlier in lambs of corresponding ages (Experiment 3.3.). Each successive GnRH injection resulted in increases in plasma LH levels to above those at the time of injection. At 6 weeks of age, peak LH levels following each GnRH injection generally occurred within 20 min, but delays of up to 60 min were recorded following GnRH treatment at 14 and 22 weeks.

At 6 weeks of age the highest peak of LH occurred following the first or second GnRH injection, but at the later ages, highest LH levels usually occurred after the third injection. At 22 weeks the fourth

TABLE 6.3

Mean pre-injection hormone levels (ng/ml) and mean total LH and testosterone responses to repeated GnRH injections and a summary of analyses of variance of this data.

	LH		Testosterone	
Age (weeks)	Pre-injection	Total	Pre-injection	Total
6	0.72	35.88	0.37	20.80
14	0.15	38.14	0.34	24.63
22	0.14	36.26	0.31	25.91

Source of variation	d.f.	Variance Ratios			
AGE					
linear	1	8.46**	0.19	0.26	5.00*
quadratic	1	2.46	17.94***	0.00	0.41
Residual mean square	21	0.06	2.86	0.06	7.84

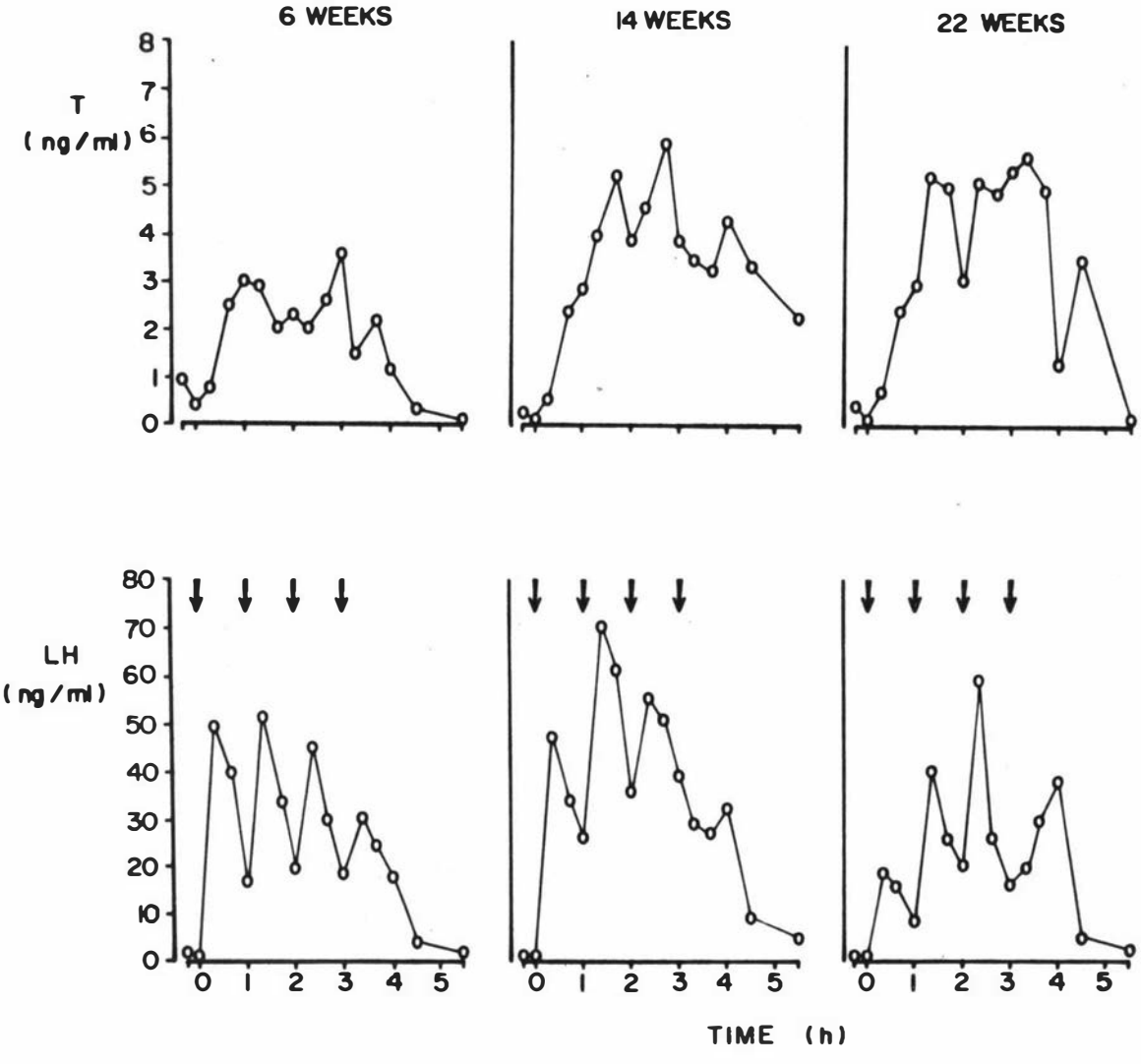


Figure 6.9: Ram 112. LH and testosterone responses following four hourly injections of GnRH (represented by arrows) at 6, 14 and 22 weeks of age.

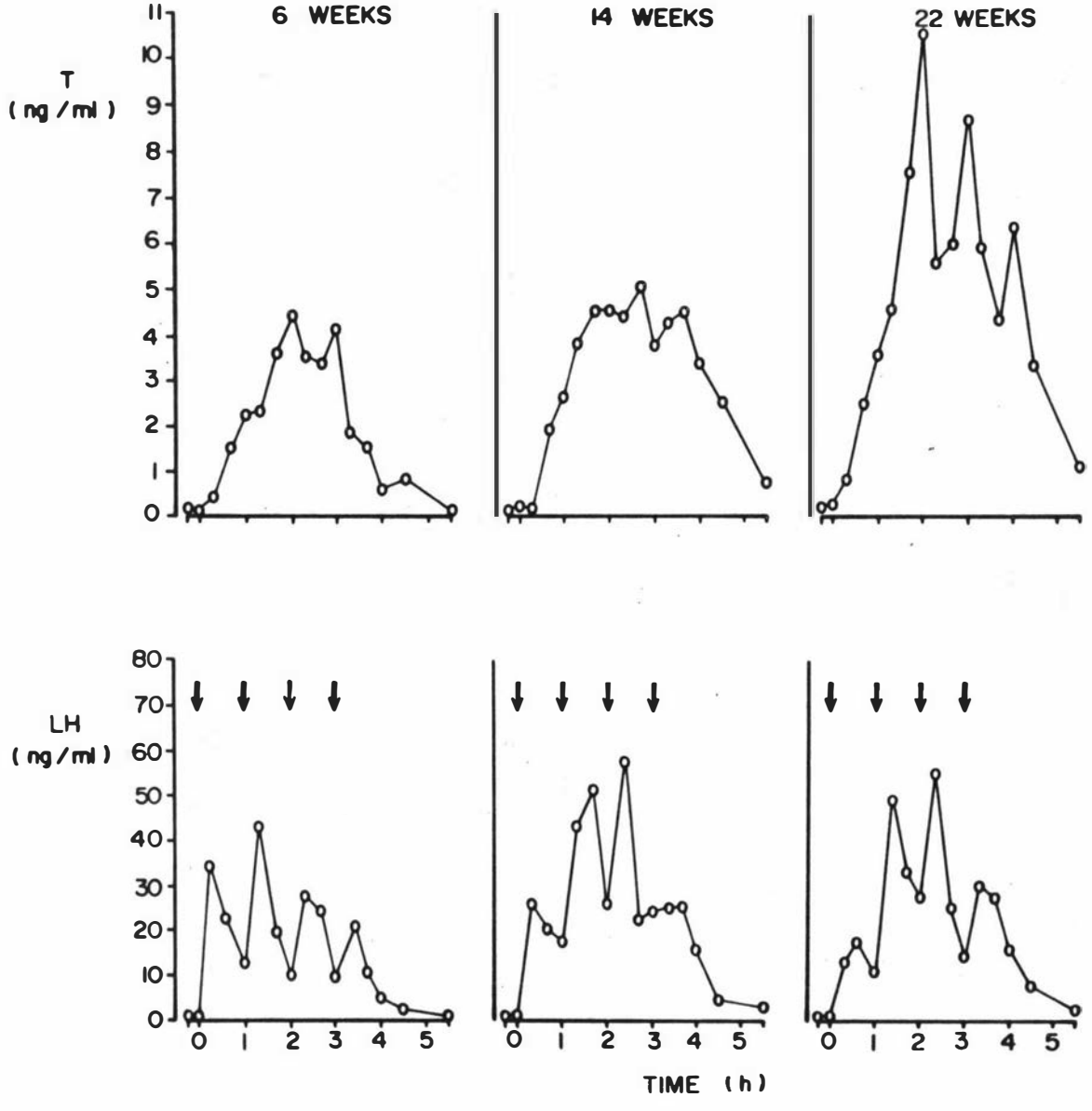


Figure 6.10: Ram 113. LH and testosterone responses following four hourly injections of GnRH (represented by arrows) at 6, 14 and 22 weeks of age.

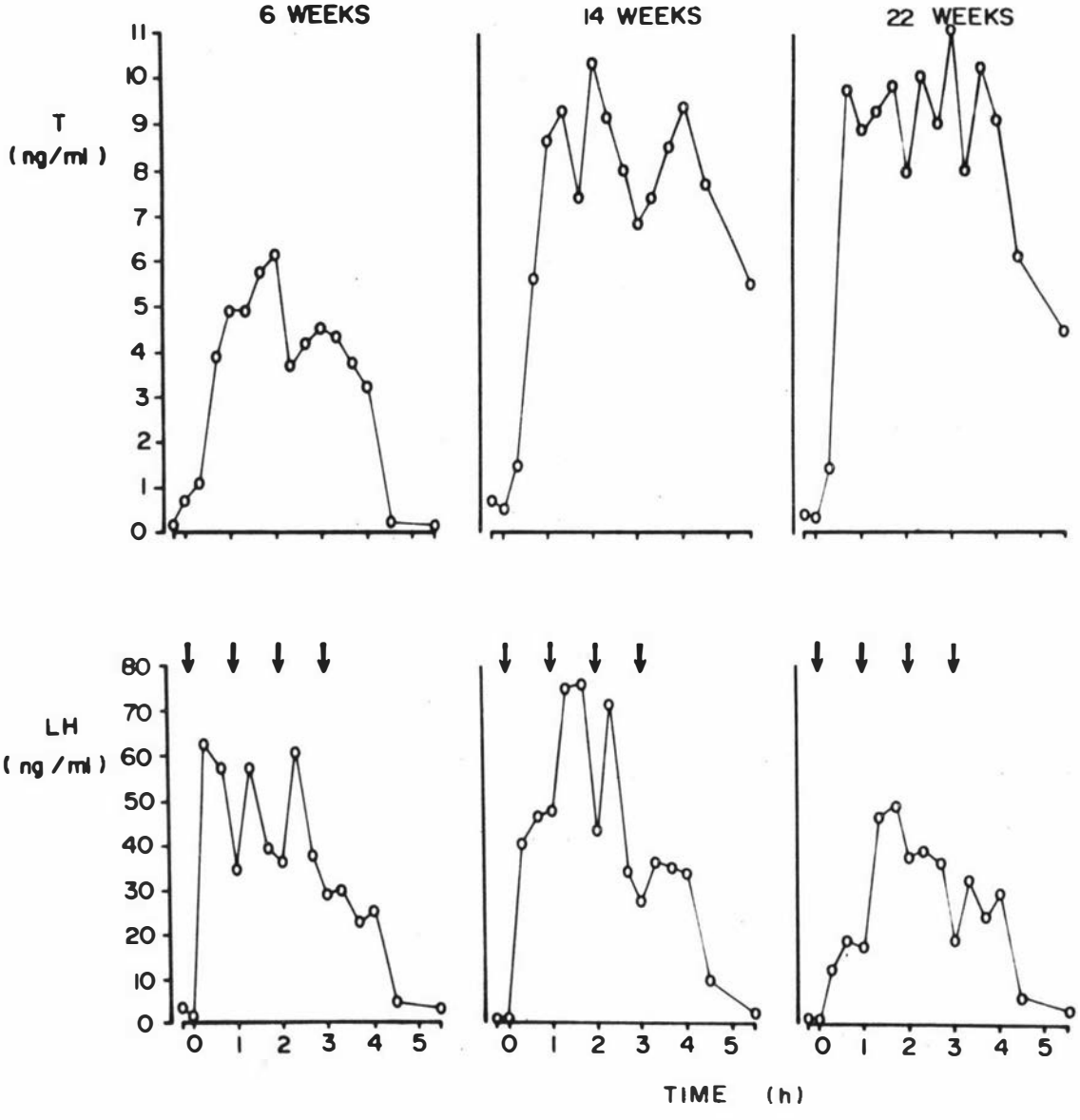


Figure 6.11: Ram 114. LH and testosterone responses following four hourly injections of GnRH (represented by arrows) at 6, 14 and 22 weeks of age.

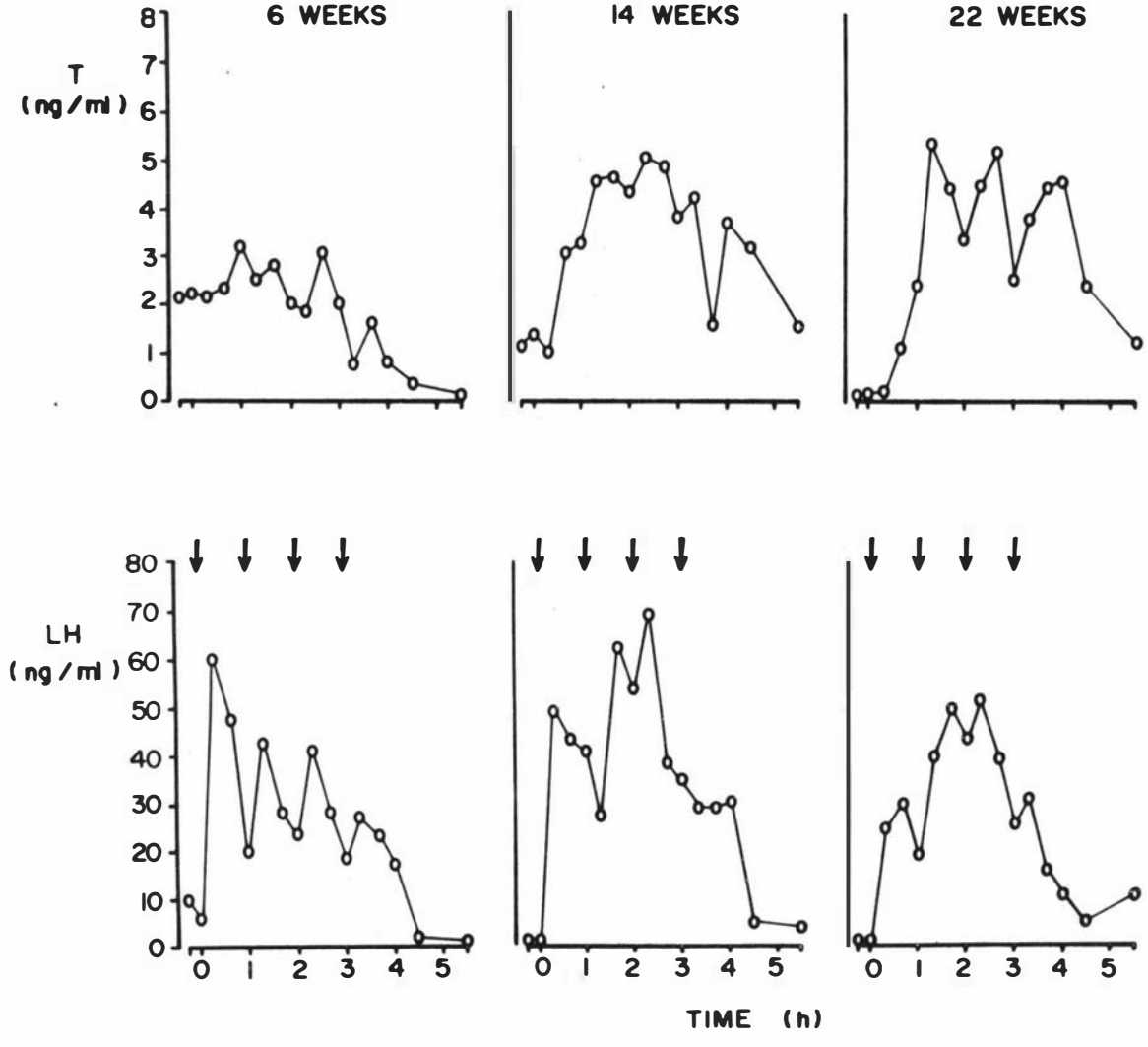


Figure 6.12: Ram 115. LH and testosterone responses following four hourly injections of GnRH (represented by arrows) at 6, 14 and 22 weeks of age.

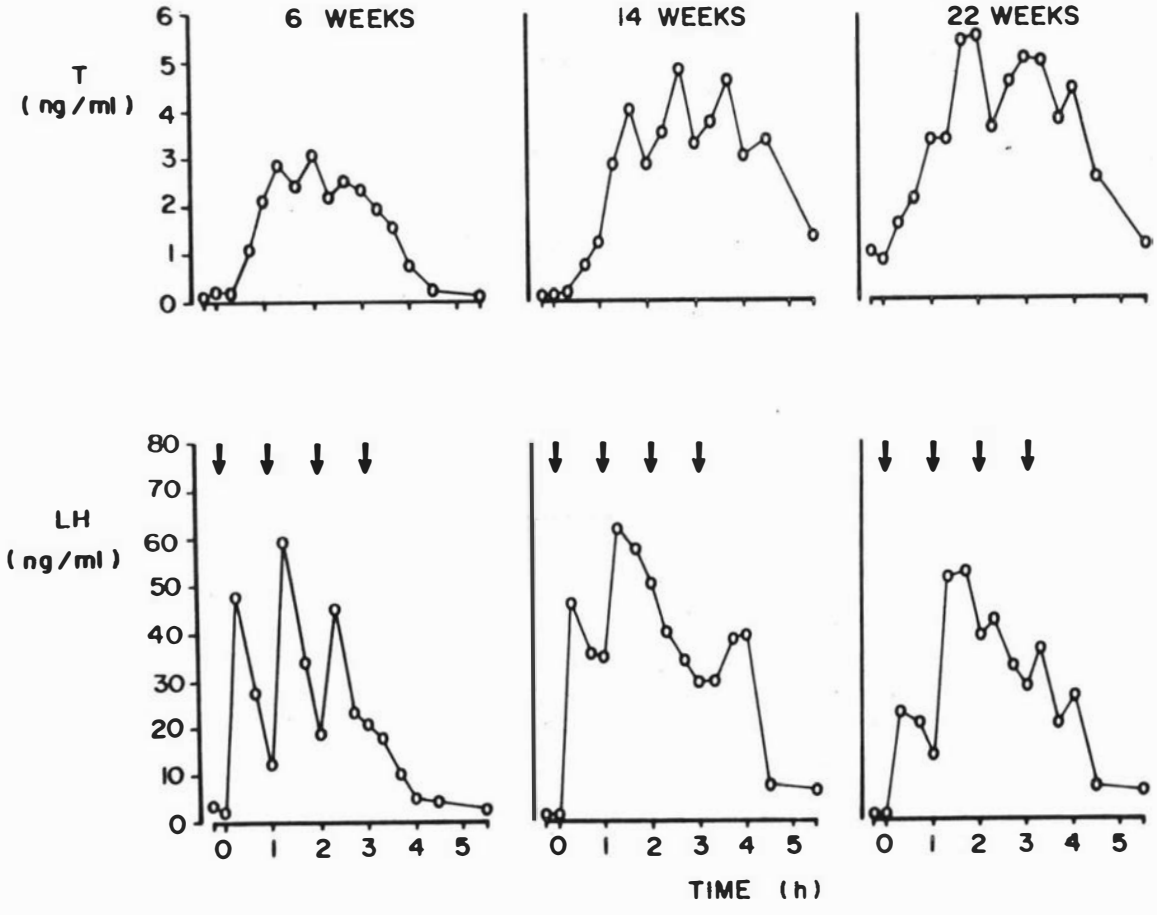


Figure 6.13: Ram 116. LH and testosterone responses following four hourly injections of GnRH (represented by arrows) at 6, 14 and 22 weeks of age.

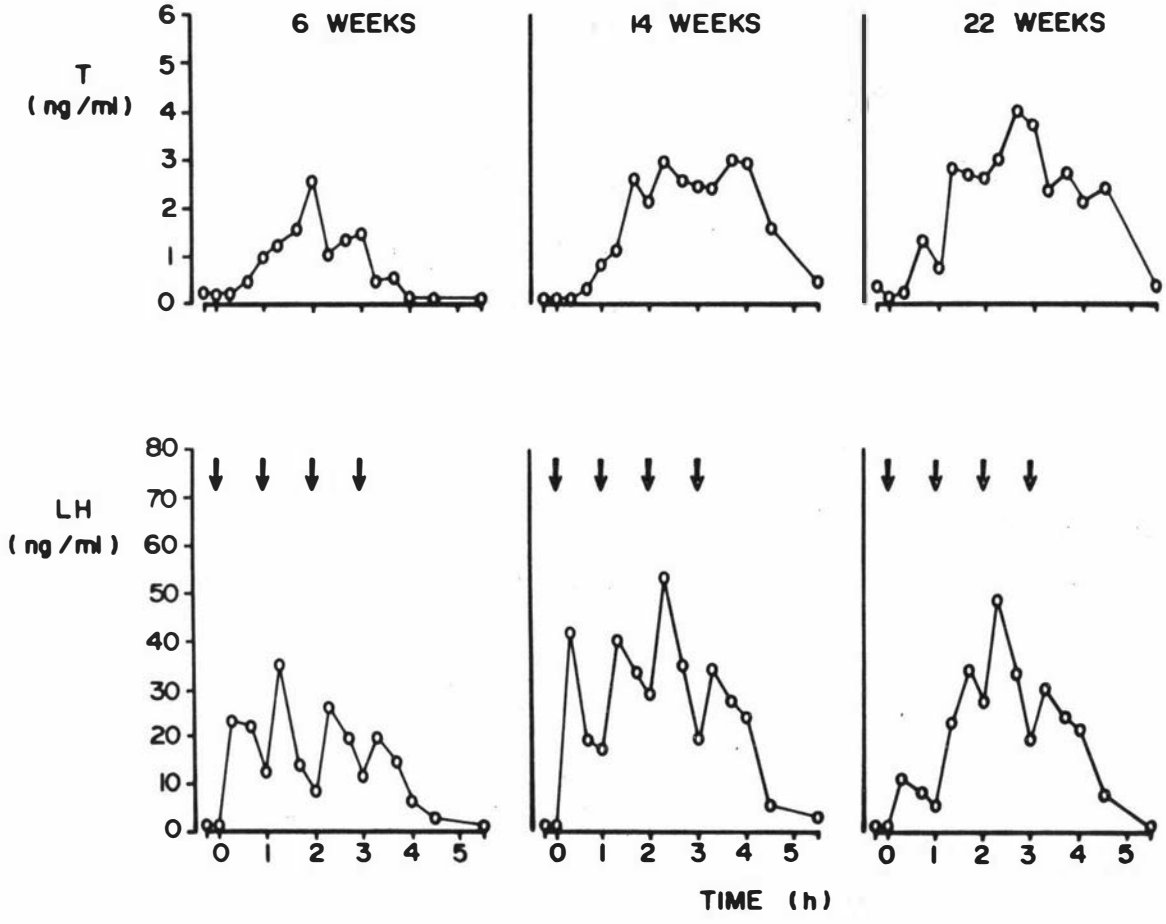


Figure 6.14: Ram 117. LH and testosterone responses following four hourly injections of GnRH (represented by arrows) at 6, 14 and 22 weeks of age.

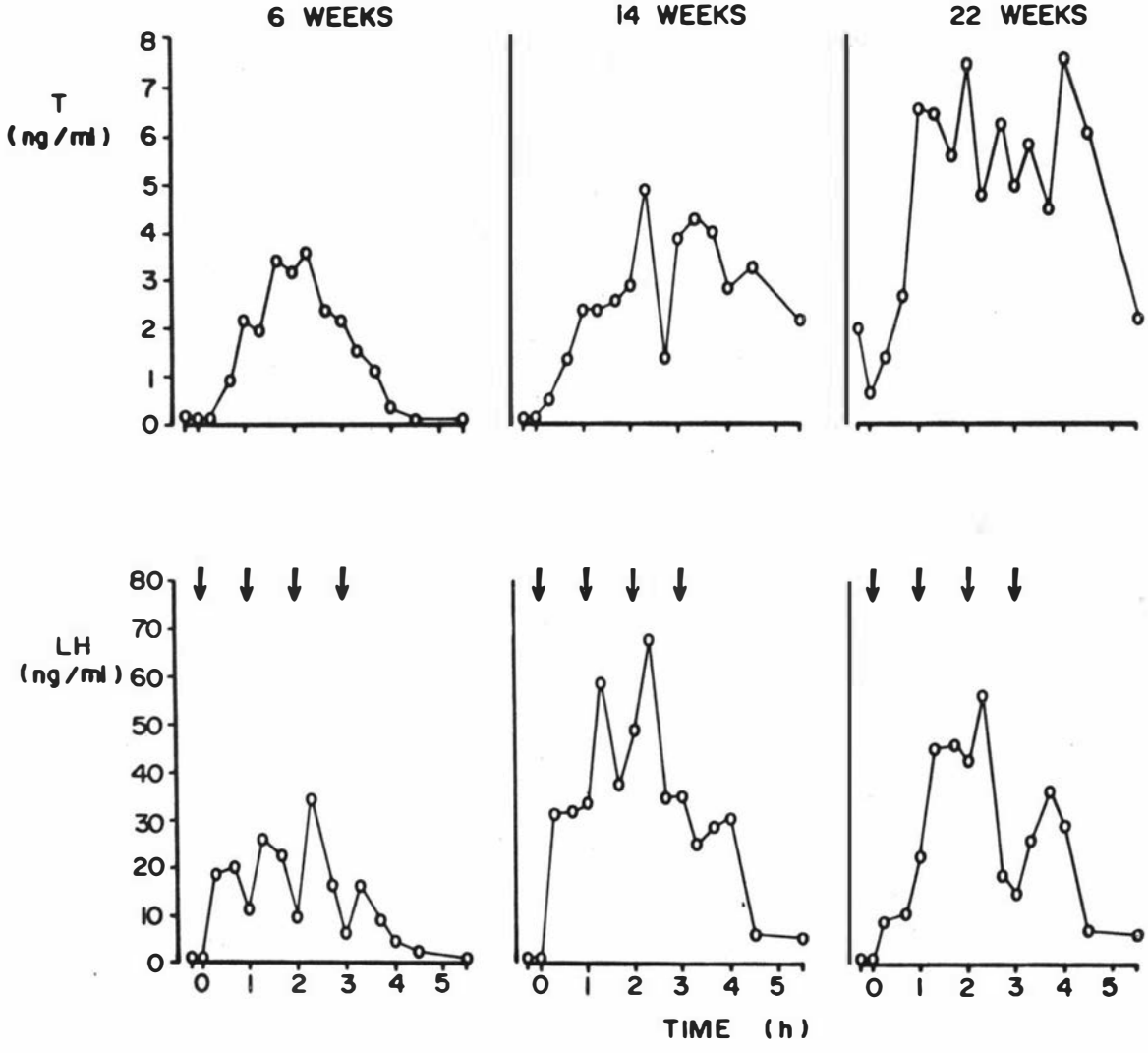


Figure 6.15: Ram 118. LH and testosterone responses following four hourly injections of GnRH (represented by arrows) at 6, 14 and 22 weeks of age.

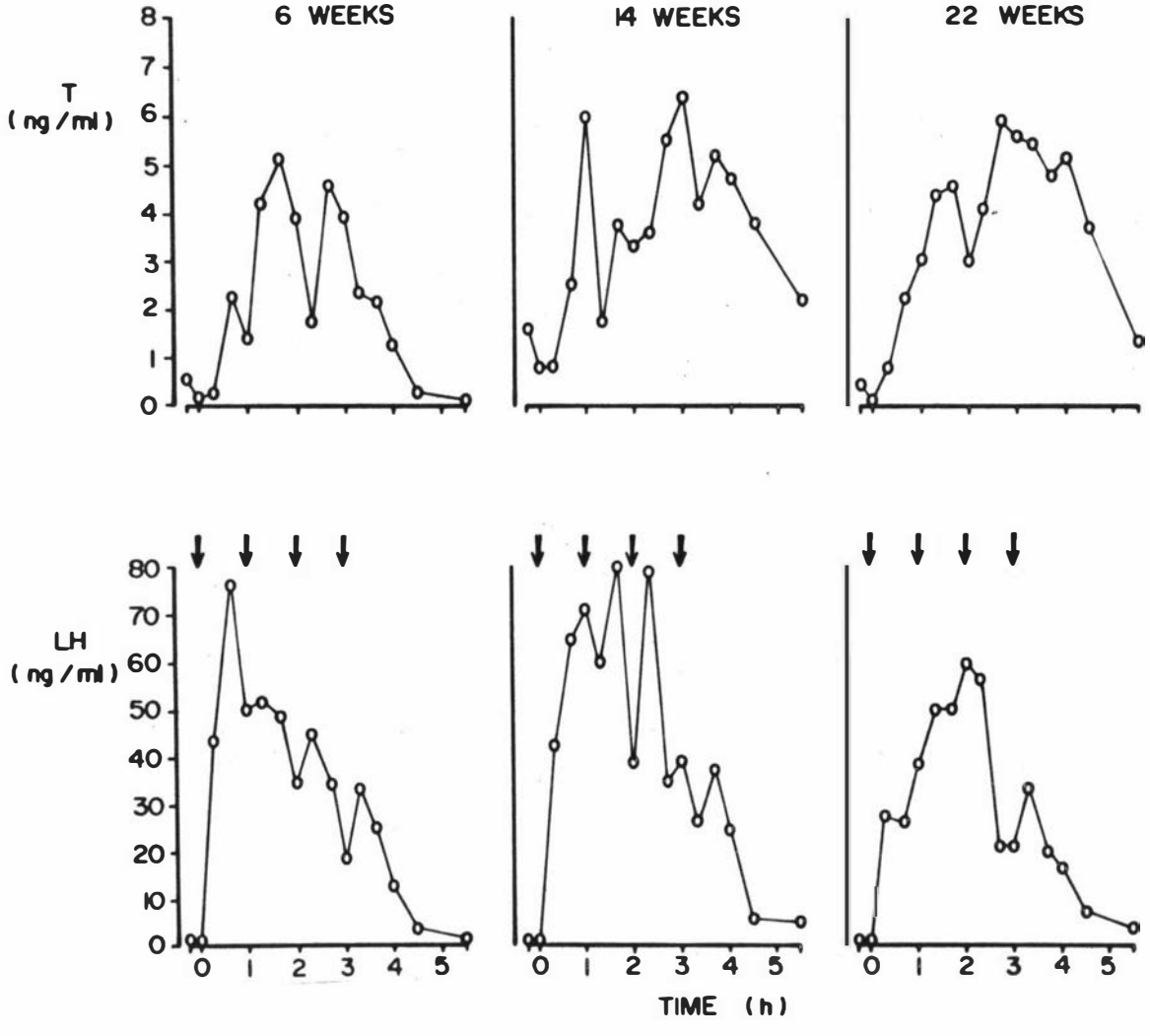


Figure 6.16: Ram 120. LH and testosterone responses following four hourly injections of GnRH (represented by arrows) at 6, 14 and 22 weeks of age.

induced LH peak was invariably higher than the first peak, a situation opposite to that at six weeks of age.

Plasma testosterone levels also rose subsequent to the initial LH elevation. At each age, maximal testosterone levels occurred following the second or third LH elevation, although testosterone levels did not increase in response to individual GnRH injections as did LH levels. LH levels at each age, and testosterone levels at 6 weeks, generally had returned to pre-injection levels 2.5 h after the final GnRH injection, but testosterone concentrations at 14 and 22 weeks mostly remained elevated 2.5 h after the last injection.

Analyses of variance of pre-injection and total hormone responses to GnRH injections (Table 6.3) showed that while pre-injection levels of testosterone did not alter significantly, pre-injection LH levels decreased linearly from 6 to 22 weeks ($P < 0.01$). The total LH response was highest at 14 weeks of age ($P < 0.001$) and the total testosterone response increased linearly from 6 to 22 weeks ($P < 0.05$).

Analysis of variance of regression coefficients of total testosterone responses on total LH responses was significant ($P < 0.001$) indicating a close relationship between the quantity of LH released and the subsequent release of testosterone.

4. DISCUSSION

From the observations of testicular development discussed in Chapter 5, it was clear that at 6 weeks all animals would have been prepubertal, those at 14 weeks would have been approximately mid-way through pubertal testicular development, and those at 22 weeks should have possessed spermatozoa in their seminiferous tubules and hence represented an early post-pubertal stage.

The existence of circadian rhythms of LH, testosterone and prolactin output were not examined in this study, but were not observed in adult rams (Experiment 1). However, because work with pubertal male human subjects (Weitzman et al., 1975) has shown that plasma concentrations of LH varied in a circadian rhythm, an attempt was made to minimize possible effects of daily variations in hormone levels by commencing sampling routines at the same time at each sampling period. To date there appears to have been no reports in the literature of circadian rhythms of LH, testosterone or prolactin plasma concentrations in pubertal rams, but M.W. Fisher (pers. comm) observed no circadian

rhythm of LH or prolactin levels in 100 day old rams or wethers.

Few other authors have examined the LH and testosterone secretion patterns in ram lambs closely, and no reports of short term prolactin secretion profiles in ram lambs have appeared in the literature. Sanford (1974) studied eight ram lambs of three different breeds (Finnish Landrace, Suffolk and Line-M) aged between 2 and 7 months. Each ram was sampled at 20 min intervals for eight hours then LH and testosterone levels compared with those of yearling and adult rams. The younger rams had significantly lower mean plasma testosterone levels than yearlings or adults and significantly higher plasma LH levels than adults. While results in this chapter generally agreed with the conclusions of Sanford (1974), the ages of his "young" rams ranged from 8 to 30 weeks and he made no attempt to distinguish changes in secretion patterns which may have occurred during this period. Other factors which limit the value of Sanford's data were the mixture of breeds utilized and the fact that samples were collected from all animals on the same day. This latter point indicated that the rams had widely varying birth dates so that it was not possible to distinguish age and seasonal influences on hormone levels.

In a second investigation Sanford (1974) followed an identical sampling procedure on eight rams aged between 74 and 92 days. Five lambs showed 1 - 3 episodic LH peaks of up to 12 ng/ml, which were followed within 60 min by well defined testosterone peaks, while three rams showed no LH fluctuations. These results were in accord with those obtained in Experiment 4.1 from 14 week and older rams. Sanford concluded: "....secretory patterns of LH and testosterone in prepubertal rams were strikingly similar to adults....". However, results from the present study conflict with this statement, since lambs at 6 weeks of age (prepubertal) did not show the quantitative or qualitative relationships between these two hormones which were observed in pubertal and adult rams. A probable explanation for this apparent disparity between results of the present, and the Canadian experiments is that Sanford appeared to make an error of judgement as to what constitutes a prepubertal lamb; data of Skinner and Rowson (1968) would support the view that 9 - 13 week old Suffolk rams were in fact pubertal rather than prepubertal.

In an experiment using Merino-Corriedale crossbred rams, late maturing animals, Lee et al. (1976a) reported that even though LH levels

did display pulsatile fluctuations, there was no correlation between LH and testosterone levels in pubertal subjects (10 - 12 months of age). Pubertal boars have been shown to exhibit pulsatile release of LH and subsequent testosterone elevations in frequent samples collected over 24 hours (Flor Cruz, 1977); similar patterns were recorded in adult boars, but prepubertal boars were not studied.

Prolactin levels showed wide variations at each age, but no differences in prolactin output were detected between the 6th, 14th and 22nd week of life. These observations were consistent with the patterns of basal prolactin levels described in Experiment 3.2; levels were high by 6 weeks of age, and did not being to fall until after 22 weeks.

Though short term LH secretion patterns resembled those of adult rams (Experiment 1), major changes in the interrelationships between LH and testosterone secretion patterns during puberty have been shown in Experiment 4.1. Despite the absence of detectable changes in LH output, testosterone output increased from 6 to 22 weeks. This increase was manifest in two ways: firstly, the episodic peaks of LH at 14 and 22 weeks (and in adults in Experiment 1) all were succeeded by testosterone peaks, yet at 6 weeks only six of eleven LH peaks were followed by testosterone peaks; and secondly, the nature of this change was depicted by the establishment of significant quantitiative relationships between LH and subsequent testosterone peaks at 14 and 22 weeks (and in adults in Experiment 1), yet no significant quantitative relationship existed between the LH and following testosterone peaks at 6 weeks of age. It may be suggested that the stabilization of this relationship is an important factor in the maturation of the testes.

Data from Experiment 4.1 supported the concept discussed in Chapter 5, that handling and sampling procedures influenced the output of prolactin and LH. Deletion of the first three samples significantly decreased coefficients of cumulative regressions of prolactin secretion profile data. Therefore it was considered appropriate to delete these samples from prolactin data for statistical analysis; a similar approach was adopted by Barrell (1976) following venepuncture blood collection from adult rams. Stress induction of prolactin output has been reviewed in Chapter 1 (p 12).

Using paired t tests, the differences between coefficients of cumulative regressions of complete and reduced LH secretion profile data were shown to be non-significant. Probably this was because only half of the LH secretion profiles displayed elevated LH levels in the first three samples. Apart from visual assessment of a sampling-induced LH elevation, combined evidence from Experiments 4.1 and 4.2 also suggested a sampling-induced elevation of LH levels: in Experiment 4.1, in which 25 samples from each animal were included in an analysis of variance, no significant differences in LH levels were detected between 6, 14 and 22 week old rams, but in Experiment 4.2, when means of only two samples (pre-injection samples representing basal LH concentrations) were included in an analysis of variance, a significant linear decrease in LH level was observed from 6 to 22 weeks of age. Therefore it could be suggested that the use of large numbers of samples for analysis tended to dampen the effect of sampling-induced LH elevations. The lower incidence of apparent induced elevations of plasma LH concentrations at 14 and 22 weeks may have indicated an adaptation to sampling and handling procedures as suggested by Sitarz et al. (1977). These authors reported a similar sampling-induced LH release in yearling bulls and provided substantial statistical evidence to support their claim.

Total pituitary LH output following repeated GnRH injections was highest during the pubertal sampling, at 14 weeks of age. This result was consistent with the findings of Skinner et al. (1968) and Courot et al (1975) who recorded increasing pituitary LH content up to approximately 70 - 100 days of age. The lower LH response at 6 weeks may have been a manifestation of a limited pituitary LH content, while that at 22 weeks was likely to have resulted from the inhibitory effects of the higher plasma levels of testosterone (see Chapter 5). The greatest output of LH, at 14 weeks, may have been due to a combination of the attainment of peak pituitary stores of LH and possible sub-maximal testosterone feedback influences, since plasma concentrations of this steroid were lower at 14 weeks than at 22 weeks. The delayed LH response to GnRH as age increased from 6 to 22 weeks, was consistent with the patterns recorded following single injections of GnRH described in Experiment 3.3, and those published by Galloway and Pelletier (1974). Courot et al. (1975) reported that the half life of LH in plasma of young rams was similar to that in adults

(see Chapter 1), therefore the factor most likely to have contributed to these delayed responses must have been the rising basal testosterone levels such as recorded in Experiments 4.1 and 3.2.

In response to repeated GnRH injections testosterone output was seen to increase from 6 to 22 weeks, which was consistent with observations following single injections as described in Experiment 3.3.

Intracarotid infusions of GnRH raised plasma LH and testosterone levels of rams aged from birth to seven months (Lee et al., 1976b) and are discussed in Chapter 5. Though Lee et al. (1976b) suggested that the pituitary sensitivity to GnRH increased between 6 and 8 weeks and that this marked the initiation of the pubertal process, it was difficult to ascertain from their data the precise nature of changes in the pituitary-gonadal axis to which they may have been alluding.

CHAPTER VII

STUDIES ON PRENATALLY ANDROGENIZED SHEEP

1. INTRODUCTION

Differentiation of the neuroendocrine and anatomical components of the reproductive system is largely dependent on the presence or absence of testosterone. The early observations providing the basis for current concepts of sexual differentiation are outlined in a widely quoted review by Harris (1964). Although that review reported some observations from the cat and rabbit, most current knowledge of sexual differentiation has been derived from studies using the rat. At present little is known about sexual differentiation in domestic animals.

This study was designed to investigate aspects of prenatal androgenization of sheep and in particular, hypothalamo-pituitary-gonadal function of androgenized offspring during puberty.

2. MATERIALS AND METHODS

(1) Animals.

Sixty adult New Zealand Romney ewes were mated during late March and April 1974. Husbandry practices are described in Chapter 2. Ewes were checked for service twice daily by observation of crayon marks from ram mating harnesses, and mating dates were recorded. Lambing took place in a sheltered field and particular care was taken to ensure that lambs were identified with their dams as soon as possible after birth, to minimize the danger of cross-fostering between treated and control ewes.

Lambs were weighed and examined at birth for the anatomical masculinizing syndrome previously described by Tarttelin (1975). Lambs were weighed weekly for 5 months.

Three groups of offspring, normal females, androgenized females and androgenized males were selected from the initial 60 ewes. Controls for androgenized males were ram lambs used in the parallel study described in Experiments 3.2 and 3.3, and which were bred from similar ewes lambed at the same time under similar conditions on the same farm. To comply with management requirements, these flocks were mated and

lambed separately but were combined shortly after lambing.

To simplify terminology, offspring from androgen treated ewes are referred to as "treated males" and "treated females" (masculinized), and those from untreated ewes termed "normal" males and females.

(2) Treatment of Ewes.

Forty ewes were selected for intramuscular injection of 200 mg of testosterone cypionate ("Depo-Testosterone", Upjohn Limited) on each of the 20th, 27th, and 40th days of gestation. In addition, six ewes not included in the current study were given the same quantity of testosterone cypionate on two occasions, two weeks apart and weekly blood samples collected for six weeks commencing at the time of the first injection. Plasma testosterone levels were measured to estimate the period of elevated testosterone concentrations following treatment of ewes.

(3) Experimental.

(i) Experiment 5.1: Basal LH and Testosterone Levels. Six normal and ten treated females, and eight normal and eight treated males were selected for a longitudinal study of hormone levels. Single blood samples were collected each two weeks from four to thirty weeks of age. From normal males, the first sample of four collected for Experiment 3.2 was used. Plasma from males and females was assayed for LH concentrations, while testosterone levels were estimated only in plasma samples from males.

For presentation of data (Figure 7.1), monthly mean LH levels were calculated, and for statistical analysis, groups were compared following analysis of variance using the coefficients shown below after weighting for disproportionate numbers within groups:

	Contrast	Normal Female	Treated Female	Normal Male	Treated Male
1.	Treated (male + female) vs Normal (male + female)	-1	+1	-1	+1
2.	Male vs Female	+1	+1	-1	-1
3.	Treatment x Sex (interaction)	-1	+1	+1	-1

Testosterone levels observed in the treated and normal males also were compared using an analysis of variance.

Analyses using body weight as a covariate was performed on LH and testosterone data from males to examine the possibility that body weight differences influenced detected differences in hormone levels.

(ii) Experiment 5.2: LH Output in Response to GnRH Administration.

At 6, 14, 22 and 30 weeks, studies using GnRH were performed with the animals and treatments summarized in Table 7.1. Animals used in this study were in addition to those used in Experiment 5.1, and since numbers were limited, only one treated male could be sampled. At 30 weeks, three entire treated males from Experiment 5.1 were included subsequent to the conclusion of that study. Castrated treated males were from a growth study run in parallel with this investigation.

Each animal was given a single jugular injection of GnRH (10 μg/ml in 0.9% saline) at a dose rate of 1 μg/Kg. Plasma samples were collected 20 min, 10 min and immediately prior to GnRH injection, and 15, 30, 60, 90, 120 and 240 min after injection. Control sheep were selected at random from each group in Experiment 5.1 at corresponding ages and were given control injections of saline, and plasma samples were collected at 0, 30 and 90 min after injection. No castrate males were available for control injections. Plasma samples were assayed for LH concentrations.

For analysis, total LH responses to GnRH were calculated (see Chapter 2) while pre-injection levels were calculated by averaging the LH concentrations of the three pre-injection samples. Variances of data thus calculated were compared by analyses of variance for all ages using coefficients shown below after weighting for disproportionate numbers within groups:

Contrast	Normal Female	Treated Female	Normal Male	Castrated Treated Male
1. Normal Male vs Castrated Treated Male	0	0	+1	-1
2. Normal Female vs Treated Female	+1	-1	0	0
3. Males vs Females	-1	-1	+1	+1

Data from the one treated male was not included in statistical analyses but variances of responses of the four rams of this group sampled at 30 weeks were compared by analyses of variance with those from other male groups using coefficients shown below after weighting

TABLE 7.1

Summary of experimental design showing numbers of animals used for investigation of responses to GnRH administration.

GnRH Treatment	Normal Female	Treated Female	Normal Male	Treated Male	Castrated Treated Male
Week 6	4	4	4	1	3
Week 14	4	4	4	1	3
Week 22	4	4	4	1	3
Week 30	4	4	4	4	3
Control Saline Injection					
Weeks 6, 14, 22 and 30.	4	4	4	4	0

for disproportionate numbers within groups:

Contrast	Normal Male	Treated Male	Castrated Treated Male
1. Treated vs Castrated Treated Male	0	+1	-1
2. Normal vs Treated Male	+1	-1	0

Regression fits of mean LH output response curves were calculated (see Chapter 2) and comparisons were made by t tests based on the coefficients used to determine differences in total and pre-injection levels described earlier in this section.

(iii) Experiment 5.3: Responses of Females to GnRH Before and After Ovariectomy. At 45 weeks of age, six normal and six treated females were selected, GnRH administered, and plasma samples collected as described for Experiment 5.2 above. Ewes were ovariectomised within the following two days and the GnRH injection and sampling procedures repeated two weeks later. Samples were assayed for LH concentrations.

Pre-injection levels and total LH responses before and after ovariectomy were compared by analyses of variance using the coefficients shown below, and regression fits of mean response curves were calculated and compared by t tests using the same coefficients:

Contrast	Pre-ovariectomy		Post-ovariectomy	
	Normal	Treated	Normal	Treated
1. Pre - vs Post-ovariectomy	+1	+1	-1	-1
2. Normal vs Treated	+1	-1	+1	-1
3. Interaction	+1	-1	-1	+1

This procedure was undertaken to determine whether prenatal exposure to androgen had interfered with oestrogen feedback receptor activity, and to provide a comparison of female responses to gonadectomy to correspond with responses of castrated treated males observed in Experiment 5.2

3. RESULTS

(1) Plasma Testosterone Levels in Ewes.

Plasma testosterone levels after testosterone cypionate administration are shown below:

Week	Number of Ewes Sampled	Number With Elevated Plasma Testosterone	Mean Plasma Testosterone Level (ng/ml ± SEM)
0	6	0	0
1	6	6	25.17 ± 4.83
2	6	6	4.89 ± 1.68
3	5	5	13.47 ± 2.41
4	6	6	6.32 ± 1.35
5	6	6	3.88 ± 1.28
6	6	1	0.53
7	6	0	0

No testosterone was detected prior to the first injection but levels were still elevated two weeks after the first injection. The second injection, at week 2, was followed by a second though less marked elevation of plasma testosterone levels then a gradual decline during the ensuing three weeks. Four weeks after the second injection, testosterone was detectable in the plasma of only one ewe. It was concluded that the androgen treatment regime employed in this study would have ensured elevated plasma testosterone levels from the 20th to approximately the 65th day of gestation.

(2) Effect of Testosterone Treatment on Ewes.

Following testosterone administration, ewes became noticeably aggressive with increased foot stamping and defensive behaviour, and many displayed mounting behaviour. Of the 59 ewes which lambed, three cases of dystocia occurred, an incidence not considered unusual for the New Zealand Romney breed. Neonatal mortality of lambs, 14% in control offspring and 6% in treated offspring, also was considered normal.

(3) Description of Offspring.

Treated males were normal at birth and continued to display normal anatomical growth and development. All treated females were indistinguishable from males at birth upon external visual examination: no external vulval opening was present, a penis, prepuce and scrotum were present and urination occurred via the penile urethra. However, no testes were palpable. Upon dissection of three treated females which died neonatally, no testes were present either scrotally or internally, but all possessed normal ovaries. Normal fallopian tubes, uterine horns and bodies, cervices and vaginae were present, along

with normal ligamentous attachments. The penis was identical to that of the normal male, including the sigmoid flexure and retractor penis muscle. No differences, macroscopic or microscopic, were observed between ovaries and internal reproductive tracts of normal and treated females following ovariectomy at 45 weeks of age (Experiment 5.3).

Diagnosis of the "intersex" syndrome was initially based on the lack of palpable testes and was later confirmed upon ovariectomy at 45 weeks.

The scrotum of the "intersex" animals did not develop and the penis and prepuce failed to progress to normal size as the animals aged. This and the failure of breakdown of preputial adhesions led to urinary scald about the prepuce. Many "intersex" animals displayed female urination postures.

(4) Hormone Data.

(i) Experiment 5.1: Basal LH and Testosterone Levels.

See Table 7.2 and Figures 7.1 and 7.2.

Mean LH levels in females during the first 7 months of life varied markedly from 0.1 to 1.4 ng/ml. LH levels were highest in normal females at 4 months and in treated females at 3 months, but no overall increase or decrease in LH levels was observed.

LH levels in males were highest during the second month of life and generally decreased during the following 5 months. The major difference shown by the analysis of variance (Table 7.2) was that treated animals produced significantly less LH than normal animals ($P < 0.001$).

Testosterone levels of normal rams increased markedly during the first 7 months (Figure 7.2) but levels in treated males showed only a small increase during the same period. Analysis of variance showed the testosterone levels in normal animals to be significantly greater than in the treated animals ($F_{1,98} = 53.19$, $P < 0.001$).

Analysis of variance of body weight data showed treated males to be significantly lighter than normal males ($F_{1,70} = 63.84$, $P < 0.001$), but covariance analysis showed hormone level differences were not attributable to body weight differences.

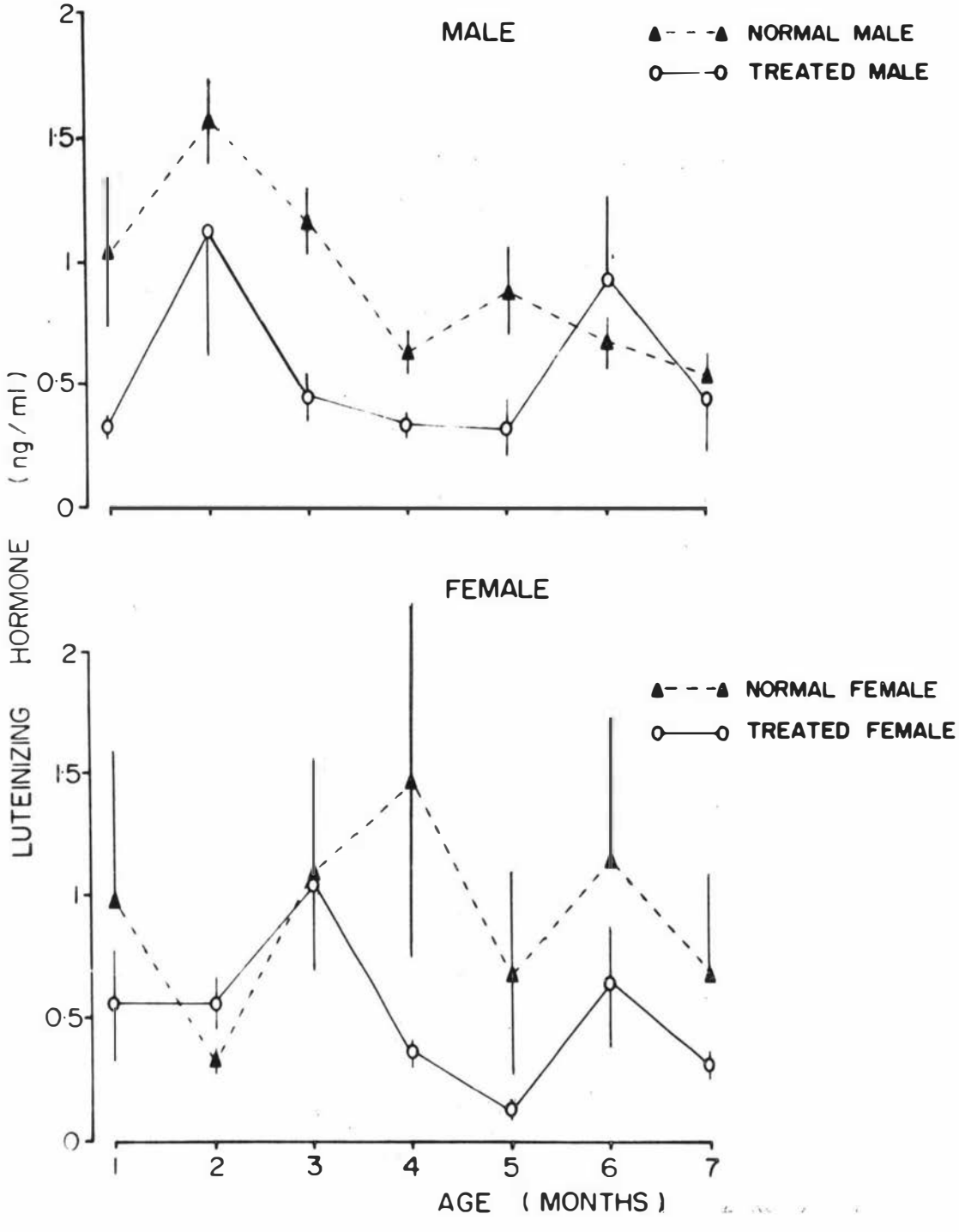


Figure 7.1: Monthly variations of plasma LH concentrations (means ± SEM) of normal and prenatal androgen treated lambs.

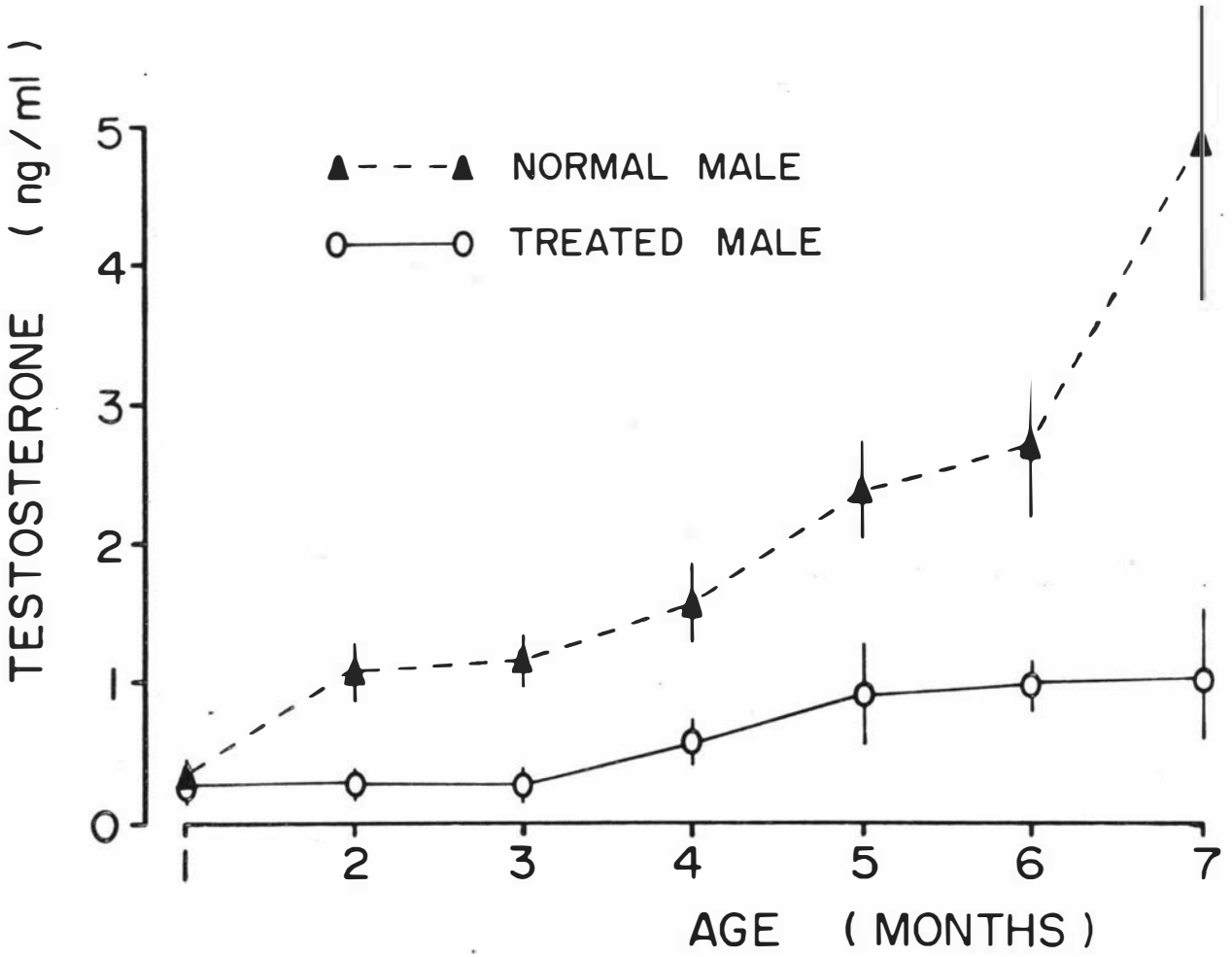


Figure 7.2: Monthly variations of plasma testosterone concentrations (means ± SEM) from normal and prenatal androgen treated ram lambs.

Table 7.2: Summary of analysis of variance of basal LH data from Normal and treated animals.

Source of Variation	d.f.	Variance Ratios
1. Male vs Female	1	11.68***
2. Normal (Male + Female) vs Treated (Male + Female)	1	24.30***
3. Sex x Treatment Interaction	1	1.34
Residual Mean Square	190	1223.9

(ii) Experiment 5.2: LH Output in Response to GnRH Administration.

See Table 7.3 and Figures 7.3 - 7.6

Each injection of GnRH resulted in a marked and rapid elevation of plasma LH concentrations while control injections of saline induced no such elevation. At six weeks of age, the peak LH concentration was reached 15 min after injection, but in the entire animals, the time taken to reach peak levels was delayed up to 30 - 60 min as the animals aged. In castrated animals, the peak LH concentration was reached within 15 min up to 22 weeks, but at 30 weeks, the mean peak was delayed because one animal displayed a biphasic output response. There were no striking differences in the pattern of LH secretion between the groups of entire males and females. The one treated male sampled at 6, 14 and 22 weeks produced quantities of LH within the range observed from normal males, while at 30 weeks, the output of LH from all four treated males was indistinguishable from that of normal males.

Analyses of variance (Table 7.3) showed that castrated treated males possessed significantly higher pre-injection LH levels and total LH output than normal males. Pre-injection concentrations and total LH responses of normal and treated females were not significantly different. As a result of the higher levels of LH recorded from the castrated males, overall results showed that males had significantly greater pre-injection LH concentrations and total responses than females.

Analyses of variance of regression coefficients showed an overall decrease of pre-injection LH levels with age ($F_{1,55} = 9.18$, $P < 0.01$) and of total LH output responses with age ($F_{1,55} = 5.78$, $P < 0.05$).

TABLE 7.3

Summary of analyses of variance performed to determine differences in pre-injection LH levels and total LH output responses to GnRH injection, and differences between components of regression fits of LH output response curves.

Source of Variation	d.f.	Variance Ratios				
		Pre-injection Level	Total	Component of Regression Fit		
				Linear	Quadratic	Cubic
1. Normal Male vs Castrated Treated Male	1	45.09***	4.80*	0.41	0.01	0.00
2. Normal Female vs Treated Female	1	0.39	0.94	0.19	0.03	0.01
3. Male vs Female	1	21.76***	11.00**	9.08**	1.41	0.50
Residual mean square	56	0.051	2.064	0.0060	0.0462	0.0084

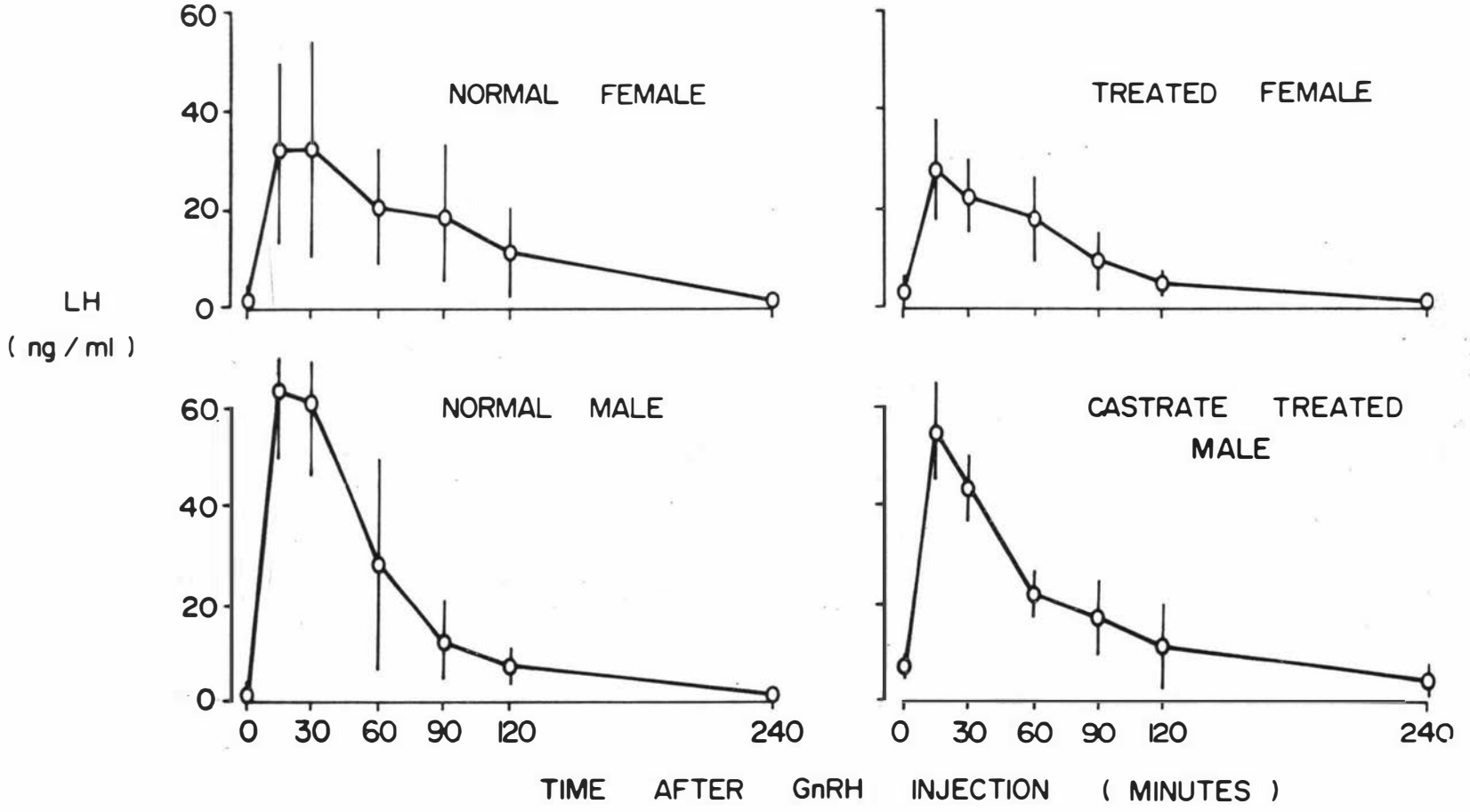


Figure 7.3: 6 weeks. Plasma LH responses (means ± SEM) following GnRH injection.

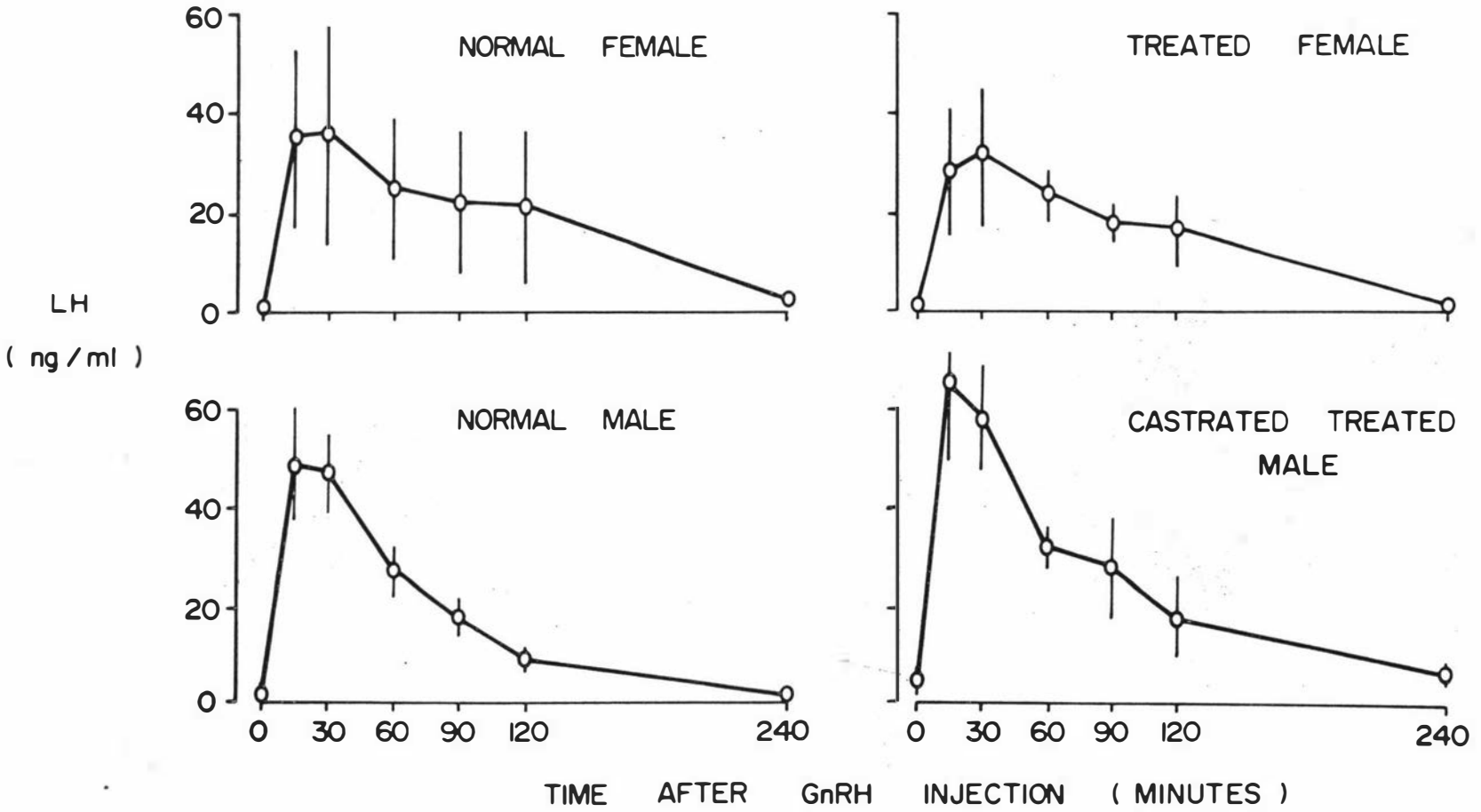


Figure 7.4: 14 weeks. Plasma LH responses (means ± SEM) following GnRH injection.

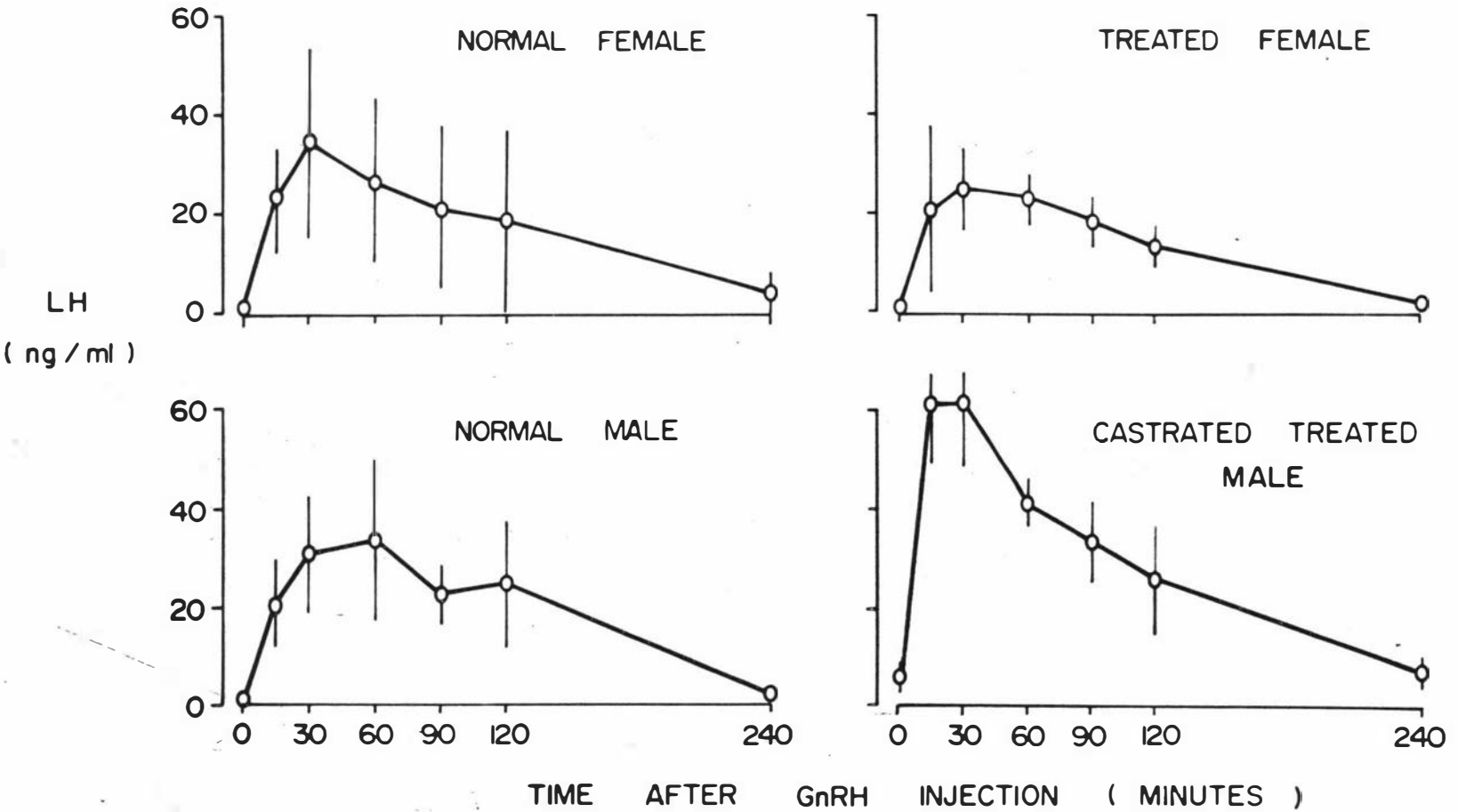


Figure 7.5: 22weeks. Plasma LH responses (means ± SEM) following GnRH injection.

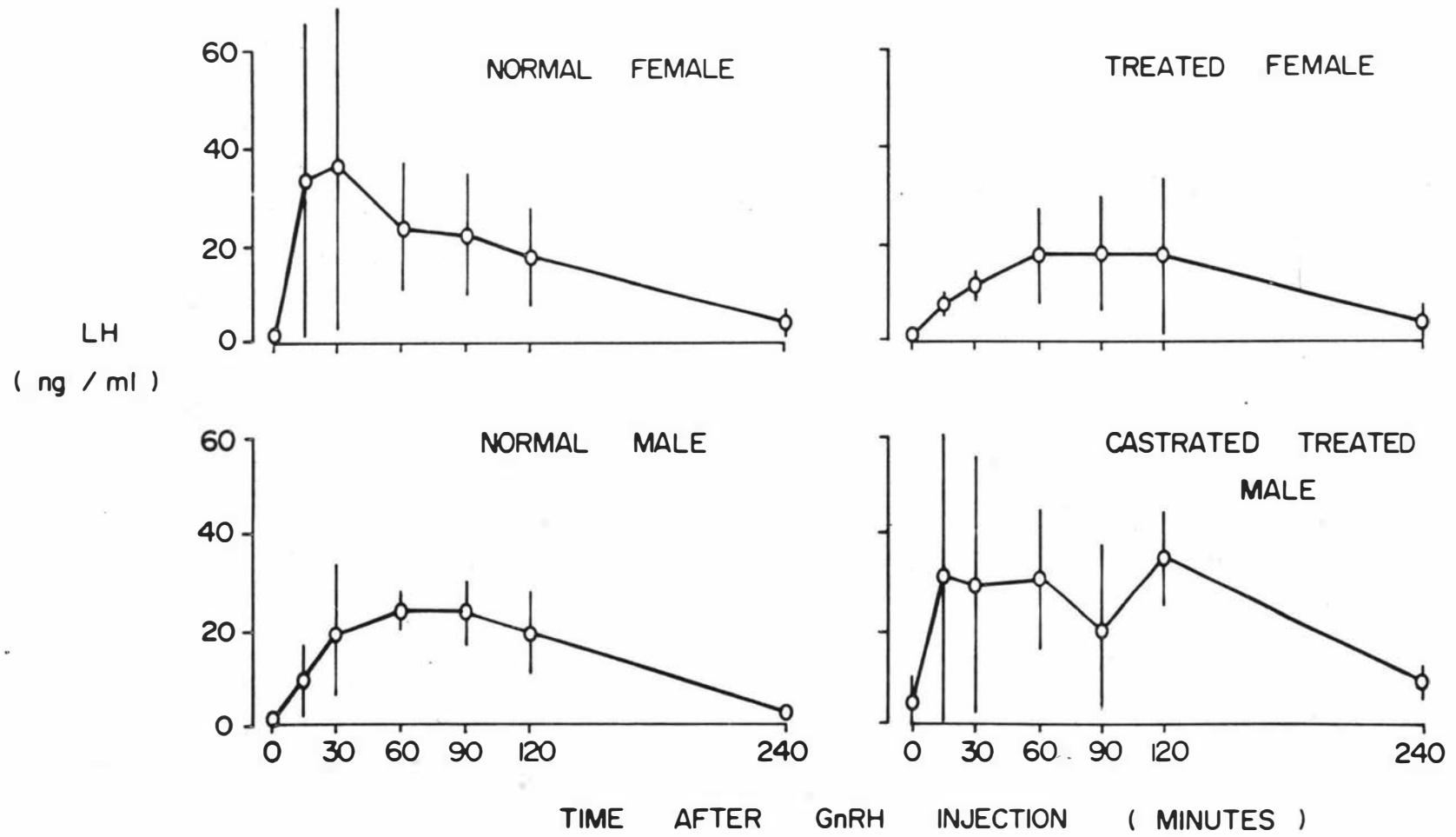


Figure 7.6: 30 weeks. Plasma LH responses (means ± SEM) following GnRH injection.

At 30 weeks, pre-injection levels and total responses of normal and treated males were not significantly different, but castrates possessed significantly greater pre-injection ($F_{1,8}$ = 18.10, P < 0.01) and total LH responses ($F_{1,8}$ = 8.63, P< 0.05) than treated males. Hence higher levels in castrate treated males could be attributable to castration rather than prenatal androgenization.

Analyses of variance of regression fits of LH output response curves (Table 7.3) showed that castrated treated males possessed a significantly greater linear component of LH output responses confirming greater LH output, but lack of differences between non-linear components showed that the shapes of all response curves were essentially the same. At 30 weeks of age, castrated treated males possessed a greater linear component of LH output than entire treated males (t_6 = 2.77, P <0.05), but the shapes of response curves from all three male groups at this age were essentially the same.

(iii) Experiment 5.3: Responses of Females to GnRH Before and After Ovariectomy.

See Table 7.4 and Figure 7.7

GnRH injection was followed by a rapid elevation of plasma LH concentrations. Analysis of variance (Table 7.4) showed that ovariectomy resulted in significantly greater pre-injection levels and total responses but that there were no significant differences in pre-injection levels or total responses of normal and treated females either before or after ovariectomy. t tests of regression fits of response curves showed there were no significant differences in the shapes of response curves between groups or as a result of ovariectomy.

Table 7.4: Summary of Analyses of Variance of Pre-injection LH levels and Total Output of LH in Response to GnRH Injection Before and After Ovariectomy.

Source of Variation	d.f	Variance Ratios	
		Pre-injection Level	Total Response
1. Pre- vs Post-ovariectomy	1	66.04***	20.25***
2. Normal vs Treated	1	0.78	0.52
3. Interaction	1	0.00	0.50
Residual Mean Square	20	0.051	0.727

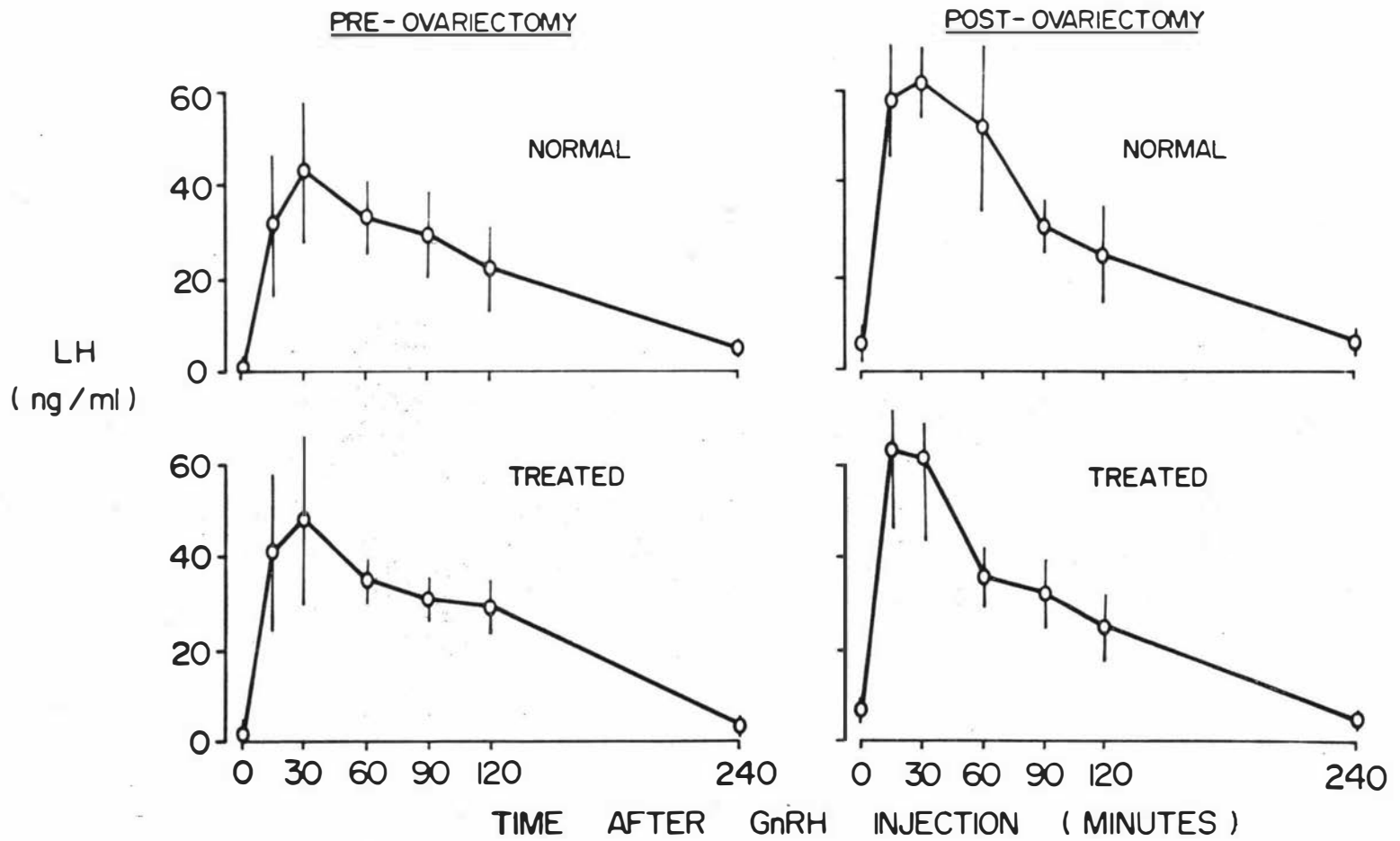


Figure 7.7: Plasma LH responses (means ± SEM) following GnRH injection before ovariectomy at 45 weeks of age, and again at 47 weeks, two weeks after ovariectomy.

4. DISCUSSION

(1) Timing of Exposure to Androgen.

Production of the anatomical masculinizing syndrome required exposure to androgen over a critical time period, but the dose, route of administration and pharmacological form of testosterone appear less critical. 1 g testosterone implants (Short, 1974), intramuscular or intrauterine testosterone enanthate with subsequent intramuscular injections (Alifakiotis et al., 1976) and intramuscular testosterone cypionate injections (Experiment 5), all produced masculinization in sheep.

Treatment of ewes starting at days 20 and 40 of gestation induced complete masculinization of female offspring, but treatment beginning at day 60 did not (Short, 1974). Clarke et al. (1976) reported that the same syndrome followed treatment between days 30 and 80 of gestation, but did not follow treatment from days 50 to 100, and Alifakiotis et al. (1976) achieved masculinization of female lambs after treatment beginning from the 25th to 45th days of gestation. It may be deduced therefore that the masculinizing syndrome is brought about by exposure to testosterone between approximately the 40th and 50th day of gestation. It is now clear that Przekop et al. (1974) failed to induce masculinization because of inappropriate timing of androgen exposure.

Zimbleman and Lauderdale (1973) failed to show anatomical or post-natal reproductive abnormalities in heifers exposed to testosterone from 80 - 133 days of gestation indicating early sexual differentiation also occurs in the bovine. Attempts to alter hypothalamic sexual differentiation in bitches, gilts and heifers by androgen treatment at birth also were unsuccessful (Zimbleman and Lauderdale, 1973).

It is evident that anatomical sexual differentiation occurs prior to hypothalamic sexual differentiation since Przekop et al. (1974) found no abnormalities of post-pubertal reproductive cycles in ewes androgenized after 84 days in utero, yet Clarke et al. (1976) produced abnormal cyclical activity in ewes androgenized both between 30 - 80 and 50 - 100 days in utero. Short (1974) observed that one ewe androgenized at day 80 in utero displayed normal cyclical activity after puberty. Hence, in sheep hypothalamic differentiation appears to occur between days 50 - 80 in foetal life.

Rats are born with genetic sex displayed phenotypically yet differentiation of the hypothalamic function occurs post-natally (Harris, 1964), hence the sequence of occurrence of anatomical then hypothalamic differentiation is likely to be common to many species. No information concerning hypothalamic sexual differentiation was determined from the present study since later endocrine and behavioural studies necessary to elucidate this aspect were outside the scope of this thesis.

Sexual differentiation normally is induced by the endogenous production of testosterone by the male foetus. Attal (1969) reported testosterone production by the male ovine foetus as early as day 30 in utero. Testicular testosterone levels rose at about 80 days, but the patterns of production were difficult to ascertain, particularly since small numbers of animals were used (Attal, 1969). It was evident that maternal treatment resulted in the transport of testosterone to the foetus, and that in the female, this mimicked male gonadal steroid production. Since endogenously produced foetal levels of testosterone were not high (Attal, 1969; Pomerantz and Nalbandov, 1975) it is likely that only small quantitites of steroid are necessary to induce anatomical sexual differentiation provided the critical timing is observed. In rats (Warren et al., 1973) and man (Reyes et al., 1973) foetal testosterone levels were highest at 18.5 days in utero, and at foetal length 7 - 10 cm, respectively, in each case these are periods of maximum Wolffian duct development.

(2) Anatomical Modifications.

The anatomical modifications described were similar to those described by Short (1974) and Clarke et al. (1976). Alifakiotis et al. (1976) also described the presence of Wolffian duct derived epididymides, ductulideferentia, and seminal vesicles and other secondary sex glands together with the external masculinizing syndrome. These structures were not noted in the present study nor that described by Short (1974), though all reports agree on the external appearance of the female offspring.

Since all reports in the literature have been based solely on neonatal anatomical examination, no studies have reported anatomical growth and development of androgenized offspring. In this study it was observed that the scrotum, penis and prepuce of masculinized female

offspring did not develop to the normal extent and penile-preputial adhesions were not broken down and this predisposed the animals to urine scald, preputial inflamation and in some cases, urinary overflow incontinence. These abnormalities were probably due to an insufficiency of testosterone, but may have been due to an inhibitory action of ovarian steroids.

The genital syndrome produced by androgenization has been likened to the freemartin condition, but Jost et al. (1973) has disagreed with this conclusion since only rarely is the freemartin condition manifest by gross masculinization of the external genitalia.

(3) Behavioural Modifications.

Short (1974) observed that all androgenized female offspring displayed male urination postures, regardless of time of exposure to testosterone, from 20 days onward. Though the present investigation did not set out to study specific behavioural attributes, it was noted that the androgenized females displayed female urination postures, a finding in direct contrast to that of Short (1974).

Aggressive tendencies of androgenized dams were reported by Short (1974) and observed in this study, supporting the concept that androgens are responsible for the initiation of many behavioural patterns.

Sexual behaviour of androgenized offspring has been studied by Short (1974) and Clarke et al. (1976). Irregular oestrous cycles have been observed in some animals androgenized between days 30 to 100 in utero, but most offspring did not exhibit oestrous cycles. Przekop et al (1974) observed no alteration of later sexual behaviour in ewe offspring androgenized from day 84 of foetal life. Short (1974) and Clarke et al. (1976) observed also that some androgenized ewes actually displayed male sexual behavioural characteristics. This evidence suggests that adult behavioural patterns may also be modified by the foetal exposure to testosterone, and indeed, this steroid may be necessary in the male foetus in order to ensure normal behavioural patterns later in life.

(4) Maternal Modifications.

Although no abnormal incidence of dystocia was noted in the current study, Short (1974) reported a "high incidence" of dystocia

from his small flock, and Alifakiotis et al. (1976) determined a statistically significantly greater incidence of foetal loss during gestation following both intrauterine and intramuscular administration of the steroid. The latter authors suggested that conversion of androgen to oestrogen within the placenta may have been a causative factor. Such conversion could have interfered with the endocrine control of parturition and have caused the dystocia reported by Short (1974). Short employed treatment regimes which elevated maternal testosterone levels beyond parturition. Lack of udder development reported by Short (1974) was countered by removal of implants prior to parturition. In the present study, maternal androgen levels returned to normal approximately three weeks after the final injection and hence interference with gestation, parturition and lactation was not evident.

(5) Hormonal Output

Prenatal exposure to androgen significantly depressed basal LH levels in both male and female offspring which suggests that depression of hypothalamic hypophysiotrophic function or of pituitary gland activity had occurred. Since a close quantitative relationship between LH and testosterone had been demonstrated (Experiments 1 and 4) particularly as the ram progressed through puberty, it was likely that the significant depression of plasma testosterone levels was secondary to the depressed LH output.

Prenatal androgen exposure however, did not significantly alter pituitary LH output following GnRH administration indicating that the depression of basal LH output observed was due to depression of hypothalamic centres controlling pituitary LH secretion rather than to direct modification of the pituitary gland. Impaired hypothalamic GnRH synthesis and/or release probably caused the lowered pituitary LH release as it has been shown (Experiments 2.1 and 2.2) that output of this gonadotrophin is GnRH dose dependent. Androgen in the early foetus may have altered the number of GnRH-producing neurons or steroid feedback receptors. Alternatively, hypothalamic steroid receptors may have been sensitized to later steroid production and hence small quantities of testosterone may have exerted a greater inhibitory effect on GnRH release in androgenized rams. The sensitivity of feedback receptors has been shown to decrease at puberty (Smith and Davidson, 1967) indicating that sexual neuroendocrine feedback receptors may

be labile components at least at some stages of maturation.

Testosterone production by the male foetal gonads begins as early as 30 days in utero (Attal, 1969). Thus the effect of exogenous androgen in suppression of later LH output must be related either to a greater dose of steroid reaching the hypothalamus than would be found in the normal male, or to exposure to androgen at a very early age, before endogenous production begins. Since exposure was commenced at day 20, the period between day 20 and 30 may be critical in determining the later hormone production.

It has already been suggested (p194) that the reduced testosterone production in androgen treated males was secondary to a diminished LH output, but also it was possible that a permanent suppression of numbers or steroidogenic capacity of Leydig cells had occurred. However, if the decreased testosterone output was due to this alone, the levels of LH would be expected to be elevated. In fact, LH levels were depressed. Though lower testosterone and LH output was not related to body weight this relationship may be misleading since it may be suggested that lowered testosterone production may have resulted in a lower body weight.

Experiment 5.2 suffered the disadvantage that insufficient treated males were available until 30 weeks of age. It was considered undesireable to employ treated males from Experiment 5.1 before 30 weeks in view of the lack of knowledge of long term effects of periodic GnRH injections, and hence this procedure may have reduced the validity of results in Experiment 5.1. At 6, 14 and 22 weeks, the single animal of this group which was sampled produced similar quantities of LH to normal ram lambs, and at 30 weeks, output from four animals sampled was not significantly different from that of normal rams. This evidence suggested that prenatal androgenization did not alter responses to GnRH in males. No difference in LH output was observed between treated and normal females. Castrated treated males produced significantly greater quantities of LH, presumably because of the lack of endogenous testosterone production which normally inhibits pituitary secretion (Pelletier, 1976).

Experiment 5.3 served to demonstrate that prenatal androgenization did not significantly alter the response to removal of the gonadal steroid feedback in the female. The increase in plasma levels of LH and the greater response to GnRH were due to removal of the negative feedback effects of ovarian steroids following ovariectomy. Since

gonadectomy also significantly elevated plasma LH levels in castrated treated males, it can be suggested that prenatal androgenization did not interfere with feedback receptor mechanisms in either sex. Hence it may be postulated that the activity of prenatally administered androgen was to depress metabolic processes in hypothalamic GnRH producing neurons, ultimately resulting in the depression of hypothalamic GnRH output, and decreased plasma LH concentrations.

CHAPTER VIII

GENERAL DISCUSSION AND CONCLUSIONS

Aspects of LH, testosterone and prolactin secretion in rams, with particular emphasis on the succession of hormonal events during sexual maturation, are described in this thesis. Three approaches were adopted to investigate secretion of these hormones : longitudinal studies of hormone output; acute studies of secretion profiles; and delineation of pituitary and subsequent gonadal responsiveness to GnRH administration.

Experiments 1 and 2 were designed to provide information about hormone secretion in adult rams, and to provide indices for the attainment of adult hormone secretory patterns and hormonal interrelationships by sexually developing rams. Experiment 2 also facilitated selection of the dose of GnRH and sample collection sequences employed in later experiments. An initial study on young animals (Experiment 3) commenced at birth and continued for 32-35 weeks, thus spanning the period during which spermatogenesis was established and progressing through the first breeding season for young rams under natural conditions. Supplementary acute studies of secretion profiles and responses to repeated GnRH injections (Experiments 4.1 and 4.2) utilized rams during prepubertal, pubertal and late pubertal phases.

Experiment 5 was undertaken to examine some aspects of sexual differentiation in sheep and was considered to be an extension of studies on endocrine parameters during puberty. Females were included to highlight interactions between the genetic sex and the foetal endocrine environment. Testicular developmental parameters of androgenized offspring were not investigated as the animals were required for other studies which extended beyond the termination of this experiment.

1. HORMONE SECRETION PROFILES

Rhythms of reproductive hormone secretion have been known since the recognition of the gonadotrophin-steroid basis of the menstrual cycle in the early 1930's (Bogumil, 1973). In the male, four

hypothalamo-pituitary-gonadal axis rhythms occur: sexual developmental, seasonal, circadian and short-term (ultradian) rhythms (Vermuelen et al., 1973). Though seasonal variations of reproductive parameters have now become established (Lincoln, 1976a), developmental, circadian and ultradian rhythms have been less extensively examined.

Prior to recent intensive studies, short-term variations in plasma hormone levels tended to be overlooked because low sampling frequencies, and conventional "steady state" concepts of endocrine control systems overshadowed their existence (Weitzman et al., 1975), hence widely discrepant hormone levels often were rejected or regarded as unimportant (Oatley, 1971).

Experiment 1 utilized a 20 min sampling interval and demonstrated conclusively that LH and testosterone were secreted in a pulsatile manner in adult rams, a finding in accord with other recent reports (Katongole et al., 1974; Purvis et al., 1974; Sanford et al., 1974a; Barrell, 1976; Lincoln, 1976a; Schanbacher and Ford, 1976). Pulsatile secretion of LH and testosterone also has been shown in young rams (Experiment 4.1 and Sanford, 1974).

The striking summer-winter difference of prolactin secretion profiles (Experiment 1) indicated that the hormone concentration changes in plasma observed in longitudinal studies (Pelletier, 1973; Barrell, 1976) reflected not only changes in baseline levels, but also alterations in the manner of secretion: in the case of prolactin, from tonically high to low but pulsatile output. An experimental approach combining longitudinal studies with frequent (e.g. monthly) secretion profile studies would be useful to elucidate the relationship between mean basal levels and short-term secretion profiles of hormones displaying seasonal variations of plasma levels.

Prolactin secretion profiles in 6, 14 and 22 week old rams (Experiment 4.1) did not differ significantly though the intervals between samplings were short compared with the six month interval used for seasonal comparisons in Experiment 1. It was noted however, that prolactin levels in the younger rams fluctuated more markedly than levels in adults at the same time of the year, though this may have been due to venepuncture sampling of the younger animals.

Clearly the secretion of prolactin in both adult and young rams requires further investigation. Secretion profile studies at close

intervals from birth under natural conditions, together with investigations employing artificially reversed and constant long and short daily photoperiods, and ultimately experimentation with prolactin inhibitory and stimulatory factors, should yield a greater understanding of prolactin secretion in rams. Studies of spermatogenesis and of the reproductive tract in conjunction with lighting and pharmacologically-induced changes in prolactin levels would also aid the determination of the physiological role of prolactin as a reproductive hormone in the sheep.

The significant quantitative relationship between episodic peaks of LH and subsequent testosterone peaks is important. Since the output of LH was in turn quantitatively controlled by GnRH (Experiment 2), it could be postulated that a quantitative hormonal amplification system emanates from the hypothalamus. The character of episodic LH spikes compared with elevations resulting from peripheral GnRH administration suggested that only a small quantity of GnRH from the hypothalamus was required and that its dissemination from the pituitary gland was rapid. Cannulation of pituitary portal blood vessels and measurement of portal blood GnRH concentrations, together with studies of intraportal administration of GnRH and measurement of peripheral LH levels, would give basic data on the dynamics of hypothalamic control of pituitary LH output in rams.

The physiological rationale for episodic output of hormones remains an enigma. Oatley (1971) suggested that pulsatile output may enhance control system integration. Indeed, episodic release may allow a more precise command of steroid output since testosterone release in response to more prolonged elevations of LH following GnRH administration (Experiment 2) was not consistently quantitative (though LH levels often were in excess of endogenous LH peak levels), whereas testosterone release following episodic LH spikes was. The target organ may respond maximally to periodic hormonal stimuli and the resulting episodic testosterone release may in turn provide a more accurate index of testicular steroidogenesis at the hypothalamic integrating centres than would a constant level of hormone. Further investigation of the nature and control of pulsatile hormone release and receptor-hormone dynamics of the pituitary and hypothalamus are necessary for an understanding of these basic neuroendocrine mechanisms.

As suggested by Oately (1971), pulsatile release of hormones exemplifies the dynamic nature of neuroendocrine systems and highlights the lack of known quantitative laws which describe such activities within an organism, and distinguishes biological systems from purely physical or chemical phenomena which are governed by strict mathematical relationships.

2. SAMPLING-INDUCED ELEVATIONS OF HORMONE LEVELS

If plasma hormone estimates are to be employed for diagnostic or experimental purposes, or for studies of hormone secretion, the importance of possible sampling-induced hormone elevations must be clarified.

Results of Experiments 3.2 and 4 indicated that sampling-induced elevations of LH and prolactin concentrations in plasma were real entities. Sampling-induced elevations of plasma prolactin levels in sheep are widely accepted (Davis, 1972; Barrell, 1976). In gilts, transportation-induced oestrus, oestrus synchronization and advancement of puberty discussed by Nalbandov (1976) may have been due to a stress induced elevation of LH, but there are no reports of sampling or stress induced elevations of LH in the sheep. Apparent discrepancies between results of Experiments 4.1 and 4.2 suggested that the use of multiple estimates of hormone concentrations dampened the effects of sampling-induced elevated LH levels which appeared to occur more frequently at 6 weeks of age than at 14 or 22 weeks. Thus interpretation of results could have differed depending on the number of samples collected. However, the LH output in response to GnRH injection during puberty (Experiment 3.3) closely followed the pattern of changing basal LH levels (Experiment 3.2), suggesting that the highest levels at 6 weeks were a real phenomenon rather than an artifact of sampling-induced origin. The pattern of basal LH levels observed in Experiment 3.2 was similar to that reported by Courot (1974).

Results suggesting sampling-induced elevation of hormone levels clearly indicated the desirability of an experimental approach designed to assess and overcome factors which may influence the output of the hormones under investigation. Research comparing venepuncture and remote catheter collection of samples during a

variety of stressful procedures such as intensive handling and noise are needed to determine both the period during which sampling-induced elevations occur and the extent to which such elevations influence subsequent hormone output. Similarly, measurement of the hormone or a metabolite in a body fluid pool such as milk or urine may be useful in overcoming any effects of pulsatile hormone output, or sampling-induced secretion, when an estimate of the mean hormone level is required.

However, the practicability of accounting for pulsatile and sampling-induced hormone secretion is difficult and appropriate sampling regimes and techniques must be devised to suit the experimental design. From longitundinal studies which employ large numbers of animals, mean hormone levels from single samples approach the theoretical population mean provided no sampling-induced elevation has occurred, but to examine short term profiles or mean levels within an individual, multiple samples are necessary. Peterson (1977), using the secretion profile data from Experiment 1, determined that at least 10 blood samples must be collected at intervals throughout the day and assayed to provide estimates of mean daily LH and testosterone levels within 10% of the 24 hour mean LH or testosterone level calculated from all samples collected (73 during the 24 hour period). The number of prolactin estimates required to provide an estimate of mean daily prolactin levels differed depending on whether secretion was constant as seen during the summer period, or pulsatile as occurred during the winter sampling period.

When a sampling-induced elevation of hormone output is observed or suspected, three alternative types of approach are available: (a) use of sampling methods which do not induce hormone elevations, (e.g. remote catheter sampling); (b) collection of multiple samples and disposal of early ones (for LH and prolactin, only the first few samples were affected), or use of a sham sampling procedure during the period of susceptability; or (c) use of a pharmacological stimulus which produces a response unaffected by the sampling and handling procedures (GnRH may prove useful in this regard). A further possibility is that animals be trained to adapt to the stresses associated with sampling as it has been suggested that animals so trained may not display induced elevations of hormone secretion (Sitarz et al., 1977), though this needs to be investigated to provide further evidence for such a claim.

3. STUDIES OF HORMONAL RESPONSES TO GnRH

Investigation of LH and testosterone output in adults (Experiment 2) in response to GnRH has proved that administration of GnRH provides a useful tool for testing both pituitary and gonadal endocrine functions.

In young animals the progressive increase in testosterone secretory response following GnRH administration during sexual maturation, despite a corresponding decline of LH output, showed major functional changes in both the pituitary gland and gonads. Rising testosterone output probably reflected a combination of increased testicular steroid content (Skinner et al., 1968), increased Leydig cell numbers (Sapsford, 1962) and possible progressively increased Leydig cell receptor formation (Odell and Swerdloff, 1976), while the decreased and delayed LH output probably was a direct result of the negative feedback effects of the elevated testosterone levels (Galloway and Pelletier, 1975). Artificial induction of a range of plasma testosterone levels followed by GnRH injections in castrated rams would clarify the respective negative and positive activities of testosterone and GnRH on the pituitary gland.

Since biphasic and monophasic output responses have been observed in rams following high and low GnRH doses, respectively, (Experiment 2 and Bremner et al., 1976) the dose chosen for an experiment should be appropriate to the requirements of the experiment. Linear increases in LH output response to logarithmic increments of GnRH dose (Experiment 2) indicated that lower doses should provide an index of pituitary sensivitity to GnRH, whereas higher doses should indicate the capacity of the pituitary gland to secrete LH.

A finding of practical importance was that there were significant between-animal differences in the LH and testosterone output responses following GnRH administration (Experiment 2). Hence GnRH may prove useful in the future for determination of differences between individuals for purposes of prediction of fertility or fecundity, or as an index for the prediction of other hormone-related reproductive parameters.

Repeated GnRH injections were used in Experiment 4.2 but did not add greatly to the understanding of pituitary function during puberty, though it was evident that large quantities of LH were

released even prepubertally. Therefore it is unlikely that the capacity of the pituitary to produce LH is a factor limiting to the onset of puberty.

Though many potential applications for GnRH in animal production have been suggested (Convey, 1973; Hoffman et al., 1975) (See Chapter 1, p 10), long term effects of the hormone are limited by its short half-life, the short half-lives of the hormones ultimately secreted following its administration, and the non-availability of slow-release formulations. However, potentially advantageous applications of GnRH have recently been indicated by Schanbacher and Lunstra (1977) who reported increased testosterone levels in plasma, greater mating activity, and increased testis size and semen quality following twice-daily injections of this hormone during the non-breeding season.

It is pertinent to note that weekly administration of GnRH did not significantly influence any of the parameters studied in Experiment 3.2, thus investigations using weekly or less frequent doses of GnRH may be performed without permanently influencing such parameters. Repeated use of the same individuals in longitudinal studies such as Experiment 3.2 and 3.3 would have simplified the experimental designs considerably.

4. SEXUAL MATURATION IN RAMS

Though some earlier studies with rams have involved hormone measurements (Lindner, 1961; Skinner et al., 1968), it was not until recently that plasma hormone profiles during puberty have been recorded (Crim and Geschwind, 1972a,b; Galloway and Pelletier, 1974; Sanford, 1974; Cotta et al., 1975; Courot et al., 1975; Ravault and Courot, 1975; Lee et al., 1976a,b). No endocrine studies of developing Romney rams have yet been reported, while few previous authors have attempted to relate reproductive organ development to plasma hormone levels.

Hormonal changes delineated by this investigation during sexual maturation in rams can be summarized:

- Basal LH concentrations increased until approximately six weeks of age, and generally decreased during the period of most rapid testicular development (10-18 weeks).

- LH secretion was pulsatile and indistinguishable from that of the adult.

- Pituitary LH output in response to single injections of GnRH decreased from 6 to 32 weeks of age, but following repeated injections, output of LH was greatest during the pubertal phase (14 weeks).

- The time of occurrence of LH peaks following GnRH injection was delayed as sexual maturation progressed.

- Basal plasma testosterone concentrations increased steadily from the day of birth.

- Testosterone levels rose following each LH spike at age 14 weeks and older, but in younger rams not all LH spikes were succeeded by testosterone elevations.

- Quantitative relationships between LH spikes and testosterone peaks were established by 14 weeks of age.

- Testosterone output following both single and repeated injections of GnRH increased as puberty progressed.

- Basal plasma prolactin levels followed a pattern similar to the seasonal pattern observed in adults.

Complementary findings of others can be summarized:

- Basal LH concentrations increased until approximately 70 days then decreased (Courot, 1974; Courot et al., 1975).

- Basal testosterone levels increased steadily during puberty (Courot, 1974; Courot et al., 1975; Lee et al., 1976a)

- Pituitary content of LH increased until 70 - 100 days and remained constant thereafter (Skinner et al.,1968; Courot et al., 1975).

- Gonadal testosterone content (Skinner et al., 1968) and Leydig cell numbers (Sapsford, 1962) increased with testis growth.

- Pituitary LH output in response to high doses of GnRH increased from weeks 1 to 20 (Galloway and Pelletier, 1974).

- FSH concentrations increased in parallel with LH levels during the first few weeks of life (Lee et al., 1976a).

- Both gonadotrophins are essential for pubertal testicular development in the ram (Courot, 1967).

An important supplementary observation was that numbers of Leydig cell receptors increased under the influence of FSH in rats, and that prolactin and growth hormone may augument the activities of this gonadotrophin (Odell and Swerdloff, 1976).

Thus the following model outlining the major endocrine events of puberty in rams is suggested:

An early increase in both LH and FSH output probably provides the basis for the onset of testicular development. The greater output of gonadotrophins probably results from the increase in volume of pituitary secretory tissue which occurs up to approximately 98 days (Skinner et al., 1968), and possibly as a result of gonadotroph receptor formation enhanced by GnRH (GnRH may stimulate gonadotroph receptors in a manner similar to FSH stimulation of Leydig cell receptors). Gonadotrophins stimulate Leydig cell steroidogenesis and receptor formation, possibly with the aid of prolactin and growth hormone. Development of an adequate Leydig cell receptor population may be the factor responsible for the establishment of regular and quantitative testosterone output in response to episodic LH elevations as puberty progresses, as the small numbers of receptors present prepubertally may be incapable of direct responses to episodic LH spikes. Together with this, the Leydig cell steroidogenic mechanisms may be promoted by the gonadotrophins. Hence, under pituitary hormonal influences, the quantity of Leydig cells and LH receptors increase and increased testosterone production and gonadal growth and development ensues. Increased testosterone output, despite falling LH output possibly is the result of increased Leydig cell and receptor numbers or receptor and steroidogenic activity. The fall in LH output and delays in responses to GnRH were probably due to increasing steroid negative feedback activity upon the pituitary gland and possibly the hypothalamus.

The role of the hypothalamus in initiation of sexual maturation is open to conjecture, although it is tempting to speculate that increasing output of GnRH may be the cause of both the increased pituitary LH content and gonadotrophin output. Probably the hypothalamus is responsible for pulsatile pituitary LH output, a characteristic of LH secretion at all ages studied. Earlier theories regarding altered sensitivities of the hypothalamus to steroids during the process of puberty(Smith and Davidson, 1967) are now being questioned in light of proposed Leydig cell receptor changes during sexual maturation (Odell and Swerdloff, 1976). Whatever its role in the onset of puberty, the hypothalamus is essential for the maintenance of pituitary function, and only further experimentation including measurement of hypothalamic and portal vessel GnRH following artificially manipulated steroid levels in vivo, further study of

hypothalamic feedback receptor dynamics *in vitro*, and *in vivo* hypothalamic lesion studies will yield answers as to its functions during puberty.

The establishment of spermatogenesis is the culmination of the series of events described, though the precise physiological activity of each hormone discussed remains to be elucidated. Study of systems subject to synergistic hormonal influences is difficult since deletion of one hormone may obscure the activities of its synergists. Quantitative studies of Leydig cell numbers and receptor formation, and *in vitro* studies of steroidogenesis of testes removed at intervals prior to and during puberty after artificial modification of plasma LH, FSH, prolactin, growth hormone and testosterone levels *in vivo*, would provide further information about cellular changes modulated by individual or groups of hormones during sexual maturation. Similar studies on sex cord and seminiferous tubule elements and pituitary endocrine cells would also aid clarification of the endocrine involvement in maturation of these components of the developing reproductive system. Immunohistochemical and autoradiographic techniques would be useful for receptor location and quantification. Thus further knowledge of factors associated with sexual maturation requires intensive investigations at both cellular and sub-cellular levels.

The demonstration that prolactin may aid the development of Leydig cell receptors, together with LH, FSH and growth hormone (Odell and Swerdloff, 1976), was a significant development in the elucidation of factors implicated in the onset of puberty. Evidence from Experiment 3.2, combined with findings of Ravault and Courot (1975), strongly suggested a role for prolactin in puberty in rams. In spring born rams, photoperiodic induction of higher prolactin concentrations in plasma earlier than observed in autumn born rams, may be an adaptation for the rapid onset of spermatogenesis prior to the first breeding season. Studies involving manipulation of daily photoperiod, as discussed earlier, should provide a better understanding of the integration of photoperiod with plasma prolactin levels during puberty.

Despite the emphasis on hormonal factors in the proposed concept of events during sexual maturation, direct attempts to relate anatomical and functional development of the reproductive system to changes in output of these hormones were not altogether successful.

No major index of development could be achieved by examining basal hormone levels (Experiment 3) although some parameters (testis and epididymal weights and epididymal spermatozoal reserves) were correlated with the increasing testosterone output following GnRH injection. The inability to relate anatomical development to endocrine changes probably was attributable to the requirements for long term exposure to the hormone for sustained organ growth and maturational changes. For example, the rising levels of LH during the first six weeks of life in ram lambs may be responsible for the major testicular changes which occur at 6 - 18 weeks of age. Abolition of the early LH rise by pharmacological means or perhaps by use of specific antisera against LH, may preclude pubertal testicular development. Inability to relate hormone levels to spermatogenesis has been noted in other studies (Lunenfield and Weissenberg, 1972; Paulsen et al., 1972), though de Kretser et al. (1974) reported correlation between FSH levels and spermatogonia, primary spermatocyte and spermatid numbers in men.

The present study was restricted by the non-availability of a radioimmunoassay for FSH as it is known that this hormone fulfils a vital role in the initiation of spermatogenesis (Courot, 1967). However the experiments designed for this thesis have provided considerable information about puberty, but upon completion of these experiments, improvements can be suggested. For a study of puberty, the availability of a more homogenous population of experimental animals is desirable. Animals used in Experiment 3.2 were born over a 4 week period and both twins and singletons had to be used since restricted numbers were available. Thus complications arose in an experimental design based on random selection for a study of changes occurring with age.

Wide variations in body weights were encountered at each age, a problem which was compounded by a drought and feed shortage during the study, and which often was reflected by large variations in testicular histological appearance in animals of the same age. Use of randomly selected animals as controls for treated groups consisting of animals sampled at each age (Experiments 3.2 and 5.1) may have influenced comparisons between control and treated animals. However, the variance of hormone levels within individuals was similar to the variance observed between animals as a result of pulsatile hormone output, hence this problem was of minimal importance. Longitudinal

studies on the same control animals would have been advantageous in determining hormone profiles within individuals, but use of randomly selected animals at each age possessed the advantage that results represented an estimate of the change in hormone concentrations in a larger population rather than in a small selected sample population in which progressive hormone levels may have been correlated. However, despite possible improvements to the experimental design employed, these studies have produced results which correspond closely with observations of others, and have greatly assisted the formulation of a model of endocrine events during sexual maturation in the ram.

Whatever the mechanism governing the process of sexual maturation, the recent words of Odell and Swerdloff (1976) highlighted the current status of understanding of factors controlling the onset of puberty"... the physiological events responsible for sexual maturation remain poorly understood in spite of considerable investigation and extensive speculation".

5. HORMONAL MODIFICATION OF ENDOCRINE PATTERNS DURING SEXUAL MATURATION

Although androgens are able to maintain spermatogenesis in the hypophysectomised adult (Lunenfield and Weissenberg, 1972), regular administration of testosterone to prepubertal and pubertal rams suppressed gonadal development and function (Experiment 3.2). Testosterone strongly inhibited FSH output in young rams (Crim and Geschwind, 1972b) and this possibly resulted in diminished synthesis of Leydig cell receptors (Odell and Swerdloff, 1976) thus limiting testosterone synthesis and release, testicular growth and associated spermatozoal production. The apparent total suppression of gonadal development in one subject is difficult to explain in view of the only partial suppression observed in three other rams which underwent the same treatment.

It has been claimed that GnRH offered a potential aid to advancement of puberty in domestic animals (Convey, 1973) since this hormone resulted in the release of both LH and FSH from the pituitary gland (Schally et al., 1972a). However, adult (Pelletier, 1976; Sanford et al., 1976) and prepubertal rams (Lee et al., 1976b) showed a variable output of FSH following GnRH injection; this fact, together

with the short half-lives of LH, FSH and testosterone, probably was responsible for the failure of weekly injections of GnRH to produce any detectable alteration in the course of sexual maturation (Experiment 3.2). However, Schanbacher and Lunstra (1977) have reported improved reproductive performance in rams after frequent administration of GnRH (see p 203), thus further investigations into the use of this hormone in the acceleration of puberty in rams are warranted. Development of slow-release formulations of GnRH may be a key development required for such experiments.

Investigations into the role of prolactin during puberty by methods discussed earlier in this chapter are indicated in view of reports that this hormone may act synergistically with other hormones such as growth hormone to promote testosterone production (Odell and Swerdloff, 1976), and in view of the findings relating to prolactin in rams in this study and by others (Ravault and Courot, 1976; Barrell, 1976). Ultimately, hormonal manipulation of puberty may involve the use of a number of hormones which possess synergistic activities in promoting various aspects of testicular development.

Early prenatal administration of testosterone to ewes has been shown to depress postnatal plasma LH levels in female offspring, and both LH and testosterone secretion in male offspring (Experiment 5). These results suggested that exposure to androgen restricted the later ability of the hypothalamus to produce GnRH and ultimately induce LH release, and indicated the likely importance of the hypothalamus in modulation of sexual development. Since LH levels were suppressed, it could be postulated that FSH output may also have been depressed, since GnRH induced output of both gonadotrophins to some extent. This offers a plausible explanation for depressed testosterone output since lack of FSH may inhibit Leydig cell receptor formation (Odell and Swedloff, 1976), and it was likely that pubertal development in prenatally androgenized subjects would have been delayed as a result.

Combined with evidence from other authors (Short, 1974; Clarke et al., 1976) Experiment 5 has confirmed that external anatomical sexual differentiation in sheep occurred between days 40 and 50 of gestation. A study using precisely timed administration of a testosterone preparation with a known duration of action, to large numbers of pregnant ewes followed by behavioural and endocrine studies

after puberty are needed to determine accurately the timing of hypothalamic differentiation in sheep. Investigation of the ability of other steroids such as oestrogens and progesterone to modify sexual development is a logical extension of this study, while further studies also are required to verify the depression of LH and testosterone in offspring which was observed in Experiment 5.1.

Though prenatal androgenization does not have immediate practical applications to the sheep industry, this technique offers a useful tool for studies of interactions between the genetic sex and the hormonal environment, and may prove a useful model for endocrine-related congenital anatomical abnormalities, as well as aberrent behaviour syndromes in man. By causing anatomical and behavioural abnormalities, androgenization may prove to be a useful method for the biological control or eradication of feral and noxious animals, provided the steroid can be given successfully by the oral route.

6. POSSIBLE APPLICATIONS OF THE FINDINGS OF THE PRESENT STUDY

Fundamental research providing a basic understanding of the long and short-term patterns of secretion of reproductive hormones is a prerequisite for determination of possible applications of hormones and hormone measurements for diagnosis of the causes of reproductive abnormalities such as anoestrus or aspermia, clinical treatment of reproductive disorders, determination of the physiological role of hormones, establishment of neuroendocrine control concepts, and the discovery of further concepts for hormone uses and hormone measurements. This study has highlighted problems of interpretation of data from hormones secreted in a pulsatile fashion and which may be influenced by factors including age, season and stress. The establishment of considerable fundamental data which may be applied to the future use of hormones and their measurement is a major potential benefit of the experiments undertaken for this thesis.

The advancement of the onset of puberty could have many advantages particularly in terms of efficient utilization of ram lambs as sires. Although good nutrition is imperative to the early attainment of puberty, and although ram lambs often can be used to a limited capacity as sires, endocrine acceleration of the attainment of satisfactory semen output and possibly libido, would enhance the

use of rams early in life, thus accelerating the progress of breeding and selection programmes. This study has provided a basis upon which to design experiments to test compounds for this purpose, and in particular has provided a basic understanding of the physiological actions of and responses to GnRH, a hormone which has shown promise in enhancing reproductive parameters in rams.

Hormone measurements may prove useful for purposes of selection of animals for reproductive parameters. Further investigations may yield relationships between hormone levels and fertility and fecundity potential of rams (Carr and Land, 1975), and may eventually enable the prediction of reproductive traits later in life, hence favouring early selection and preferential managemental procedures. GnRH may prove useful in tests of pituitary and gonadal function for these purposes. Findings from this thesis may also prove useful in other domestic species such as pigs and cattle used in commercial farming enterprises, and in the human.

This study proposes further areas of research involving: the role of prolactin in reproductive development and seasonality in rams; physiological studies designed to elucidate the cause of and reasons for pulsatile secretion of hormones; the use of hormones and hormone measurements as diagnostic and therepeutic aids in domestic animal reproduction; and the elucidation of cellular and subcellular changes which occur during the process of sexual maturation in rams. Although this research has not yielded information of direct and immediate practical benefit to the agricultural industry, it has provided a foundation of knowledge about the output of three hormones of importance in the ram upon which an understanding of the ability of the animal to produce spermatozoa can be constructed.

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Addenda:

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