IMMUNOMODULATION OF REPRODUCTIVE FUNCTION IN DOMESTIC RUMINANTS

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To Mel

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Chapter 4 The Effects of Active Immunisation against GnRH on Reproductive Function in Heifer Calves

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ABSTRACT

Active immunisation against GnRH inhibits reproductive function by inducing a hypo-gonadotropic condition associated with gonadal atrophy. Despite economic, ethical and environmental advantages of GnRH immunisation in cattle over conventional castration methods, the technology has not yet been commercially adopted. Primarily because of the requirement for numerous booster vaccinations because of the reversibility of physiological effects, the commercial efficacy of immunocastration is currently poor. However, neonatal GnRH immunisation in sheep can result in a permanent suppression of reproduction (Brown *et al.*, 1994; 1995; Clarke *et al.*, 1998). These findings and a study in pigs (Molenaar *et al.*, 1993) indicate that, the hypothalamic/pituitary gland unit (HPU) may be particularly susceptible to GnRH antibodies during a specific window of development in the pre-pubertal animal, but no long-term studies in cattle have been conducted. Therefore the primary objective of this project was to determine the effect of neonatal immunisation against GnRH in cattle. Beef cross bull (n=9; Chapter 3) and heifer calves (n=9; Chapter 4) were vaccinated against a newly developed (Pfizer®) GnRH construct vaccine at ~2, 6 and 13 weeks of age. Nine calves of each sex served as negative controls, receiving saline injections only. The GnRH vaccine had proved effective (Dr. A.R. Peters, personnel communication 2000) in inducing immune responses and reducing variation between animals in unpublished industrial studies, compared to earlier vaccines, and hence was reasoned to be capable of raising GnRH antibodies despite the relative immaturity of the neonatal immune system. Following vaccination, circulating GnRH antibodies and reproductive hormones, such as FSH (Chapters 3 and 4), testosterone (Chapter 3), progesterone (to assess onset of puberty) and oestradiol (Chapter 4) were measured and additional intensive serial bleeds were carried out to assess LH parameters up to and beyond puberty (puberty defined by testes circumference in bulls). Gonadal (antral follicles and testes growth) and accessory gland development was quantified throughout the trial using ultrasound scanning. Sexual behaviour (Chapter 3) was studied from 38 weeks of age, while an assessment of sperm quality (Chapter 3), and anabolic response to vaccination was also performed post-mortem (Chapters 3 and 4).

GnRH immunisation in neonatal calves did not permanently impair reproduction. A temporary suppression in reproductive function was evident through the disruption of pituitary gland function, as indicated by a reduction of LH pulse amplitude and mean plasma LH concentrations (Chapters 3 and 4). In addition, a reduction in medium-

sized follicle numbers, testes growth, plasma testosterone concentration, vesicular gland length and juvenile aggression occurred. Some beneficial anabolic effects were observed e.g., carcass composition grades. Changes all occurred subsequent to increased GnRH antibody titres in immunised cattle. Despite some evidence of prolonged effects on LH amplitude and circulating testosterone after anti-GnRH titres had dissipated, all inhibited parameters, except carcass quality, returned to levels comparable to control animals by 72 weeks of age. No treatment effects on FSH concentrations, large follicle numbers, reproductive tracts (post mortem) or peri- and post-pubertal behaviours were observed following treatment. Sperm morphological abnormalities tended to be more prevalent in GnRH immunised bulls.

A significant increase in GnRH antibody titres occurred at ~23 weeks of age (Chapter 4), this may have been a rebound in antibody titre, possibly caused by an anti-idiotype immune response (antibody response to GnRH antibodies), or due to significant maturational changes in immune function at this time causing a delayed response to vaccination. Alternatively a novel "auto-immune" response may have been detected, which if confirmed/repeatable might be incorporated into an immunisation protocol to act as a "self-booster". However, no previous reports of such an event have been published and further investigation is urgently required.

A more prolonged or permanent suppression of reproductive function may be possible following an earlier, greater and more sustained elevation of antibody titres during the neonatal period. Further development of GnRH vaccines and/or protocols (prime-boost, cytokine modulation vaccines, concomitant passive and active immunisation and pregnant cow GnRH vaccination), and studies of performance and GnRH antibody mechanism(s) of action in cattle are required. Chapters 3 and 4 provide a comprehensive study on pubertal development and neonatal GnRH vaccination, thus contributing significantly to knowledge in these fields. Currently, the vaccine used in this trial may be used to delay puberty in older calves or transiently suppress reproductive function to aid management.

The economical viability of animal production systems such as beef and lamb are closely related to rates of reproduction. The Fec B gene in ewes increases ovulation rate and litter size, possibly through the development of precocious follicles, which can switch their primary dependence from FSH to LH. As a result, more follicles are selected to continue growth to an ovulatory size. The precise mechanisms by which these processes occur have recently been shown to involve oocyte follicle

interactions (see section 1.1.5). Follicle development is modulated by GH/IGF and inhibin, however attempts to increase follicular development and ovulation through active inhibin immunisation alone have been variable and hence not commercially attractive. To develop successful protocols to induce twin ovulations in cows and ewes, without superovulation, a clearer, more details understanding of follicullogenesis is required. The objective of the current study was to better understand these mechanisms through investigating interactions of GH/IGF and inhibin in the ovary, follicle development, steroidogenesis, and receptor populations using an anoestrous sheep model.

Spring born Mule x Charolais ewe lambs were actively immunised (n=8) against porcine inhibin α-C 1-26 peptide conjugated to KLH in NUFCA (primary and 3 boosters (NUFA)), while 8 served as negative controls. Seven days following the final booster, the ewes were subdivided to give four groups: (1) controls + saline (n=4); (2) controls + rbGH (4ml s.c; 1mg. ml^{-1}; n=4); (3) inhibin immunised + saline (n=4); and (4) immunised + rbGH (n=4). Recombinant bovine growth hormone (rbGH) was given (i.m.) for 6 days. On day 4 GnRH (Receptal®; 1ml) was injected s.c, to all animals to initiate the beginning of a new follicular wave. Blood samples were collected fortnightly to measure inhibin antibody titres, IGF-I, FSH and steroids. On the seventh day ensuing slaughter serum antibodies and ovaries were harvested. Left ovaries were intended for ISH (mRNA for $P450_{arom}$) and/or immunohistochemical analysis. Follicles from right ovaries were dissected out, counted, measured and cultured in M199 at 37°C for 2 hours. Culture media was then assayed for oestradiol. Follicle shells were stored at –180°C for LH receptor binding studies.

This work reports on the influence of different treatments on follicle populations. All immunised animals produced antibodies, which bound to 125I-inhibin. Using ANOVA to compare treatments it was observed that, Inhibin immunisation significantly ($p<0.05$) increased the number of follicles >3.5mm in diameter, but did not affect the smaller <3.5mm population. In contrast, rbGH administration led to a significant ($p<0.05$) elevation of follicles <3.5mm, without increasing the >3.5mm follicle numbers. These findings are in agreement with previous research. The molecular studies of left ovaries are not presented herein as due to time constraints the work was not completed and is currently on going.

In conclusion, additional results of this study are required to meet the objectives of the experiment. Further research is required on dominant follicle selection if

superovulatory programmes in both livestock and humans are to be more precisely controlled and readily accepted.

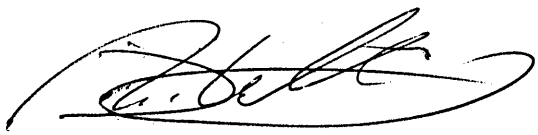
An increased twinning rate in beef suckler cows is acknowledged as an optimal means of improving production efficiency (Diskin *et al.*, 1987), and preliminary observations (Campbell, Webb and Gong, unpublished) suggests that following oestrous cycle synchronisation, a passive inhibin immunisation protocol gives a more predictable twin ovulation response than active inhibin vaccination. Therefore, using inhibin antiserum raised in ewes (Chapter5), the final thesis objective was to investigate the efficacy of a bolus and booster antiserum administration protocol in heifers and the effects of passive inhibin immunisation on hormone parameters (FSH and progesterone), folliculogenesis (*in vivo*) ovulation rate and conception.

Beef cross heifers (n=18; used earlier in Chapter 4 study) were randomly allocated to receive inhibin antiserum or NSS (controls). Following the Chapter 4 study, and 4 months summer break at pasture, heifers were considered to be exhibiting typical cyclic reproductive function based on hormone measurements and scanning of ovaries. Oestrous cycles were synchronised using 2 injections (i.m.) of PG 11 days apart and antiserum 'bolus' (108ml) was injected into the jugular vein 4 days after 2nd PG treatment. Subsequently, at 24h intervals for 5 days 14ml booster infusions were administered. Five days after ovulation, as confirmed by twice daily scanning, a 3rd PG was given and 72 and 96h later, heifers were A.I. Blood samples were collected every 8 hours from 2nd PG to 2nd A.I.

Dominant follicle number and ovulation rate was unaffected by treatment due to inadequate inhibin antibodies in antiserum compared with the earlier trial (Campbell, Webb and Gong, unpublished), however, recruited follicle numbers were enhanced in inhibin immunised heifers. In addition, further evidence that inhibin antibodies act at the ovarian level to enhance recruitment, or reduce atresia was provided, as FSH concentrations were similar between groups. Higher inhibin antibody titre may be necessary to over-ride the strong dominant follicle selection mechanism in heifers. In addition, the results of this and a previous trial demonstrate that a threshold inhibin antibody titre range is required to obtain a reproducible increase in ovulation rate on which future passive inhibin immunisation studies may be based. Finally, this approach of a bolus followed by subsequent small antiserum injections has successfully been shown to give a steady and consistent concentration of circulating inhibin antibody titres.

DECLARATION

I hereby declare that the studies undertaken herein, except where due to acknowledgement is made by reference, were the unaided work of the author. No part of this work has been previously submitted in candidature for another degree. I also acknowledge all assistance given to me during my preparation of this thesis.



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Abbreviations

π	Pi (approx 3.142)
^{125}I	Radioactive Iodine
3βHSD	3beta Hydroxy Steroid Dehydrogenase
A.I.	Artificial Insemination
AbT	Antibody titre
AKA	Also Known As
ANOVA	Analysis of Variance
ANS	8-Anilio-1-Naphthalene Sulphonic acid
APC	Antigen Presenting Cell
Arg	Argenine
BBB	Blood Brain Barrier
bGH	Bovine growth hormone
BMP	Bone morphogenetic protein
BMP15	Bone morphogenetic protein 15 (AKA; GDF9β)
BSA	Bovine Serum Albumin
BSE	Bovine Spongiform Encephalopathy
BVH1	Bovine Herpes Virus (Simplex 1)
C	Celsius
cAMP	Cyclic Adenosine Mono-phosphate
CD40	CD System number 40 receptor (expressed on B Cells)
CD86	CD System number 86 receptor (expressed on B Cells)
CG	Chorionic gonadotrophin
CH	Corpus Haemorrhagicum
CHAPS	3-[(3 Cholamidopropyl) dimethylammonio]-1-propane-sulphonate
CIDR	Controlled Intra-vaginal progesterone releasing device
CL	Corpus luteum
cm	Centimetre
CNS	Central Nervous System
CO2	Carbon dioxide
CON	Control
CPM	Counts Per Minute
CSP	Charcoal Stripped Plasma
DAG	Diacylglycerol
DAR	Donkey Anti-Rabbit
DASG	Donkey Anti- Sheep Goat
DC	Dendritic Cell
DDA	Dimethyl Dioctadecyl Ammonium Bromide
DEAE-D	Diethylaminoethyl-dextran
DF	Dominant Follicle
DNA	Deoxyribonucleic acid
DPX	DPX Mountant
E2	Oestradiol
EBSS	Earle's Balanced Salt Solution
eCG	Equine Chorionic Gonadotrophin
EDTA	Ethylenediaminetetra Acetic Acid
EGF	Epidermal Growth Factors
ESA	Equine Serum Albumin
FA	Freund's Adjuvant
FCA	Freund's Complete Adjuvant
FCE	Feed Conversion Efficiency
FecB	Booroola Fecundity Gene
FecXH	Hanna Gene Mutation

FecXI	Inverdale Gene Mutation
FGF	Fibroblast Growth Factor
FMD	Foot and Mouth Disease
FSH	Follicle stimulating hormone
FSH-R	Follicle stimulating hormone receptor
G	Gravity
GABA	Gamma Amino Butyric Acid
GDF	Growth Differentiation Factor
GDF9β	Growth Differentiation Factor 9β (BMP15)
GGE	Greenhouse-Geisser Epsilon
GH	Growth hormone (somatotrophin)
Glu	Glucine
Gly	Glycine
GnRH	Gonadotrophin-releasing hormone
H	Heat (oestrus)
h	Hour
His	Histamine
HOS	Hypo-osmotic Swelling
HOST	Hypo-osmotic swelling test
HPG	Hypothalamic-Pituitary gland-Gonadal (axis)
HPO	Hypothalamic/pituitary gland ovarian (axis)
HPU	Hypothalamic-pituitary gland Unit
HRE	Hot Recovery Estimates
HRT	Hot Recovery Totals
HSA	Human Serum Albumin
IFN-γ	Gamma Interferon
IgA	Immunoglobulin (A series)
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding protein
IGF-I/II	Insulin-like growth factor –I (or II)
IgG	Immunoglobulin (G series)
IL-12	Interleukin 12
IMM	Immunised
IMS	Industrial strength Metholated Spirit
IPS	Inositol Phosphates
ISH	In Situ Hybridisation
KDa	Kilo Dalton
KLH	Keyhole Limpet Haemocyanin
L.A.	Long Acting
Leu	Leucine
LH	Luteinising hormone
LH-R	Luteinising Hormone Receptor
m	Metre
MARC	Meat Animal Research Centre
ME	Median Eminence
MHC-I/II	Major Histocompatability – I (or II)
MLC	Meat and Livestock Commission
mm	Millimetre
mRNA	Messenger Ribonucleic Acid
MW	Molecular Weight
Na_2CO_3	Sodium Carbonate
$NA_2S_2O_5$	Sodium Metabisulphite
NaCL	Sodium Chloride
NaHCO3	Sodium Hydrogen Carbonate

ng ml^{-1}	Nanograms per Millilitre
NK	Natural Killer Cell
NRS	Normal Rabbit Serum
NSB	Non Specific Binding
NSS	Normal Sheep Serum
NUA	Non Ulcerative Adjuvant
NUFA	Non Ulcerative Freund's Adjuvant
NUFCA	Non Ulcerative Freund's Complete Adjuvant
O	Oestrus (heat)
O2	Oxygen
OE378	Oestrus Ewe 378 (high titre positive control inhibin antiserum)
oFSH	Ovine Follicle Stimulating Hormone
oLH	Ovine Luteinising Hormone
OV	Ovulation
OVA	Ovalbumin
OVX	Ovarectomy/ovarectomised
$P450_{AROM}$	P450 Aromatase
$P450_{SCC}$	P450 Side Chain Cleavage
PAPP-A	Pregnancy Associated Plasma Protein A
PBS	Phosphate Buffered Saline
PCGB	Phosphate Citrate Buffer with Gelatin
PEG	Polyethylene Glycol
PG	Prostaglandin
PGF	Prostaglandin (F series)
PGF2α	Prostaglandin F2alpha
PIs	Phosphoinositides
PO_4	Phosphate
Pro	Proline
Pyro	Pyrosine
QC	Quality Control
QS	Quillaha saponin (adjuvant)
RAS	Rabi Adjuvant System
rbGH	Recombinant Bovine Growth Hormone
rGH	Recombinant growth hormone
RT	Room Temperature
s.e.d	Standard error of differences
s.e.m	Standard error of mean
SAPU	Scottish Antibody Production Unit
Ser	Serine
TC	Total Counts
TGF-β	Transforming growth factor beta
Th1	Helper T Cell type-1
Th2	Helper T Cell type-2
Trp	Triptophan
Tyr	Tyrosine
VG	Vesicular Gland (AKA; Seminal Vesicles)
WHO	World Health Organisation

CHAPTER ONE

REVIEW OF LITERATURE

1.1 ENDOCRINOLOGICAL CONTROL OF REPRODUCTION

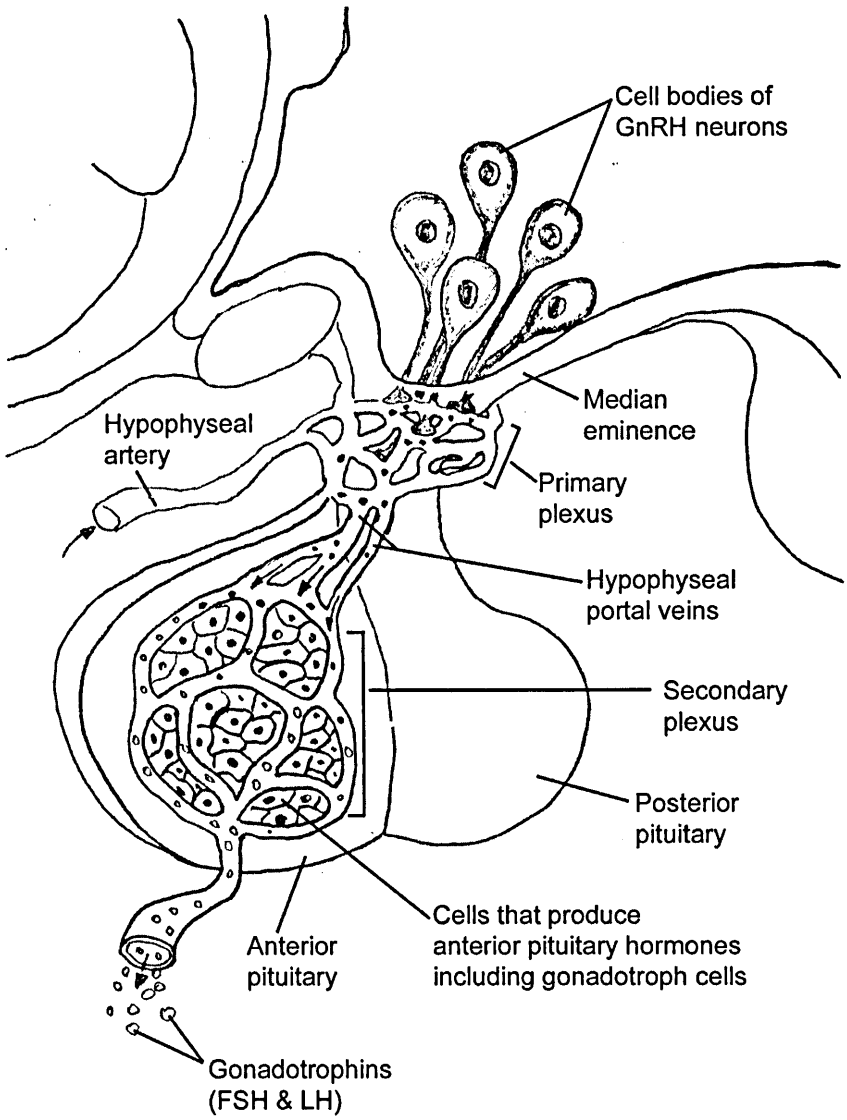
Spermatogenesis and folliculogenesis are controlled by complex relationships between the hypothalamic-pituitary-gonadal (HPG) feedback axis (Figures 1.3 and 1.9), together with intra-gonadal steroids, growth and extra-gonadal factors (see Sharpe, 1990; Campbell, Scaramuzzi and Webb, 1995; Campbell *et al.*, 1999a).

1.1.1 GnRH and Hypothalamus

Gonadotrophin releasing hormone (GnRH) is a low molecular weight (1183) decapeptide released from the basal hypothalamus (Fraser, 1980), and is highly conserved among mammals (Schally, Arimura and Kastin, 1973). GnRH was first isolated and characterised from sheep (Burgus and Guilleman, 1970) and pig hypothalami (Schally *et al.*, 1971), and was shown to have the same structure in cattle (Currie *et al.*, 1971). GnRH was synthesised by Matsuo *et al*, (1971) and shown to possess the same biological activity as hypothalamic extracts. The GnRH peptide is acknowledged as the primary controlling hormone of reproductive activity in both sexes, as it affects gonadotrophins, synthesis and release, and steroids and gonadal proteins either directly or indirectly. GnRH synthesis and secretion is regulated by numerous neurotransmitters from higher brain centres (see Smith and Jennes, 2001; Goodman *et al.*, 2002), as well as by ovarian (Williams *et al.*, 2000; Tsai and Legan, 2002; Looper *et al.*, 2003) and testicular steroids (Tilbrook *et al.*, 1991; Hileman and Jackson, 1999). Gonadal steroids act both centrally to control GnRH secretion and also directly at the pituitary level to modulate gonadotroph responsiveness to GnRH (Evans *et al.*, 1991a; Williams *et al.*, 2000; Looper *et al.*, 2003; see Evans *et al.*, 2002; McNeilly *et al.*, 2003).

GnRH has been detected in several areas, both within the hypothalamus and in other regions of the brain, with high concentrations in the mid-brain, appreciable amounts in the cerebellum, brainstem and cerebral cortex (Wilber *et al.*, 1976). In addition, GnRH producing cells and GnRH receptors were identified in the medial amygdala

Figure 1.1 Schematic diagram of the hypothalamic/pituitary gland portal system. Key: dark dots represent GnRH and white dots gonadotrophins.



and accessory olfactory bulb (Lehman *et al.*, 1986), the gonads (Hsueh and Jones, 1981), placenta (Khodr and Siler-Khodr, 1980; Iwashita, Evans and Catt, 1986) and lactating mammary glands (Seeburg *et al.*, 1987). Hypothalamic function is influenced by hormonal and neural signals from extra-hypothalamic tissues. Furthermore, the mid-brain has the ability to increase or decrease gonadotrophin output depending on the site of stimulation (Sharpe and Fraser, 1978).

1.1.1.1 GnRH synthesis

GnRH synthesis occurs in the cell bodies of hypothalamic neurons (Thompson Jr., 2000), which are found in relatively high concentrations such as the arcuate nucleus (McCann, 1983), the medial pre-optic area (Lehman et al., 1986) and

suprachiasmatic nuclei. These tissues are therefore considered to be the major sites of GnRH synthesis (Silverman, 1987). GnRH is synthesized as a large precursor molecule, made up of 92 amino acids in the human (MW 10,000; Seeburg and Adelman, 1984). The GnRH precursor encodes a signal sequence (23 amino acids), the GnRH peptide sequence (Pyro-Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2), a cleavage sequence (Gly-Lys-Arg) and a long (56 amino acid) C-terminal peptide called GnRH associated protein (GAP; Seeburg and Adelman, 1984; Adelman *et al.*, 1986; Seeburg *et al.*, 1987). Following its synthesis, GnRH is transported via the respective long and short axons, to the terminal buttons, which synapse on the vessels within the median eminence (Kainer, 1993; Thompson Jr. 2000). It is during axonal transport that the pro-hormone is cleaved (Seeburg and Adelman, 1984).

1.1.1.2 GnRH release

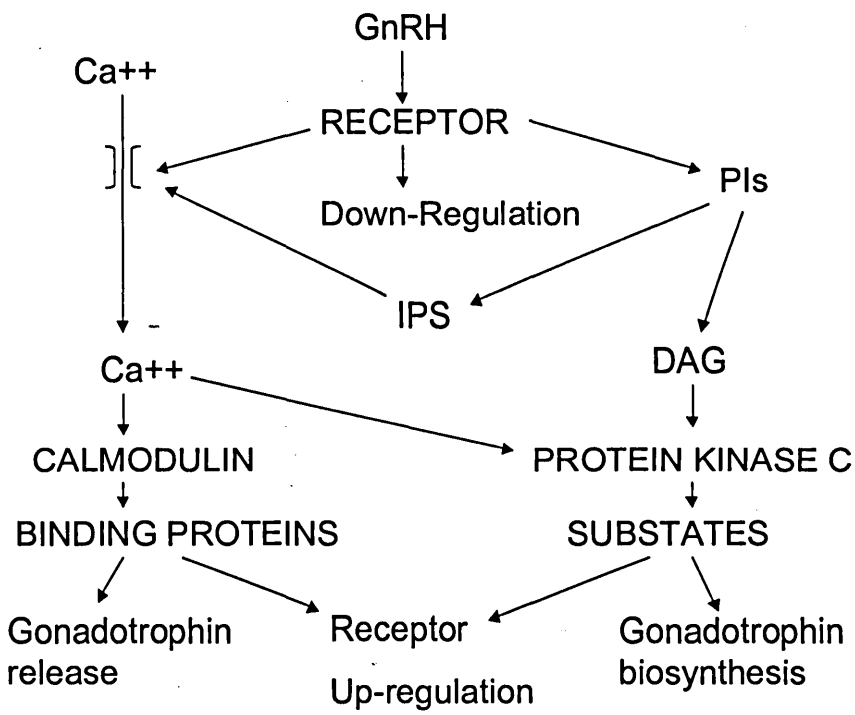
Excited GnRH neurons secrete the peptide from granules into the extra-cellular space, where GnRH diffuses into the capillaries and travels via the hypophysial portal system to the sinusoidal capillary plexus within the adenohypophysis. Here GnRH exits the capillaries and binds to receptors on the anterior pituitary gland cells known as gonadotrophs. Pioneering studies involving hypophyseal portal blood sampling from ewes conclusively showed that GnRH release is pulsatile (Clarke and Cummins 1982; Levine *et al.*, 1982), i.e., relatively large amounts of GnRH are released over a short period of time, followed by a period of low secretion and subsequently another pulse of GnRH.

1.1.1.3 GnRH receptors

The response of pituitary gonadotrophs to GnRH correlates directly with the concentrations of GnRH receptors on the cell surface (Norwitz *et al.*, 2002). On binding to GnRH, G-protein coupled receptors on gonadotrophs microaggregate and are internalised (Hazum and Conn, 1988; D'Occhio, Aspden and Trigg, 2001; McArdle *et al.*, 2002). The second messenger signal transduction pathway is activated (Figure 1.2) inducing the release of stored LH and FSH and *de novo* synthesis of both gonadotrophins (Huckle and Conn, 1988; Hawes *et al.*, 1992). The GnRH receptor population increases with exposure to GnRH (see Clayton and Catt, 1981; Popkin and Fraser, 1985). Endogenous GnRH is essential for the maintenance and expression of pituitary gland receptors (Fraser *et al.*, 1982; Popkin and Fraser, 1985; Brown *et al.*, 1994). In sheep, GnRH receptor mRNA appears to be increased by rising follicular phase oestradiol (Brooks and McNeilly, 1994). However, further

investigations are required to confirm the relationship between GnRH receptor expression and oestrogen receptors on gonadotrophs (see McNeilly *et al.*, 2003).

Figure 1.2 Pathways of GnRH action. (PIs = phosphoinositides; IPS = inositol phosphates; DAG = diacylglycerol) (adapted from Huckle and Conn, 1988).



1.1.1.4 GnRH priming

After an initial exposure of the pituitary gland to GnRH, an additional exposure results in a much larger release of LH. Several researchers have explained the phenomenon whereby small pulses of GnRH prime the pituitary gland to respond with increased efficiency to subsequent larger pulses by stimulating GnRH receptor formation (Foster, 1978; Padmamabhan, Kesner and Convey, 1981; Clayton, 1982; Catt *et al.*, 1985). GnRH stimulates gonadotroph expression of its receptor and promotes gonadotrophin synthesis (Fraser *et al.*, 1974). For example, a reduction in gonadotrophin α and β-subunit mRNA, pituitary LH and FSH content, and GnRH receptors was recently reported in three to four year old ewes, which had diminished GnRH secretion following neonatal immunisation against GnRH (Clarke *et al.*, 1998). Gonadotrophs, like many hormone-responsive cell types, can become desensitised to GnRH or GnRH analogues, following long-term administration (see McArdle *et al.*, 2002). Desensitisation can result in virtually the complete cessation of episodic gonadotrophin release, with a concomitant decrease in sex steroid production

(chemical castration; Evans and Rawlings, 1994; Gong *et al.*, 1995; D'Occhio, Aspden and Whyte, 1996; Chandolia, Evans and Rawlings, 1997; Jimenez-Severiano *et al.*, 1998; 2003; see D'Occhio and Aspden, 1999).

1.1.2 Gonadotrophins and the Anterior Pituitary Gland

FSH and LH are synthesized in the basophil cells of the anterior pituitary gland. Gonadotrophin structure was described first by Reichert (1962) and Reichert and Jiang (1965). Both are glycoprotein hormones (MW 28,000) consisting of two glycosylated polypeptide (α and β) chains. The α chains (92 amino acid) are identical in both molecules, but the β chains differ. The FSH and LH β chains consist of 111 and 121 amino acids respectively. It is the characteristics of the β chain, which confers the hormones functional properties. The two sub-units are bound non-covalently and they become inert when dissociated. Vaitukaitis *et al.* (1976) demonstrated that re-association of the units could be achieved and biological activity restored by incubating equimolar concentrations of the isolated complementary subunits. Later it was proposed that the two sub-units are synthesised separately within the pituitary gland (Landefeld and Kepa, 1979; Godine, Chin and Habener, 1980). In all species examined to date, both LH and FSH are present in the same gonadotrophs, however it is not clear whether all gonadotrophs release both FSH and LH (see McNeilly *et al.*, 2003). After synthesis, LH predominantly enters storage granules and is released in response to GnRH stimulation. In contrast, FSH release is dependent on the synthesis and hence gene expression of FSH β-subunit. Alternate intracellular packaging and secretory pathways explain how gonadotrophs release LH and FSH differentially, and why basal LH levels, are not inhibited by chronic GnRH agonist (Gong *et al.*, 1996a; Jimmenez-Severiano *et al.*, 2003) and GnRH immunisation (Finnerty *et al.*, 1996), while these treatments inhibit LH pulse frequency and amplitude (McNeilly, personal communication 2003). It is still not known how gonadotrophs respond to multiple positive and negative signals to achieve the differential pattern of gonadotrophin secretion that is essential to reproductive function (see McNeilly *et al.*, 2003).

1.1.2.1 Gonadotrophin release

By sampling hypophseal portal blood in conscious sheep, Clarke and Cummins (1982) confirmed the "neurohumoral theory" of Harris (1955) by showing that GnRH secretion is pulsatile and each pulse of GnRH induces a pulsatile secretory episode of LH. Proving Harris's theory had been difficult due to the short half-life (4-7 minutes) of GnRH in the peripheral circulation. Clarke and Cummins (1982) discovered that

each pulse of LH preceded a GnRH pulse. However, GnRH pulses were not necessarily followed by an LH episode (see Clarke, 2002).

Interestingly, FSH seems to be differentially regulated by GnRH. For example, after hypothalamic disconnection from the pituitary gland in sheep (Clarke, Cummins and de Kretser, 1983), GnRH immunisation (active; Clarke, Fraser and McNeilly, 1978; McNeilly, Jonassen and Fraser, 1986; passive; Fraser and McNeilly, 1983), or down regulation of GnRH receptors with GnRH agonists (ram; Lincoln and Fraser, 1987; heifer; Gong *et al.*, 1996a) FSH secretion continued for days, but at a decreased rate as FSH synthesis does depend on GnRH input (Mercer *et al.*, 1989; Gong *et al.*, 1995; 1996a), whereas LH secretion had stopped within 48-72h (Findlay and Clarke, 1987).

Measurements of metabolic clearance of circulating FSH in sheep (Akbar *et al.*, 1974; Findley and Cumming, 1976) support the notion that the changes in FSH secretion that occur throughout the cycle, are due to changes in secretion of FSH by the pituitary gland and not removal of hormone from blood. Clarke *et al.* (1986) concluded that, unlike LH, FSH secretion in sheep is not pulsatile. However, small-dose GnRH injection studies in beef cows (Walters *et al.*, 1982) and other FSH studies in heifers (Ireland and Roche, 1982a) and cows (Schallenberger and Peterson, 1982; Walters, Schams and Schallenberger, 1984) indicate that in this species FSH release may be pulsatile. However, the pulsatility of FSH in blood samples may be obscured in some studies due to the relatively long half-life of FSH of about 300 minutes in cattle (Laster, Glimp and Gregory, 1972) and 100 minutes in sheep (Akbar *et al.*, 1974; Fry *et al.*, 1987). By comparison shorter half-lives for LH in cattle (Katangole, Naftolin and Short, 1971; Steinberger, 1981) and sheep (Geschwind and Dewey, 1968), of between 26 and 35 minutes have been reported.

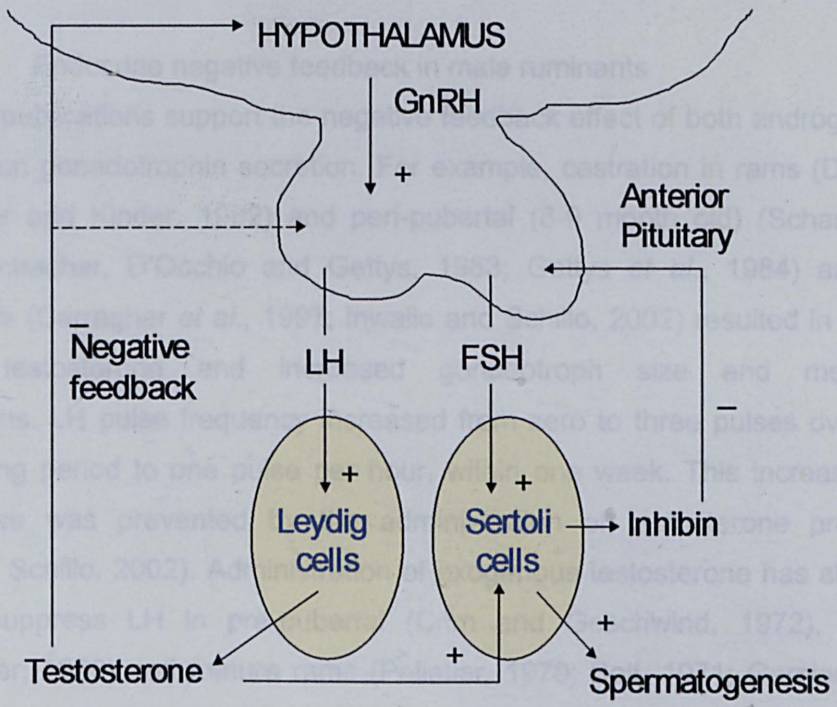
A recent study in sheep indicated that 93% of FSH pulses were associated with GnRH/LH pulses (Padmanabhan *et al.*, 1997). The remaining portion of FSH is released independently of GnRH pulses (McNeilly, 1988; Farnworth, 1995; Padmanabhan, Karsch and Lee, 2002). Recent reviews have addressed in detail the arguments for and against the existence of a separate hypothalamic FSH releasing factor (see Padmanabhan and McNeilly, 2001; Padmanabhan and Sharma, 2001). The bulk of the evidence indicates that such a factor does not exist (Padmanabhan and McNeilly, 2001; McNeilly *et al.*, 2003).

1.1.3 Gonadotrophin Control of Gametogenesis I: Spermatogenesis

The development of the reproductive system and the gonadotrophin influence on pre-pubertal development are discussed in section 1.4.

The testes can be divided functionally into the interstitium, containing testosterone producing Leydig cells bathed in interstitial fluid, and the seminiferous tubules, were Sertoli cells aid spermatogenesis. Several hormones of the HPG axis regulate spermatogenesis (Figure 1.3).

Figure 1.3 Schematic illustration of hormonal regulation of reproduction in the male (adapted from Intervet® Compendium of Reproduction, 1993).



1.1.3.1 Functions of Leydig cells and LH on spermatogenesis

LH binds to its receptors on the cell membrane of Leydig cells, causing a rise in intracellular cyclic adenosine monophosphate (cAMP), which promotes steroidogenesis. The development of assays for LH and testosterone enabled Katangole, Naftolin and Short, (1971) to publish that testosterone pulse release follows within 90 minutes of each LH pulse, and despite just two bulls and infrequent sampling, Thibier, (1976) later confirmed the results. From the Leydig cells in the interstitium, testosterone diffuses into the Sertoli cells in the seminiferous tubules where the steroid stimulates further Sertoli cell FSH and testosterone receptor expression (Verhoeven and Cailleau, 1988). Therefore, testosterone is critical for the initiation and maintenance of spermatogenesis (Kerr, Maddocks and Sharpe, 1992;

Maddocks, Kern and Setchell, 1995). While the development of spermatids from spermatogonia appears to be androgen independent, the maturation of spermatids to spermatozoa (spermiogenesis) depends on androgens acting on Sertoli cells in which the developing sperm are embedded. Intra-testicular testosterone concentrations must be concentrated 100 to 300 fold, compared to peripheral concentrations, for spermatogenesis (Sharpe, Donachie and Cooper, 1988; Maddocks and Sharpe, 1989; Maddocks, Kern and Setchell, 1995). As testosterone is freely diffusible, the relatively high intra-testicular concentrations are achieved by binding to albumin or androgen binding proteins which aid transport and retention of the androgen in the seminiferous tubule (Maddocks and Setchell, 1988) and by movement of testosterone from venous to arterial blood vessels within the pampiniform plexus.

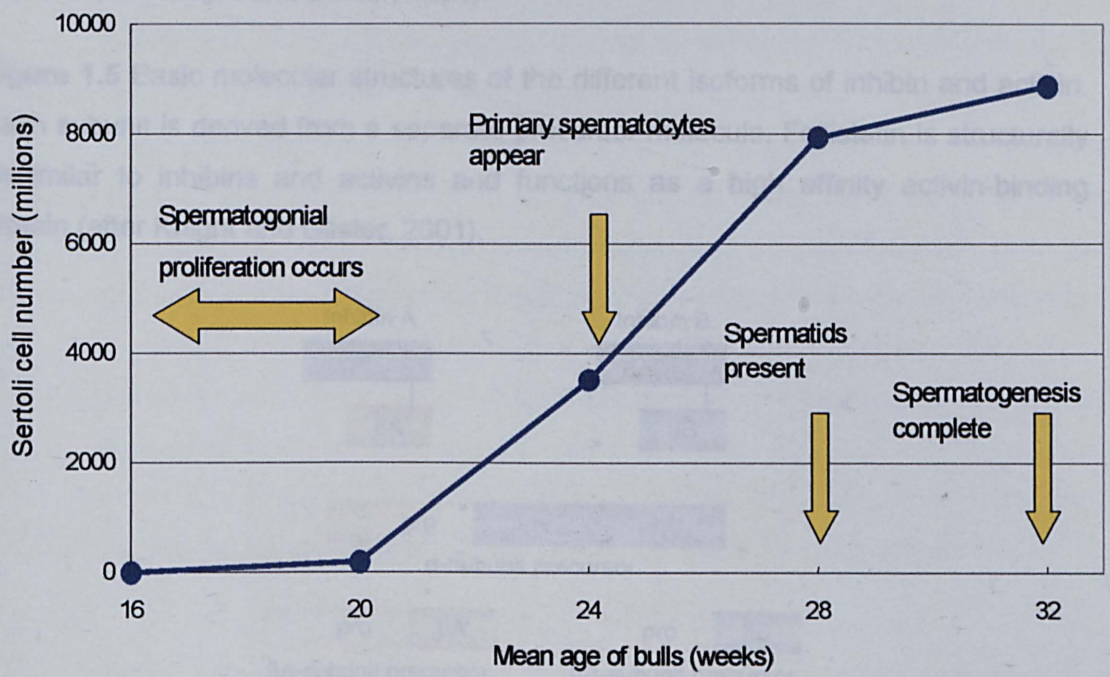
1.1.3.2 Endocrine negative feedback in male ruminants

A myriad of publications support the negative feedback effect of both androgens and oestrogens on gonadotrophin secretion. For example, castration in rams (D'Occhio, Schanbacher and Kinder, 1982) and peri-pubertal (8-9 month old) (Schanbacher, 1982; Schanbacher, D'Occhio and Gettys, 1983; Gettys *et al.*, 1984) and post-pubertal bulls (Carragher *et al.*, 1997; Inwalle and Schillo, 2002) resulted in reduced peripheral testosterone and increased gonadotroph size and mean LH concentrations. LH pulse frequency increased from zero to three pulses over a 12-hour sampling period to one pulse per hour, within one week. This increase in LH pulse release was prevented by the administration of testosterone propionate (Inwalle and Schillo, 2002). Administration of exogenous testosterone has also been shown to suppress LH in pre-pubertal (Crim and Geschwind, 1972), pubertal (Schanbacher, 1980) and mature rams (Pelletier, 1970; Bolt, 1971; Garnier, Terqui and Pelletier, 1977; Schanbacher and Ford, 1977; Bremner *et al.*, 1980; D'Occhio, Schanbacher and Kinder, 1982). Furthermore, as testosterone implanted wethers had reduced LH response to a GnRH challenge, D'Occhio, Schanbacher and Kinder, (1982) concluded that testosterone negative feedback could possibly occur at both the hypothalamic and pituitary level in the ram (Figure 1.3). As a result of the suppression of both LH parameters and the presence of steroid receptors in the pituitary gland (Schanbacher, D'Occhio and Gettys, 1983; Schanbacher, 1984) and hypothalamus (Pelletier, Carrez-Camous and Thiery, 1981; Glass, Amann and Nett, 1984), it was concluded that negative feedback effects of testicular steroids occur at both sites. However, more recent evidence in rams (Tilbrook *et al.*, 1991) suggests that the principal negative feedback effect is at the hypothalamic level, with only minimal effects on the anterior pituitary gland.

1.1.3.3 FSH and Sertoli cells

FSH stimulates Sertoli cells (Bicsak *et al.*, 1986) to synthesise and secrete inhibin (McCullogh, 1932; Steinberger and Steinberger, 1976). In addition, Sertoli cells proliferation and function is influenced by FSH, testosterone and several growth factors including IGF-I (Orth, Higginbotham and Salisbury, 1984). Furthermore, the negative feed back role of testicular oestrogens during pubertal development (see Sharpe, 1994) was clearly shown by Land, Baird and Carr, (1981), when oestradiol and oestrone immunised ram lambs exhibited elevated FSH concentrations and associated testicular growth relative to control animals. The increased numbers of Sertoli cells with advancing age towards puberty and key stages of spermatogenesis are illustrated in Figure 1.4.

Figure 1.4 Increase in Sertoli cell number in young Holstein bulls as puberty approaches (adapted from Curtis and Amann, 1981).



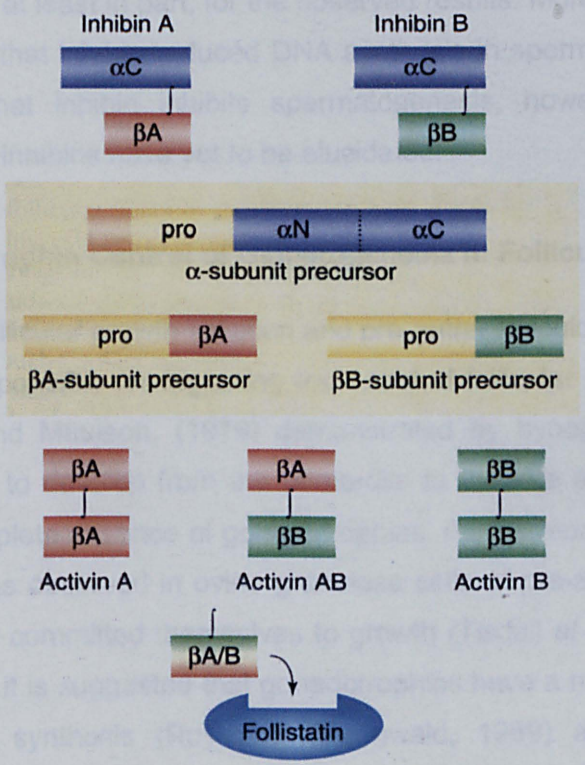
1.1.3.4 Inhibin and negative feedback

McCullogh, (1932) first used the term inhibin to describe a testicular factor, which inhibited anterior pituitary gland FSH release. Later, testicular extracts (Setchell and Jacks, 1974; Franchimont *et al.*, 1975) and bovine ovarian follicular fluid (De Jong and Sharpe, 1976) were found to contain high levels of a substance, which suppresses FSH secretion. Later still, that substance, inhibin, was purified from

bovine (Robertson *et al.*, 1985; Fukuda *et al.*, 1986), ovine (Leversha *et al.*, 1987) and porcine (Ling *et al.*, 1985; Miyamoto *et al.*, 1985) follicular fluid.

Inhibin, like other members of the transforming growth factor β (TGF-β) superfamily, is a disulphide-linked dimer of homologous subunits that contain a characteristic set of seven cysteine residues, which form a cysteine knot within each subunit (Robertson, Hertan and Farnworth, 2000). Inhibin consists of α and β subunits (Glencross, 1992). As there are two forms of the β-subunit, βA and βB, inhibin may exist as either inhibin A, α subunit combined with the βA-subunit, or inhibin B, α subunit combined with the βB subunit (Figure 1.5). Knight *et al.* (1989) showed that much of the immuno-reactivity detected in the first RIAs was free α sub-unit. Confirmation of the biological significance of inhibin was clarified with the development of 2-site assays specific for both inhibin forms (Groome *et al.*, 1996). Different post-translational processing gives rise to several different variants of inhibin A and B (see Knight and Glister, 2001).

Figure 1.5 Basic molecular structures of the different isoforms of inhibin and activin. Each subunit is derived from a separate precursor molecule. Follistatin is structurally dissimilar to inhibins and activins and functions as a high affinity activin-binding protein (after Knight and Glister, 2001).



Simple castration studies indicated the involvement of inhibin in FSH regulation (Amman and Walker, 1983; MacDonald, Deaver and Schanbacher, 1991), and in support of the earlier findings, active immunisation of intact pre-pubertal bulls against the inhibin α-subunit resulted in a 2-3 fold increase in peripheral FSH (Schanbacher, 1991; Martin *et al.*, 1991). The higher increase in peripheral FSH after inhibin immunisation compared to castration was explained following rodent trials, and results from neutralisation of pituitary inhibin (Roberts *et al.*, 1989), which may locally regulate FSH release (Bilezikjian *et al.*, 1996). Some research in bulls stated that following a rise in FSH testes size increased, spermatid numbers were elevated (Schanbacher, 1991; Martin *et al.*, 1991), and sperm output enhanced (Bame *et al.*, 1999). In contrast Schanbacher (1991) found no effect on pituitary gland weight, and no testicular hypertrophy. Interestingly, peripheral testosterone concentrations and live weight were increased (Bame *et al.*, 1999), and LH reduced (Martin *et al.*, 1991) with long-term inhibin immunisation.

In addition to "long-loop" FSH regulation, elegant experiments in rodents indicate that inhibin is a prime candidate for autocrine/paracrine regulation of spermatogenesis. For example, intra-testicular injections of bovine follicular fluid reduce spermatogonia populations in the treated, but not the contralateral, rat testis (van Diessel-Emiliani *et al.*, 1989). However, other components of follicular fluid, such as follistatin, may have been responsible at least in part, for the observed results. More recently, Hakovirta *et al.* (1993) stated that inhibin reduced DNA synthesis in spermatogonia. Evidence to date indicates that inhibin inhibits spermatogenesis, however, the exact intra-testicular roles of inhibins have yet to be elucidated.

1.1.4 Gonadotrophin Control of Gametogenesis II: Folliculogenesis

1.1.4.1 Follicular growth initiation and pre-antral folliculogenesis

The factor(s) responsible for triggering the onset of follicular growth remain elusive. Dufour, Cahill and Mauleon, (1979) demonstrated by hypophysectomy that ovine follicles are able to develop from the primordial to the late antral (~2mm diameter) stage in the complete absence of gonadotrophins. Furthermore, mRNA encoding the FSH receptor was observed in ovine granulosa cells of pre-antral follicles only after they had already committed themselves to growth (Tisdall *et al.*, 1995; see Fortune, 2003). However, it is suggested that gonadotrophins have a modulatory role, as FSH stimulates DNA synthesis (Roy and Greenwald, 1989) and steroidogenesis in neonatal rodents. Furthermore, hypophysectomy resulted in a decline in the number

of follicles growing at all stages of development (Wang and Greenwald, 1993). Indeed FSH receptors are present in early pre-antral follicles (Wandji *et al.*, 1992), at a time when follicles respond to FSH administration with elevated cAMP and granulosa cell proliferation (Ralph, 1996; see Webb *et al.*, 1999a). During antrum formation, when follicles measure 0.8-2mm and 1-3mm in sheep and cattle respectively, follicles may be referred to as (gonadotrophin responsive) (see Scaramuzzi *et al.*, 1993; Draincourt, 2001). These gonadotrophins promote antrum formation, increase follicular growth and protect follicles from early atresia (Wang and Greenwald, 1993).

1.1.4.2 Gonadotrophin dependent follicle recruitment

From the group of slow growing (gonadotrophin responsive) follicles rising peripheral FSH concentrations (Ginther, Kot and Wiltbank, 1995; Ginther *et al.*, 1996) recruit a group of 5-10 medium-sized follicles (Adams *et al.*, 1993b:c; Adams, Evans and Rawlings, 1994; Sunderland *et al.*, 1994). The "recruitment window" lasts for 1 day in the ewe and 2 days in the cow (see Draincourt, 2001). The process is also described as follicular wave emergence (Ginther *et al.*, 1996), and it is at this stage that the antral cavity becomes fully formed. For recruitment to proceed, FSH must reach a minimum threshold, which is believed to vary between animals (Picton and McNeilly, 1991) and follicles (Fry and Draincourt, 1996; see Campbell, 1999).

It would appear that approximately 4mm and 9mm diameters are key stages in bovine follicular development. Between 4-9mm diameter FSH is required for follicle growth. A reduction or withdrawal of FSH below normal concentrations by means of hypophysectomy (Dufour, Cahill and Mauleon, 1979; Draincourt *et al.*, 1987; Wang and Greenwald, 1993), chronic GnRH agonist treatment (buserelin; Picton and McNeilly, 1991; Gong *et al.*, 1996a), or GnRH immunisation (Crowe *et al.*, 2001a;b) is associated with no growth of follicles larger than 4mm. This is supported by the re-initiation of growth of follicles 4-8mm following exogenous FSH administration in immuno- (Crowe *et al.*, 2001a;b) and chemical (Picton and McNeilly, 1991) castrates. This is further supported by observations that, circulating FSH concentrations in the cow display a wave pattern, each FSH peak closely preceding a wave of follicle development (Ginther, Kot and Wiltbank, 1995; Ginther *et al.*, 1996).

FSH stimulation has numerous effects on ovarian follicles by binding to its receptor on the granulosa cells:

1. Induces aromatase activity (Saumande, 1990), enabling them to produce oestradiol. *In situ* hybridisation experiments in cattle have shown that $p450_{scc}$ mRNA expression is first detected at a follicle diameter of 4mm, with increasing $P450_{arom}$ mRNA in healthy follicles (Xu *et al.*, 1995a;b). More recently the key role that FSH plays in the induction of follicular growth and differentiation has been demonstrated (Gaverick *et al.*, 2002). A slightly prolonged FSH infusion, in GnRH agonist induced hypogonadotrophic heifers, stimulated medium-sized follicle growth and induced precocious expression of mRNAs for $P450_{scc}$ and $P450_{arom}$ in granulosa cells from small follicles and up regulated the expression of these enzymes from granulosa cells from recruited follicles.
2. Stimulates the production of inhibin, follistatin and activin (Knight, 1996), but increases the ratio of activin to follistatin and activin A to inhibin A i.e., increases 'activin tone' (Glister, Groome and Knight, 2003)
3. Suppresses IGFBP-2 production (Armstrong *et al.*, 1998; Webb *et al.*, 2003a).
4. Stimulates protease (PAPP-A), which degrades IGFBP-4 (see Draincourt, 2001; Webb *et al.*, 2003a).

Hence, follicle recruitment represents the "starting gun" in a race, during which follicles must proliferate and differentiate under the influence of FSH, to render them best able to respond to selection. Dominance may be seen as the end of the race, although the true "finish line" is ovulation (see Draincourt, 2001).

1.1.4.3 Dominant follicle selection and LH

Ginther *et al.* (1996) defined dominant follicle selection in mono-ovulatory species such as cattle, as the beginning of a difference, or "deviation", in growth rates between the two largest developing follicles in a wave. Another popular definition, as stated by Webb *et al.* (1999a), is that selection is a process whereby only some of the recruited follicles are selected to continue growth further with the eventual establishment of a dominant follicle(s). The exact selection mechanism is not fully elucidated however, it is known that selection occurs 36-72 hours after recruitment (Bao *et al.*, 1997a; b), and when, during the follicular phase, FSH concentrations are falling and LH pulse frequency is increasing (Campbell *et al.*, 1999b; see Baird 1983; Scaramuzzi *et al.*, 1993; Armstrong and Webb, 1997; Draincourt, 2001).

Prior to selection, LH principally binds to theca interna cells (Webb and England, 1982). In addition numerous researchers, utilising *in vivo* studies, have demonstrated a requirement for pulsatile LH in follicles >8mm. For example, when GnRH agonist is used to suppress LH pulses, but FSH concentrations are maintained, follicle

development does not proceed beyond 9mm (Gong *et al.*, 1995; 1996a). More recently, Crowe *et al.* (2001a;b) reported similar findings after GnRH immunisation and exogenous FSH treatment. However, FSH alone can stimulate follicle selection (Gong *et al.*, 1995; 1996a). Furthermore, a single high dose LH infusion has been observed to be as effective at inducing selection as pulsatile LH infusions (Campbell, unpublished observations). Indeed, it is the fall in FSH concentrations, coupled with the availability of LH high frequency pulses, which necessitates the "switch" in gonadotrophin dependence from FSH to LH in the dominant follicle (see Campbell *et al.*, 1999b).

Follicle selection is associated with a number of changes in the presumptive (7 to 9mm) dominant follicle (Mihm *et al.*, 2000). In medium-size follicles, up to ~7mm in cattle, and 2-3mm in sheep, LH receptors (LH-R) are only expressed on the theca interna cells and oestradiol and androstenedione levels in follicular fluid are low, relative to testosterone. In selected follicles (ovine: >3mm, bovine: >8mm) oestradiol production increases rapidly, while testosterone concentrations fall (Carson *et al.*, 1981). Importantly, as LH stimulation increased, LH-R numbers on theca cells also increase. This newfound "oestrogenic status" coincides with the appearance of LH-R on the granulosa cells, and only occurs in the ovulatory follicle(s) (Carson *et al.*, 1979; Webb and England, 1982; Ireland and Roche, 1983). These morphological observations are supported by gene expression data, which show that, mRNA expression of LH-R (Xu *et al.*, 1995b) and 3βHSD (Bao *et al.*, 1997a;b) in bovine granulosa cells can only be detected when follicles are ≥ 9mm.

1.1.4.4 Endocrine negative feedback in females

The selected follicle(s) produces the majority of the oestradiol (Straigmiller and England, 1982; Henderson *et al.*, 1984) and inhibin (Schwall *et al.*, 1990; Knight and Glister, 2003) that is found in the circulation. Robust evidence exists for the negative feedback action of both oestradiol and inhibin (Webb and Morris, 1988; Baird *et al.*, 1991; Campbell, Scaramuzzi and Webb, 1995). This has been demonstrated by treating animals with exogenous oestradiol and inhibin, which suppresses follicular development in both sheep and cattle via reduced gonadotrophin secretion (Johnson and Smith, 1985; O'Shea *et al.*, 1994; Bo *et al.*, 1995; Campbell *et al.*, 1995; see Webb *et al.*, 1992a:b). Conversely, inhibin immunised animals exhibit elevated FSH levels, which were increased further by concomitant immunisation against oestradiol in both sheep (Mann *et al.*, 1989) and cattle (Kaneko *et al.*, 1995). Furthermore, inhibin-binding sites, with high affinity and specificity, have been identified on ovine

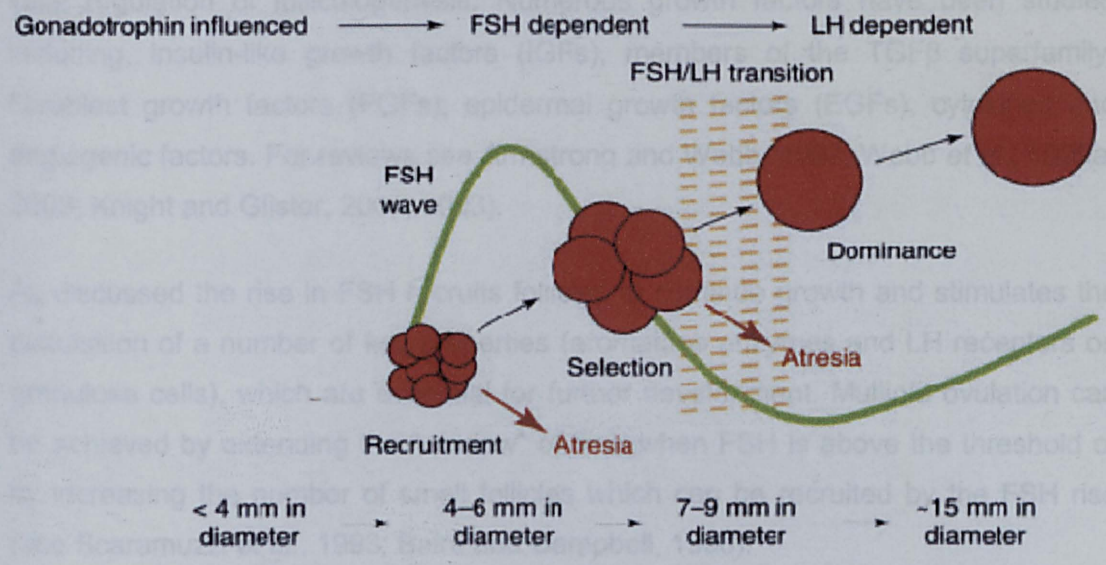
pituitary cells, consistent with the presence of a putative inhibin receptor and inhibin action on the pituitary gland (Hertan *et al.*, 1999).

Around the time of follicle selection, and as a result of negative feedback actions of these hormones on the anterior pituitary gland, FSH release is reduced (Kaneko *et al.*, 1991; Gibbons, Wiltbank and Ginther, 1997; Mihm *et al.*, 1997) to levels insufficient for the survival of the subordinate follicles (Straigmiller *et al.*, 1982; Webb and Morris, 1988; Beard *et al.*, 1989; Baird *et al.*, 1991; Campbell, Scaramuzzi and Webb, 1995). Hence, the dominant follicle(s) must differentiate in a way, which will allow it to sustain growth in an environment of lower circulating concentrations of FSH (Fortune, 1994). The expression of LH-R on the granulosa cells is believed to give the dominant follicle(s) an advantage, enabling development to continue by utilising LH, while other follicles in the cohort undergo atresia, starved of FSH on which they depend (Draincourt, 1991; Campbell *et al.*, 2000; see Figure 1.10). Despite switching dependency from FSH to LH, the dominant follicle(s) still requires a low concentration of FSH in order to survive (Fortune, 1994; Gong *et al.*, 1995; 1996a). The way the dominant follicle achieves this is described in section 1.1.5-1.1.5.2.1. After dominance is established growth typically continues for about 3-5 days until the dominant follicle reaches a size of ~15-20mm in cattle and ~ 6-7mm in sheep; see Draincourt, 2001).

1.1.4.5 Follicular waves

Follicular waves are the recurrent succession of recruitment, selection and dominance (see Figure 1.6). Follicular waves are preceded by a peak of FSH (Rawlings *et al*, 2003) in cattle commence prior to puberty (Hopper *et al.*, 1993; Adams, Evans and Rawlings, 1994), within 2 weeks postpartum, with each wave lasting 7-9 days (Evans, Adams and Rawlings, 1994a:b). Follicle waves continue throughout the animals life, stopping during the last 3 and 4 weeks of pregnancy in the cow (Ginther *et al.*, 1996) and ewe (Draincourt *et al.*, 2000), respectively. Waves resume about 10 days after calving, and finally cease when the primordial pool is exhausted within the ovaries. The function of repeated waves of follicle growth is to provide a continuous source of follicles for ovulation should fertilisation or pregnancy fail. The length of each wave, and hence the number per oestrous cycle may be influenced by LH release. For example, increased LH pulse frequency results in the maintenance and development of the dominant follicle (Sirios and Fortune, 1990).

Figure 1.6 Schematic representation of the requirement for gonadotrophins at different stages of ovarian development in cattle (after Webb *et al.*, 2003a).



Approximately 80% of cattle have 3 waves of follicular development during each oestrous cycle (Savio *et al.*, 1988; see Webb *et al.*, 1999a). The first wave starts at the time of the second FSH rise, which typically begins on day 2 of the oestrous cycle. Subsequent waves begin around days 9 and 16 post-oestrus (Sirios and Fortune, 1988). The dominant follicle of the third wave is usually the one that ovulates. Sheep typically have 3 or 4 waves (Ginther, Kot and Wiltbank, 1995; see Evans *et al*, 1999). In cattle, as the animal ages, the diameter of the dominant follicle increases (8.5 to 12mm), making dominance more pronounced, as the difference between dominant follicle(s) and subordinates increases. After the fourth month of pregnancy in the cow the dominant follicle decreases to about 9mm in diameter (see Draincourt, 2001).

1.1.5 Intra-Ovarian Regulators of Folliculogenesis

Studies on the hypothalamic-pituitary gland-ovarian (HPO) feedback system have been unable to explain, in full, the follicle selection mechanism and dominance. For example, similar sized follicles exposed to the same gonadotrophic drive respond differently, supporting the concept of intra-ovarian regulation (Campbell, Scaramuzzi and Webb, 1995; Monget and Monniaux, 1995; Webb *et al.*, 1999a; Armstrong and Webb, 1997). This section introduces and summarises some intra-ovarian regulators of folliclulogenesis. These factors are discussed in more detail in Chapters 5 and 6.

The development of serum-free culture systems, which maintain follicular phenotypes of bovine (Guitierrez *et al.*, 1997b) and ovine (Campbell, Scaramuzzi and Webb, 1996) granulosa and thecal cells have enabled the analyses of factors involved in the local regulation of folliculogenesis. Numerous growth factors have been studied including, insulin-like growth factors (IGFs), members of the TGFβ superfamily, fibroblast growth factors (FGFs), epidermal growth factors (EGFs), cytokines and angiogenic factors. For reviews see Armstrong and Webb, 1997; Webb *et al.*, 1999a; 2003; Knight and Glister, 2001; 2003).

As discussed the rise in FSH recruits follicles to continue growth and stimulates the acquisition of a number of key properties (aromatase enzymes and LH receptors on granulosa cells), which are essential for further development. Multiple ovulation can be achieved by extending the "window" of time when FSH is above the threshold or by increasing the number of small follicles which can be recruited by the FSH rise (see Scaramuzzi *et al.*, 1993; Baird and Campbell, 1998).

Draincourt (2001) suggested that in species with a "loose control" of ovulation rate (some sheep) selection is controlled by endocrine mechanisms (reduction of FSH support for follicles) only. However, in species with a more defined selection process, (cattle), products derived from the largest follicle may directly inhibit development of other cohort follicles. Indeed, steroid and inhibin depleted follicular fluid can inhibit follicular development in sheep (Campbell *et al.*, 1991b; O'Shea *et al.*, 1994) and cattle (Law *et al.*, 1992; Wood *et al.*, 1993). Partially purified follicular fluid fractions containing negligible amounts of inhibin, have been observed to inhibit the proliferation and aromatase activity of cultured ovine, porcine and bovine granulosa cells (Baxter *et al.*, 1995; Picton and Hunter, 1995; Rouillier *et al.*, 1998; see Armstrong and Webb, 1997; see Webb *et al.*, 1999a). Furthermore, administration of inhibin free follicular fluid by Turzillo and Fortune, (1990) after ovulation, postponed subsequent follicular wave by 2 days. The discovery by Ralph, Wilmut and Telfer, (1995) of a bovine oocyte secreted factor along with other evidence (see Webb *et al.*, 1999a; Eppig, 2001) indicates that the oocyte may influence follicular development. Dong *et al.*, (1996) demonstrated that, in mice deficient in growth differentiating factor-9 (GDF-9), follicles only developed one layer of granulosa cells and the theca failed to differentiate, although development at this stage appeared normal (see Webb *et al.*, 1999a).

More recently, the importance of local factors in follicle development has been revealed in prolific sheep breeds. For example, in the Booroola Merino a single gene mutation named the Fec^B gene has been identified in the intracellular kinase domain of the bone morphogenetic protein receptor-1β (BMP-1β, also known as ALK-6; Wilson *et al.*, 2001; Souza *et al.*, 2001; Mulsant *et al.*, 2001; Fabre *et al.*, 2003). The gene is expressed in oocytes, and granulosa cells of primordial and pre-antral follicles, and in granulosa and theca cells of all antral follicles as well as CLs (Souza *et al.*, 2002). All the ligands for the BMPR-1β are yet to be discovered (Galloway *et al.*, 2002). Furthermore, research to date is inconclusive (see Fabre et al., 2003) as to whether the mutation acts by up or down regulation of the BMPR-1β, however it is thought that, decreased signalling by BMPR-1β, leads to physiological changes in foetal development, pituitary and ovarian function, including precocious follicular development, which results in increased ovulation rate and litter size (Smith *et al.*, 1993; Boulton *et al.*, 1995; McNatty *et al.*, 1995; 2001; Montgomery *et al.*, 2001; Fabre *et al.*, 2003). Further evidence comes from Romney sheep in which gene carriers of the single point mutation named Fec^{XH} (Hanna) results in oocytes, which do not produce BMP15 (also known as GDF-9β), while the Inverdale mutation renders the BMP15 inactive. As BMP15 is essential for granulosa proliferation during pre-antral development, homozygous carriers exhibit arrested oocyte development (Galloway *et al.*, 2002). However, heterozygous carriers Fec^{XH} (Hanna) and Fec^{XI} (Inverdale) exhibit increased antral follicle populations (McNatty *et al.*, 1995) as granulosa cells develop a greater responsiveness to FSH (Souza et al., 2002) and acquire LH receptors at a smaller diameter, with each "oestrogenic follicle" containing fewer granulosa cells (Shackell *et al.*, 1993). It has been hypothesised that the oocyte has a significant influence on the number of follicles that proceed to ovulation (McNatty *et al.*, 2001; Eppig, 2001). The latest research suggests that BMP signalling has a key role in folliculogenesis (Otsuka *et al.*, 2001a;b). Fabre *et al.* (2003) stated that, reduced BMP signalling could positively affect the number of ovulatory follicles. For reviews of recent developments in prolific breeds of sheep see Montgomery *et al.* (2001), McNatty *et al.* (2001; 2003), Mulsant *et al.* (2001), Shimasaki *et al.* (2003) and Souza *et al.* (2003).

1.1.5.1 The Insulin-like growth factor (IGF) system

Experiments in IGF-I gene knockout mice (Baker *et al.*, 1996) have observed reduced ovarian weight and anovulation, demonstrating an absolute requirement for the protein. However, species-specific patterns of granulosa IGF-I mRNA expression have been reported and controversy exists over the expression of mRNA for IGF-I in

ovine granulosa and thecal tissue (see Armstrong and Webb, 1997). In cows, IGF-II expression is restricted to the thecal tissue of antral follicles (Armstrong *et al.*, 2000) and it is generally agreed that IGF-II is the major intra-ovarian IGF (see Webb *et al.*, 2003b). However, IGF-I may act in an endocrine manner and shows a positive correlation with follicle growth after recombinant bovine growth hormone (rbGH) treatment (see Webb *et al.*, 1999a). Echternkamp *et al.* (1994) and Austin *et al.* (2001) observed that IGF binding protein-2 (IGFBP-2), and possibly IGFBP-4 and –5 concentrations are higher in the follicular fluid of follicles prior to selection and in large atretic follicles. Thus, leading them to suggest that, IGFBPs have a regulatory role in bovine follicle development in cattle. Yet these binding proteins are significantly reduced in the follicular fluid of large or dominant follicles (Nicholas *et al.*, 2002). Expression of IGFBP-2 and -4 mRNA has been demonstrated in both granulosa and thecal tissue of healthy bovine antral follicles up to 9mm in diameter (Armstrong *et al.*, 1998). Furthermore, IGFBP-2 mRNA expression was undetectable in granulosa tissue from large dominant follicles. Based on recent findings it has been suggested that FSH may indirectly act to inhibit IGFBP-2 expression (see Webb *et al.*, 2003a;b). These findings indicate that low IGFBP-2 concentrations and an increase in LH receptors on granulosa cells may be critical to the dominant follicle selection mechanism. A reduction in follicular fluid IGFBP-4 concentrations, coupled with an increase in oestradiol concentrations, has also been reported in the dominant follicle in cattle (Mihm *et al.*, 2000). Overall, these results indicate that intra-follicular IGF bioavailability is a key factor controlling selection and maintenance of the dominant follicle in cattle (Webb *et al.*, 2003a;b). Furthermore, during follicle development, post-translational modification of IGFBPs may occur, which affects the affinity of the binding proteins for IGFs. For example, IGF-dependent regulation of IGFBP-4 has been demonstrated in bovine follicular fluid (Mazerbourg *et al.*, 2000). Furthermore, IGFBP-4 proteolytic activity was reported to be maximal in the dominant follicle (Rivera *et al.*, 2001). The IGFBP-4 degrading protease has recently been identified as pregnancy-associated plasma protein A (PAPP-A; Monget *et al.*, 2002; see Webb *et al.*, 2003a;b; Armstrong, Gong and Webb, 2003).

1.1.5.2 TGF-β superfamily

TGF-β superfamily members are expressed by oocytes, granulosa cells and theca cells in a developmental-stage related manner and function as intra-ovarian regulatory molecules involved in follicle recruitment and atresia, granulosa and theca cell proliferation, steroidogenesis, oocyte maturation, ovulation and luteinisation. The TGF-β superfamily comprises over 30 structurally related, but functionally diverse,

proteins which include TGF-β isoforms (TGF-$\beta_{1,2,3}$), anti-mullerian hormone, inhibins, activins, some 20 bone morphogenetic proteins (BMP-1 to BMP-20) and at least nine growth/ differentiation factors (GDF-1 to GDF-9) which are closely related to the BMP sub-group (Massague and Wotton, 2000). The TGF-β superfamily is discussed in more detail in other sections of this thesis. For a recent review of the TGF-β superfamily ligands, receptors and ancillary binding proteins see Knight and Glister, (2003). The remainder of this section summaries the local actions of inhibins and activins in the ovary.

1.1.5.2.1 *Local actions of inhibins and activins*

On the basis of mRNA expression and immunohistochemical evidence, it has been proposed that the relative balance between follicular activin and inhibin production shifts throughout follicle development. Generally, small follicles primarily produce activins ('high activin tone') and selected large antral follicles show 'high inhibin tone' i.e., increased inhibin A production (Schwall *et al.*, 1990; Yamoto *et al.*, 1992; Roberts *et al.*, 1993). There is a paucity of information on the intra-follicular contents of different assembled dimeric inhibin /activin isoforms, particularly in pre-antral and small antral follicles (see Knight and Glister, 2003).

Ginther *et al.* (2002) recently showed that following dominant follicle ablation, which provided the stimulus for selection of a new dominant follicle, the future dominant follicle showed a transient elevation in follicular fluid activin and oestradiol concentrations. These responses occurred after the transient FSH rise, subsequent to ablation of the dominant follicle. The lack of between-follicle differences for progesterone, androstenedione and inhibins (A and B) indicates that, an FSH induced rise in activin A by granulosa cells may be an early marker of follicular dominance (Knight and Glister, 2003). Studies using cultured bovine granulosa cells have shown that both FSH and IGF-I promote secretion of activin A and oestradiol in medium-sized follicles (~5mm; Glister *et al.*, 2001). Furthermore, the stimulatory actions of FSH and IGF-I on oestradiol secretion may be mediated by endogenous activin A (Glister *et al.*, 2001). Moreover, the role of activin in enhancing granulosa cell proliferation has also been demonstrated in rodents following studies in follicle cell culture experiments (Li, Philips and Mather, 1995; Miro and Hillier, 1996; Zhao *et al.*, 2001) and inhibin α-subunit (Matzuk *et al.*, 1996) and activin type IIB receptor (Matzuk *et al.*, 1996) gene knockout mice. Enhanced antral follicle development following inhibin immunisation in both sheep (Tannetta *et al.*, 1998) and cattle (Knight, Tannetta and Glencross, unpublished observations; as cited in Knight and

Glister, 2003) has been shown to increase intra-follicular activin A concentrations. Thus, providing further evidence, albeit indirect, supporting a local role of activin in amplifying or regulating FSH action. Furthermore, experiments on bovine granulosa cells have shown that activin can enhance $P450_{AROM}$ activity and oestradiol production, while inhibiting progesterone secretion (Hutchinson *et al.*, 1987). These observations suggest a role for activin in delaying the onset of atresia and/or luteinisation, possibly by up regulating FSH receptors (Hasegawa *et al.*, 1988). Follistatin binds activin, neutralising it and therefore opposes these effects of activin. Indeed, transgenic mice, which over express follistatin exhibit arrested follicular development (Guo *et al.*, 1998).

Despite this growing evidence (see also Chapters 5 and 6) for an intra-follicular action of inhibin, evidence for role of inhibin in granulosa cell development is inconsistent. For example, infusion of inhibin into the ovarian artery of ewes with an auto transplanted ovary reduced ovarian output of oestradiol (Campbell and Scaramuzzi, 1996). In agreement, Jimenez-Krassel *et al.* (2001) reported that inhibin suppressed oestradiol secretion by bovine granulosa cells isolated from dominant follicles, whereas inhibin antibodies raised oestradiol secretion. In contrast, Campbell and Baird, (2001) found that inhibin A enhanced FSH induced oestradiol secretion *in vitro*. Moreover, the addition of inhibin antiserum reduced FSH induced oestradiol production. The ability of inhibin A to enhance LH induced androgen production from isolated bovine thecal cells *in vitro* has been demonstrated in several species (see Chapters 5 and 6). Furthermore, these findings may reflect an important intra-follicular positive feedback mechanism for ensuring that the granulosa cells of selected follicles are provided with adequate substrate for oestradiol production. In cattle, oestradiol also promotes LH induced oestradiol production by theca cells (Wrathall and Knight, 1995). These actions of inhibins are opposed by activin (see Knight and Glister, 2003).

Oocytes express both type I and type II activin receptors (Cameron *et al.*, 1994). Furthermore, studies in both primates and rodents have revealed that activin can accelerate meiotic maturation of oocytes (see Knight and Glister, 2003). In cattle, the ability of the oocyte to form blastocysts following fertilisation was enhanced by activin (Silva and Knight, 1998). In contrast, there is evidence that inhibin, or free α-subunit, may have a negative influence on oocyte maturation (O *et al.*, 1989) and developmental competence (Silva *et al.*, 1999; see Knight and Glister, 2003). Following activin receptor stimulation, cytoplasmic signalling intermediaries, known

as receptor (R)-Smads associate with further co-Smad (Smad 4). The activated R-Smad-co-Smad complex then translocates to the nucleus to promote altered gene expression. Additional regulation is provided by inhibitory Smads (Smad 6 and 7). Therefore, the action of ligands on activin receptors may be further regulated at the cellular level. For a recent review see Knight and Glister, (2003).

As discussed, GH/IGF and inhibins have significant effects at the ovarian level, however, the literature lacks reports on the interaction between these two families, and thus the occurrence of such interactions is studied in Chapter 5.

1.2 REPRODUCTIVE DEVELOPMENT AND PUBERTY IN RUMINANTS

Due to differences in the definitions used by researchers for the stages of development, the age at which endocrine parameters change during development varies between publications. For example, Rodriguez and Wise (1989) defined the period during which LH concentrations rise as pre-pubertal, whereas others define this as the juvenile period and class the pre-pubertal period as the time during which LH levels are inhibited due to hypersensitivity to androgen negative feedback (Kinder *et al.*, 1995). To describe reproductive development, in this thesis, four periods of pubertal maturation (see Figure 1.7) and one termed post-pubertal and sexual maturation will be used.

1.2 1 Endocrinology of the young ruminant

1.2.1.1 Pre-natal and infantile development

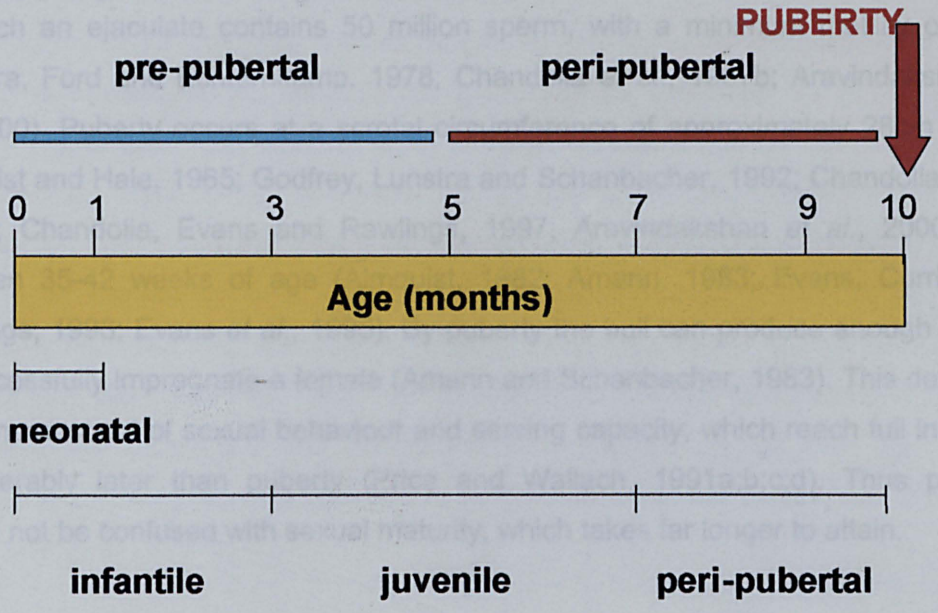
Comprehensive foetal studies in both sheep (Mesiano *et al.*, 1991; Matwijiw and Faiman, 1991; Caldani *et al.*, 1995) and cattle (Leshin *et al.*, 1988) agree that, half way through gestation GnRH neural systems are similar to those of adult animals (Caldani *et al.*, 1995). Indeed, by day 60 of gestation bull foetuses, exhibit rising LH concentrations until the end of the second trimester (Rodriguez and Wise, 1989). The gonads respond to LH stimulation with increased steroid output, which is essential for reproductive tract development, testicular descent (concluded by Day 140 in bulls) and sexual differentiation of GnRH neural networks (Danzer, McMullen and Rance, 2001). Testosterone has a major influence on neural organisation of the GnRH pulse generator and sexual dimorphism is apparent as gonadotrophin levels are higher in female fetuses compared to males, suggesting that the testes, but not ovaries exert negative feedback by mid-gestation. Moreover, castration of the male, but not the female, foetus at Day 110 resulted in increased LH and FSH output in rams (Matwijiw and Faiman, 1991). The effects of castration on LH decline by Day 130 of gestation,

to values similar to intact males and females, suggest that the suppressive effects of testicular hormones on GnRH (Rodriguez and Wise, 1989) and gonadotrophic output are superseded in late gestation by other factors (Matwijiw and Faiman, 1991; Mesiano *et al.*, 1991), possibly placental steroids.

Concentrations of androgens and oestrogens are low in the infant relative to the adult (McCarthy, Convey and Hafs, 1979). An observation that led Amann and Walker, (1983) to conclude that low levels of GnRH, LH and FSH at birth are due to hypersensitivity of the hypothalamus to gonadal steroid negative feedback. Later, Amann *et al*, (1986) stated that LH pulses are infrequent at 6 weeks of age, before being established at 8 weeks of age, as GnRH pulse frequency increased from 2-12 weeks of age (Rodriguez and Wise, 1989). However, castration at birth or shortly after (ram; Schanbacher, 1980; bull; McCarthy and Swanson, 1976; Bass *et al.*, 1977; Wise, Rodriguez and Kelly, 1987) results in a delayed increase in LH release. These data suggest that gonadal steroid negative feedback may not totally account for the changes in LH secretion observed during early development in the male. Indeed, function of the GnRH pulse generator might await maturation of some component of the CNS. Study comparisons indicate that, post-castration response e.g., LH rise is not seen until 7 and 12 weeks of age, and is not maximal until 13 and 20 weeks of age, in the bull (Wise, Rodriguez and Kelly, 1987) and ram (Schanbacher, 1980), respectively. This indicates a species difference in the maturation of the GnRH pulse generator and in the response to gonadal steroid negative feedback. The apparent earlier maturation in the bovine contributed to the decision to vaccinate calves against GnRH at 2-3 weeks of age (Chapters 3 and 4) rather than at 3-4 weeks (Brown *et al.*, 1994; 1995; see section 1.5.3.2 and 3.2.2).

The increased GnRH pulse frequency from 2-12 weeks increases gonadotroph expression of the GnRH-R by 400% between 6-8 weeks of age (Rodriguez and Wise, 1989). It also causes increased mRNA synthesis of LHβ-subunit and increases the available LH stores four fold (Amann *et al.*, 1986; Rodriguez and Wise, 1989). Together this increase in pituitary gland GnRH-R and LH stores leads to an increase in LH pulse frequency and amplitude, which peak during the juvenile period, between 12-20 weeks of age (Amann and Walker, 1983; Amann *et al.*, 1986).

Figure 1.7 Illustrates the common categories used to describe stages of reproductive development in cattle. Throughout this thesis peri-pubertal (adolescence) will refer to ≥7 months of age to attainment of puberty. Note however, that some authors define peri-pubertal as ≥5 months of age to puberty.



1.2.1.2 Juvenile development (3-7 months of age)

During the juvenile period, there is a four-fold increase in GnRH release (Amann, 1983). Evans and O'Doherty (2001) hypothesised that central control mechanisms governing GnRH release mature at this age, however, the regulatory systems are still immature, resulting in a relatively uninhibited increase in GnRH release. Alternatively, it may be that the responsiveness of the pituitary gland to low level steroid inhibition decreases. Either way, LH pulse frequency, pulse amplitude and basal LH concentrations increase dramatically (Amann and Walker, 1983). This increased LH output is essential in initiating normal testicular growth and development in bulls (McAndrews *et al.*, 1994; Chandolia, Evans and Rawlings, 1997; Chandolia *et al.*, 1997b).

1.2.1.3 Peri-pubertal development (7 months to puberty)

The increase in LH during the juvenile period stimulates gonadal development, which results in the increased production of androgens (Amann, 1983). By 12-24 weeks of age, the high levels of steroids produce a negative feedback effect on the hypothalamus (McAndrews *et al.*, 1994). This reduces the LH output and marks the beginning of the peri-pubertal (adolescent) period (Amann and Walker, 1983; McAndrews *et al.*, 1994; Chandolia, Evans and Rawlings, 1997). The gonadostat and

steroid independent hypotheses on pubertal development are addressed in sections 1.3.3.1 and 1.3.3.2, respectively.

1.2.2 Puberty

Puberty in the bull has several definitions, but is generally acknowledged as the time at which an ejaculate contains 50 million sperm, with a minimum motility of 10% (Lunstra, Ford and Echternkamp, 1978; Chandolia *et al.*, 1997b; Aravindakshan *et al.*, 2000). Puberty occurs at a scrotal circumference of approximately 28cm (Wolf, Almquist and Hale, 1965; Godfrey, Lunstra and Schanbacher, 1992; Chandolia *et al.*, 1997b; Chandolia, Evans and Rawlings, 1997; Aravindakshan *et al.*, 2000) and between 35-42 weeks of age (Almquist, 1982; Amann, 1983; Evans, Currie and Rawlings, 1993; Evans *et al.*, 1995). By puberty the bull can produce enough sperm to successfully impregnate a female (Amann and Schanbacher, 1983). This definition takes no account of sexual behaviour and serving capacity, which reach full intensity considerably later than puberty (Price and Wallach, 1991a;b;c;d). Thus puberty should not be confused with sexual maturity, which takes far longer to attain.

1.4.3 Post-pubertal Bulls and Sexual Maturity

Semen from bulls, which have recently attained puberty, is of poor quality and unsuitable for freezing (Chicoteau *et al.*, 1990). As a bull matures the number and quality of spermatozoa per ejaculate increases greatly beyond those values required for attainment of pubertal status (Lunstra, Ford and Echternkamp, 1978). Bulls used in A.I. programmes attain maximum breeding efficiency by two years of age, followed by a gradual decline with each advancing year (Tanabe and Salisbury, 1946; Collins *et al.*, 1962). With increased age, spermatogenesis is reduced, incomplete sperm maturation occurs more frequently and inter-tubular connective tissue becomes more prevalent (Grier and Marion, 1970). However, fertility has been reported in a bull aged 19 years of age (Bishop, 1970).

1.2.4 Maturation of the Reproductive Tract in the Bull

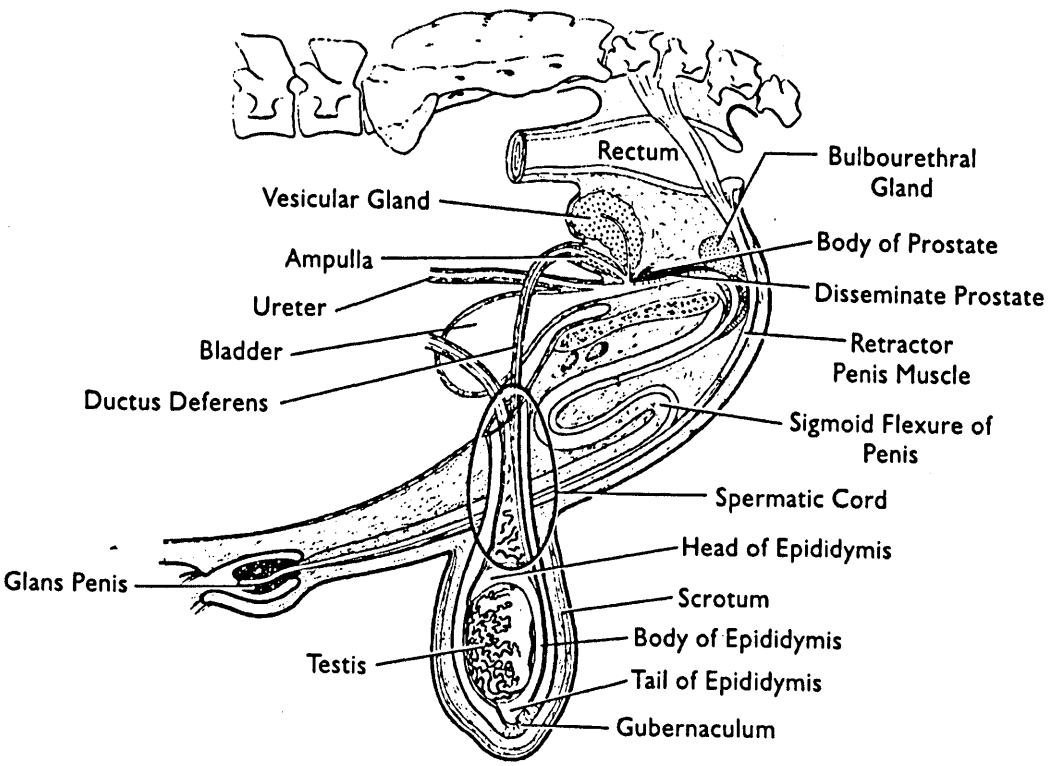
The growth and development of the reproductive tract is a gradual process, which is secondary to body growth. Several environmental factors (e.g., nutrition, social cues, photoperiod) and genetics influence pubertal development. The reproductive tract of the adult bull is presented diagrammatically in Figure 1.8.

1.2.4.1 Testicular growth and development

As early as Day 42 of gestation Leydig cells in the bovine embryo are functional and release testosterone to establish male sex ducts (Josso *et al.*, 1979), but they

regress, before re-emerging prior to puberty (Hooker, 1970). Following adequate LH stimulation, which occurs at around 12-16 weeks after birth (Schams *et al.*, 1981; Evans, Currie and Rawlings, 1993; Rawlings and Evans, 1995), Leydig cells become functional. Subsequently numerous maturational changes take place, which include; pre-Sertoli cells differentiating to form Sertoli cells, the lumen in the seminiferous tubules forms and spermatogonia differentiate to primary spermatocytes. As a result, there is a marked acceleration in the growth of all testicular components (Van Demark, 1956; Grier and Marion, 1970) at 16-21 weeks of age (Abdel-Raouf, 1960; Macmillan and Hafs, 1969). In horses, Leydig cell development appears first in the left, then the right testis, resulting in greater left testis weight 80% of the time, relative to the right (Nishikawa and Horie, 1955; Johnson, 1991). Bratton *et al*, (1959) reported similar, but less pronounced findings in the bull. However, others (Almquist and Amann, 1961; Macmillan and Hafs, 1969) found only small differences in testis weight of any given bull and no differences between the left and right testis. Ultrasound scanning recently confirmed that both testes are of similar size in the bull (Chandolia *et al.*, 1997b).

Figure 1.8 A schematic illustration of the bull reproductive tract in the sagittal plane (after Senger, 1997).



By around 38-40 weeks testicular growth slows (Abdel-Raouf, 1960; Macmillan and Hafs, 1969). Final testis weight is closely correlated to live weight, and fertility (Van Demark, 1956). Positive correlations have been reported between Sertoli cell number, total tubular length (Hochereau-de Reviers and Courot, 1978) and daily sperm production (Berndtson, Igoboelli and Parker, 1987) and scrotal circumference and sperm production (Willet and Ohms, 1957; Foote, 1978; Johnson, 1991).

1.2.4.2 Accessory glands

After spermatogenesis, sperm are released from the Sertoli cells (spermiation) and pass from the testis into the epididymis. On the 11 (bull) or 13 (tup) day journey, as sperm move through the epididymis they acquire motility and continue to mature (Holtz and Smidt, 1976). Relaxin from the prostate gland may further improve motility (Ganong, 1996). Interestingly, testosterone slows the passage of sperm through the epididymis (Sunjarit and Pholpramool, 1985), whereas frequent ejaculation increases it by up to 20% (Johnson, 1991). Sperm are stored in the caudal epididymis until ejaculation. At ejaculation the sperm exit the epididymis and travel along the vas deferens. During this passage the accessory glands add their contributions to the ejaculate.

Following establishment of the penis, the prostate develops from the urethral epithelium between Day 50-53 of gestation in the bull. Vesicular glands (seminal vesicles) and Cowper's (Bulbourethral) glands develop at about 60 days from evaginations at the base of each vas deferens, and from the urethral duct, respectively (see Figure 1.11). The establishment, growth and function of accessory glands are influenced by testosterone. For example, protrusion of the penis during mounting and separation of the penis and sheath typically occurs, in all breeds, between 8-9 and 9-10 months, respectively (Ashdown, 1960; 1962; Wolf, Almquist and Hale, 1965). However, sheath penis detachment occurs earlier in bulls with higher mean testosterone concentrations in the circulation (Lunstra, Ford and Echternkamp, 1978). The size of the prostate and vesicular gland is particularly androgen dependent (Rocha *et al.*, 1994; Chandolia *et al.*, 1997a;b) throughout the life of the bull; yet despite this fact and the advent of scanning enabling relatively easy assessment of vesicular gland growth, monitoring following GnRH immunisation in the live bull has not been performed to our knowledge prior to Chapter 3, herein.

1.2.5 Behavioural Changes Associated with Puberty

The expression of sexual and aggressive behaviours (see Table 2.1) precedes the full development and maturation of the testes in ruminants (Brown *et al.*, 1994). Male

calves become dominant over heifers at about 24-26 weeks of age, around the time of the rise in testosterone concentrations (McCarthy, Convey and Hafs, 1979). Bulls and heifers should be separated by about 28 weeks of age (see Bonneau and Enright, 1995), since by 39 weeks, 93% of Hereford bulls were found to consistently mount oestrus females (Price and Wallach, 1991a). The exact role of testosterone on behaviour is not fully elucidated, however, the influence of gonadal steroids on behaviour on bull behaviour is well known (Blockley, Ade and Galloway, 1978; Jago *et al.*, 1995; 1996; 1997). For example, both oestradiol and testosterone administration to steers and masculinised heifers (freemartins) increased aggressive characteristics (Greene, Mogil and Foote, 1978; Dykeman, Katz and Foote, 1982; Huxsoll, Price and Adams, 1998). In contrast, reducing circulating testosterone concentrations below a threshold concentration in rams or bulls (Blockley, Ade and Galloway, 1978) by surgical castration or GnRH immunisation (Robertson, Wilson and Fraser, 1979; Finnerty *et al.*, 1996; Jago *et al.*, 1995; 1996; 1997; Huxsoll, Price and Adams, 1998) has been reported to reduce the expression of these behaviours. The observations of Blockley, Ade and Galloway, (1978) led them to conclude that testosterone was required for sexual behaviour. In contrast, a recent study (Inwalle and Schillo, 2002) found that castration of sexually experienced bulls fails to suppress mounting behaviour. This report agrees with findings in other species such as the dog (Beach, 1970) and man (Rousseau *et al.*, 1998), in which sexual experience delays the eventual loss of male sexual behaviour (copulation to the point of ejaculation) for months or even years (Meisel and Sachs, 1994). Based on this evidence, Inwalle and Schillo (2002) suggested three explanations. Firstly, the control of sexual behaviour may be androgen independent in the sexually experienced adult. Secondly, castrates may have increased sensitivity to low levels of adrenal androgens, which would explain the delay in loss of libido. Finally, neural systems involved in regulating sexual behaviour may be organised prior to birth, or in the neonatal period, and exposure to testosterone during this critical period may be essential for the normal expression of reproductive behaviours in the adult animal. Evidence in other species supports the latter explanation. In addition, Godfrey, Lunstra and Schanbacher (1992) concluded that steroidal programming of sexual behaviour occurs before one month of age in bulls. This was part of the reasoning behind the neonatal immunisation studies in Chapters 3 and 4 herein, as elevated GnRH antibody titres during month one of life may suppress gonadal steroid levels in addition to possibly permanently impairing the ability of GnRH neurons to release GnRH.

1.3 REPRODUCTIVE DEVELOPMENT, PUBERTY AND OESTROUS CYCLES

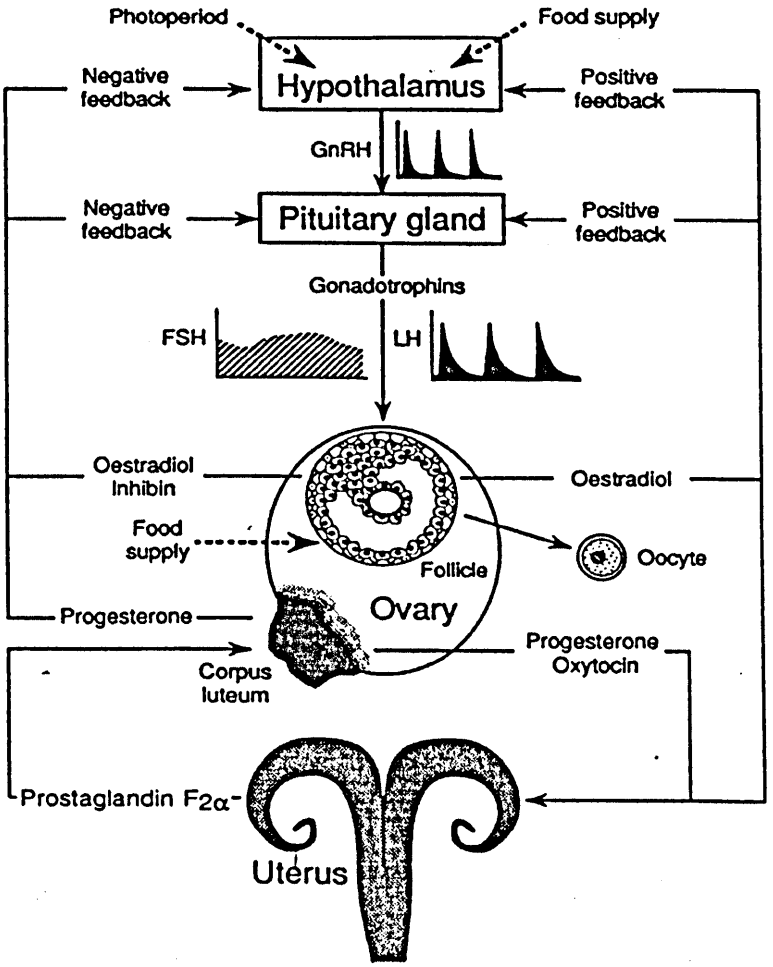
1.3.1 Endocrinology of the heifer calf

The pre-pubertal period in the heifer is characterised by an absence of cyclical activity and ovulation. Gradually, under stimulation of rising episodic LH and follicle stimulation hormone (FSH), developing follicles secrete increased levels of oestradiol (Ginther *et al.*, 2000). High amplitude, high frequency pulses of oestradiol stimulated mainly by LH, induce the pre-ovulatory gonadotrophin surge (see Peters, 1985). It is this combination of events that occur at puberty and are the culmination of a series of maturational changes within the hypothalamo-hypophyseal-gonadal axis leading to the overall induction and subsequent regulation of the oestrous cycle (see Figure 1.9).

Changes in episodic LH frequency have been studied, the lowest of which is apparent at one month of age (Schams *et al.*, 1981; Nakada *et al.*, 2002). There is then a gradual increase in LH pulse frequency from 4 months of age (Schams *et al.*, 1981; Day *at al.*, 1984), and LH amplitude and FSH from 1.5 to 6 months (Evans, Corrie and Rawlings, 1992; Evans, McNeilly and Webb, 1994; Honaramooz et al., 1999) with peak LH output occuring at about 50 days prior to puberty (Schams *et al.*, 1981; Day *at al.*, 1984; Day *et al.*, 1987; Evans, McNeilly and Webb, 1994). Surprisingly, McLeod *et al.* (1984) produced conflicting evidence, as they were unable to detect an increase in episodic LH release from birth to puberty in beef heifers. Other studies in cattle observed that as LH pulse frequency and amplitude increased as did mean circulating LH concentrations (Kinder, Day and Kittok, 1987; Day *et al,* 1984; Dodson *et al.*, 1988; Schams *et al.*, 1981). Similar findings have also been reported for ewe lambs (Rawlings and Churchill, 1990) and gilts (Lutz *et al.*, 1984).

Many studies have demonstrated that both pituitary gland and ovarian activity occurs throughout the pre-pubertal period (see Draincourt, 2001). For example, changes in the number of follicles have been examined in heifers slaughtered at intervals from birth to 12 months of age (Desjardin and Hafs; 1969). No antral follicles were visible macroscopically at birth, but their numbers appeared to increase to a maximum at 4-6 months, decrease at 8 months and remain constant thereafter.

Figure 1.9 The hypothalamic-pituitary-ovarian-uterine axis. The oestrous cycle is regulated by inter-relationships between the hypothalamic (GnRH) pituitary gland (FSH and LH), follicular (oestradiol and inhibin), luteal (progesterone and oxytocin) and uterine (prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)) hormones. GnRH drives the sequence of endocrine events that regulates the oestrous cycle. The subsequent release of gonadotrophins stimulates follicular development, ovulation and corpus luteum (CL) formation. Oestradiol, inhibin and progesterone have a negative feedback effect at the hypothalamic and anterior pituitary gland levels. Following ovulation, the remnants of the ovulated follicle, forms the CL, which secretes progesterone, which prevents ovulation and is required for pregnancy. In the absence of pregnancy, or maternal recognition of pregnancy, oxytocin from the CL causes luteolysis by releasing $PGF_{2\alpha}$ from the uterus and a new oestrous cycle ensues. External factors such as photoperiod and nutrition affect the oestrous cycle and ovulation at the hypothalamic level. Nutrition and metabolic factors also act at the ovarian level directly (after Scaramuzzi *et al.*, 1993).



Hopper *et al.* (1993) and Adams, Evans and Rawlings, (1994) observed that in pre-pubertal heifers, follicular development occurs in waves similar to that in post-pubertal heifers. However, the growing phase of follicles was shorter than that in post-pubertal animals. Progresterone levels are low in prepubertal heifers (Adams *et al.*, 1992), which are thought to prolong follicular growth. The authors suggested that this might be because the immature hypothalamic-pituitary gland unit (HPU) in pre-pubertal animals cannot produce the concentrations of LH found in mature animals, despite low progesterone concentrations. Indeed, a recent study (Nakada *et al.*, 2002) demonstrated an increase in LH response to GnRH challenge with age.

Large follicles of calves, age 12 weeks exhibit a ten-fold reduction in their ability to produce oestradiol, relative to cows, due to reduced aromatase activity of granulosa cells (Draincourt, Reynaud and Smitz, 2001). Quantitative differences in calf and cow follicular fluid proteins have also been reported, with more proteins in cow follicular fluid than in calves. In addition, calf follicular fluid is missing the 34KDa α inhibin subunit, although activin is relatively high in calf follicular fluid. However, further investigation is required to determine whether some of these differences can influence follicle and/or oocyte maturation (Draincourt, Reynaud and Smitz, 2001). Bergfeld *et al.* (1994) evaluated pre-pubertal ovarian development during the later pre-pubertal period and reported that dominant ovarian follicles were large as first ovulation approached, compared with earlier in the prepubertal period. This increase in follicle size was correlated with increased oestradiol-17β secretion. These changes may result from the decreased responsiveness of the HPU to oestradiol negative feedback, allowing increased LH and FSH secretion, subsequently resulting in follicle maturation. Furthermore, the largest increase in follicle size occurs during the last 30 days before puberty, which also corresponds to an increase in LH pulse frequency, although at this time FSH concentrations remain relatively stable (Evans, Adams and Rawlings), increasing only slightly if at all (Melvin *et al.*, 1999; Rawlings *et al.*, 2003). Bergfeld *et al.* (1994) concluded that increased LH pulse frequency may stimulate the development of dominant ovarian follicles in heifers promoting their continued growth and prolonged maintenance, which is in agreement with Foster and Ryan (1981), Adams *et al.* (1992) and Stock and Fortune (1993). This is the gonadostat or steroid dependent theory of puberty.

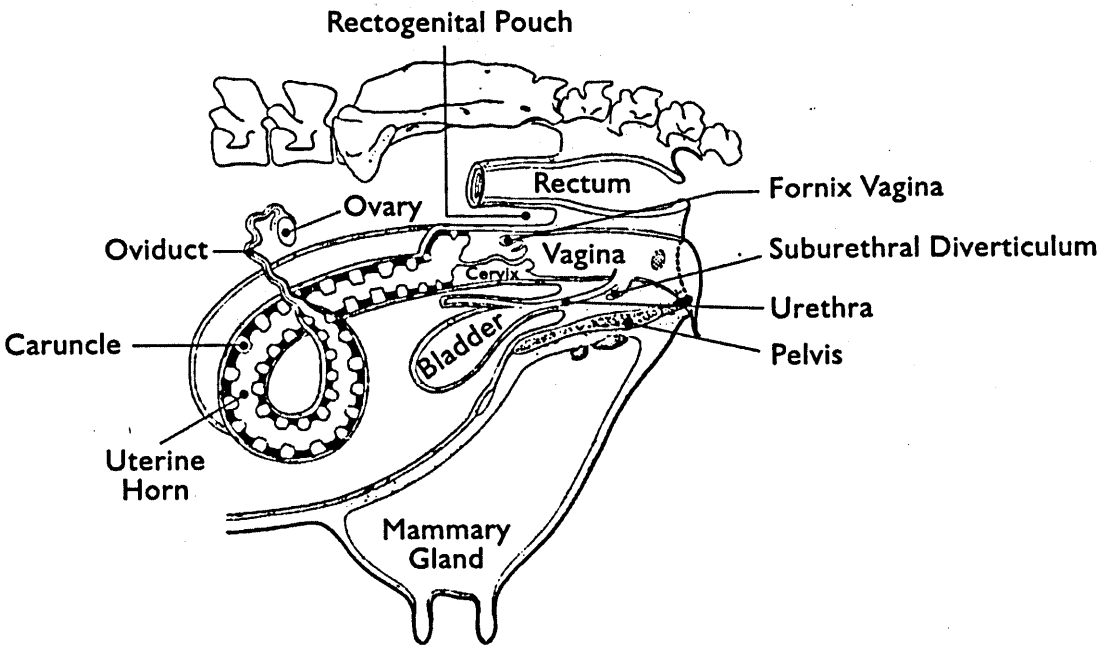
The hypothalamus is known to be the final component of the reproductive endocrine system to mature (Schams *et al.*, 1981; Foster *et al.*, 1986; Kinder, Day and Kittok,

1987). Hypothalamic maturation allows for the onset of function of the pituitary gland, ovaries and uterus in the adult. Changes occur in the hypothalamus that result in an increase in frequency of pulses of GnRH into the hypothalamic-portal vessels during peri-puberty (Rodriguez and Wise, 1989). Therefore, as concluded by Kinder *et al.* (1995) that prior to puberty GnRH is released, the pituitary gland can respond to GnRH by releasing LH and FSH and the ovary can respond to LH and FSH before puberty. This is shown by repeated administration of GnRH increasing LH pulse frequency, inducing follicular maturation and causing a preovulatory gonadotrophin surge in immature animals (Schams *et al.*, 1981; Foster, Ryan and Popkoff, 1984; McLeod *et al.*, 1985; Skaggs, Able and Stevenson, 1986). The uterus probably has the ability to respond to gonadal steroids, oestradiol and progesterone before puberty. However, limited release of GnRH from the hypothalamus results in maintenance of the prepubertal state until physical maturation of the animal is appropriate (Kinder *et al.*, 1995).

1.3.2 Maturation of the Reproductive Tract

Puberty is the result of a gradual process of sexual maturation, which is initiated prior to birth and continues throughout the pre-pubertal period of development. It is essentially a period of transition after which follicular development advances followed by ovulation and initiation of the first oestrous cycle. The reproductive tract of the cow is illustrated in Figure 1.10. Reproductive tract development is a gradual process and oestrus is not shown until 8-13 months of age (Evans, Adams and Rawlings, 1994a;b). Following monthly slaughter of Holstein heifers, ovarian weights were shown to increase to 4 months, plateau until 8 months, then increase further as heifers started to cycle and developed corpora lutea (Desjardins and Hafs, 1969). Maturation of the ovaries was found to occur between 6–12 months of age, although using ultrasound scanning, peak antral follicle number was observed recently at 4 months (Rawlings *et al.*, 2003). The uterus increases in weight from birth and though functional by puberty, continues to mature until at least 3 years of age (Willie, 1944). The results presented in Chapter 4 make a significant contribution to knowledge of follicular development, acquisition of puberty and associated hormone patterns in heifers.

Figure 1.10 A midsagittal view of the reproductive tract in the cow (modified from Ellenberger and Baum, 1943; after Senger, 1997). Note the proximity of the tract to the rectum.



1.3.3 Onset of Puberty

As described above, during the prepubertal period, gonadotrophin secretion is reduced. Although at present, endocrine mechanisms that result in onset of puberty still remain to be clearly defined (MacDonald and Page, 1986; Evans, Adams and Rawlings, 1994a;b), there are two theories on how this inhibition is exerted. The first is the classical "gonadostat" hypothesis, which is steroid dependent and the other is independent of sex steroids and attributed to "intrinsic" central nervous system inhibitory influences.

1.3.3.1 Gonadostat hypothesis- steroid dependent

This theory states that a decrease in sensitivity of the hypothalamus and pituitary gland centres, controlling gonadotrophin secretion, in response to oestradiol negative feedback is necessary for the onset of puberty (Ramirez and McCann, 1963). Indeed, decreased sensitivity to steroid negative feedback in the rat, allows increased pituitary gonadotrophin secretion, which subsequently results in a more pronounced follicular dominance and ovulation. Evidence to support this theory has been reported in lambs (Foster and Ryan, 1979), heifers (Schillo, Dierschke and Hauser, 1982; Day

et al., 1984) and gilts (Bernadinelli *et al.*, 1984), where exogenous oestradiol suppressed LH to non-detectable concentrations in ovariectomised (OVX) animals until beyond the onset of puberty in age-matched intact controls. Using OVX oestradiol supplemented heifers Day *et al.* (1987) went on to conclude, that in the pre-pubertal heifer LH secretion is responsive to oestradiol negative feedback, which decreases during the pre-pubertal period, because of a decline in the concentration of binding sites and/or oestradiol receptors in the HPU. This may be the mechanism responsible for the pre-pubertal decline in negative feedback of oestradiol on LH secretion.

1.3.3.2 Steroid independent hypothesis

Alternatively, since pre-pubertal rats shown no major decrease in sensitivity to oestradiol feedback after the first pre-ovulatory LH surge, Andrews, Advis and Ojeda, (1981) suggested that sensitivity to oestradiol negative feedback was a consequence, rather than a controlling mechanism, of pubertal onset. Indeed, certain aspects of the gonadostat theory may not be applicable to all species (Day *et al.*, 1984). Dodson *et al.* (1988) suggested that the heifer may be similar to the rat, but collection of data immediately prior to first ovulation is problematic because there is no indication of when ovulation will occur. Research to date indicates that, the preovulatory gonadotrophin surge, the increase in oestradiol that precedes the surge and the increase in progesterone after ovulation, are not responsible for initiating this change in sensitivity to ovarian steroids. All of these situations have been artificially induced in the pre-pubertal heifer, but none were followed by puberty (Gonzalez-padilla *et al.*, 1975; Barnes *et al.*, 1980; McLeod *et al.*, 1985; Skaggs, Able and Stevenson, 1986). Dodson *et al.* (1988) therefore postulated that the central mechanisms controlling the release of gonadotrophins are not responsive to ovarian steroids until just prior to puberty. Therefore puberty awaits the final development of some central component involved in gonadotrophin secretion.

1.3.4 The Post-pubertal Cow

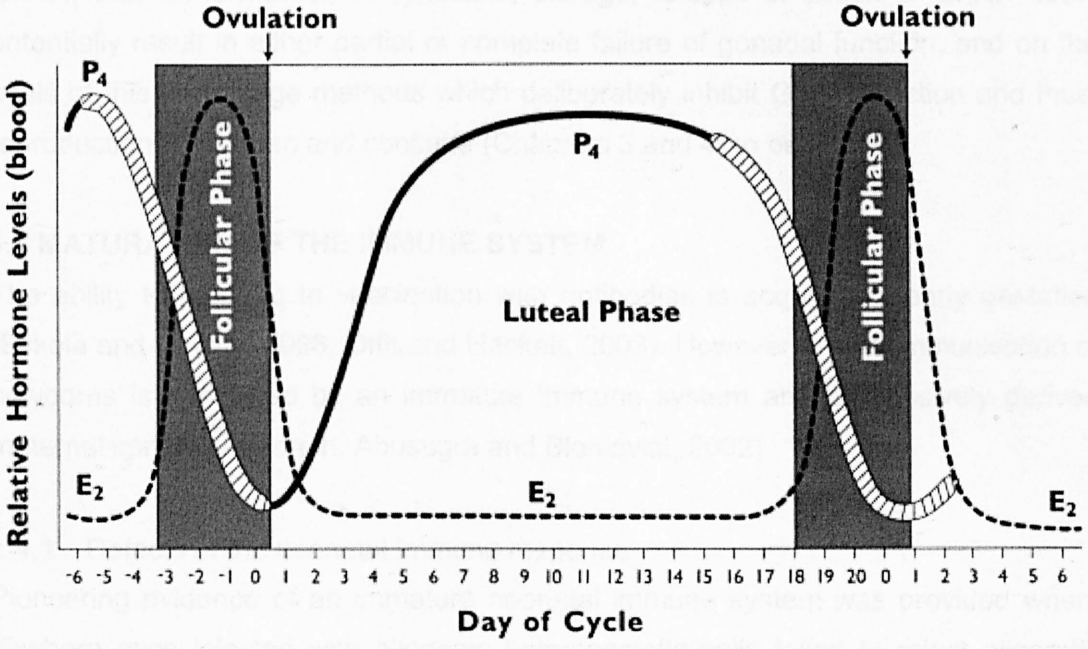
1.3.4.1 General oestrous cycle characteristics

Oestrous cycles occur about every 21 days in the non-pregnant cow with 84% of these cycles falling into a range of 18-24 days (Hafez and Sugie, 1963; Asdell, 1964; Robinson and Shelton, 1991). Each oestrous cycle may be divided into two phases; the follicular phase (regression of the CL, ovulation and the formation of the next CL) and the longer luteal phase (life of the CL). The external manifestation of the

oestrous cycle can be recognised by behavioural characteristics, the follicular phase by a short period of intense sexual activity or "oestrus", the luteal phase being a longer period of behavioural anoestrus.

The day on which behavioural oestrus is manifested is conventionally called Day 0 of the oestrous cycle. Oestrus is of short duration, approximately 18 hours (Trimberger, 1948). However, more recent investigation shows that the period of sexual receptivity can be considerably shorter than 18 hours (Hurnik, King and Robertson, 1975; Walton, Veenhuizen and King, 1987; Rodtian *et al.*, 1996). Walker, Nebel and McGilliard, (1996) used a sensor transmitter patch to monitor the period of oestrus and subsequently reported that oestrus usually lasted 9.5 hours, which is in close agreement with the results of Walton *et al.* (1987). Behavioural signs of oestrus include, restlessness (Liu and Spahr, 1993; Maatje, Loeffler and Engle, 1997), chin resting, increased licking, sniffing and mounting (Hurnik and King, 1987). However, standing to be mounted by others is the strongest sign that a cow is in oestrus (Williamson *et al.*, 1972; Foote, 1975). Several factors including parity, season, temperature, stage of lactation, breed, nutrition and stress can all influence the duration of oestrus (Trimberger, 1948; Gangwar, Branton and Evans, 1965; Wishort, 1972; Moberg, 1987; Lamothe *et al.*, 1995; Rodtian *et al.*, 1996).

Figure 1.11 Schematic changes in blood plasma hormone concentrations during the oestrous cycle (after Senger, 1997).



The end of oestrus is followed within 10-15 hours by ovulation (Schams *et al.*, 1977), approximately 28-31 hours after standing oestrus (Hunter and Wilmut, 1984). Ovulation is spontaneous, but can be hastened by mating (Marion *et al.*, 1950). Interestingly, the right ovary tends to ovulate more frequently, (~60% of ovulations) than the left (Asdell, 1964). Only one ovum (egg) is released in 96% of ovulations (Wiltbank *et al.*, 2000) in cattle.

1.3.4.2 Control of the ovarian cycle

The essential regulatory systems involved in the oestrous cycle are the endocrine system and the brain. Stimuli from the environment (smell, sight, food etc) are registered by the senses and transmitted to the central nervous system (CNS) via sensory neurones. The brain then responds accordingly by modulating gonadal function via the HPG axis. Numerous neuromodulators of GnRH neurones regulate GnRH output these include, neuropeptide Y, β-endorphin, galanin, γ-amino butyric acid (GABA), neurotensin, glutamate, 5-hydroxy-tryptamine, vasoactive intestinal polypeptide and noradrenaline. Molecular and neuroanatomical evidence suggests that oestradiol influences the activity of these 'regulators' within the hypothalamus to drive reproductive cyclicity. For a recent review see Smith and Jennes, (2001) and Goodman *et al.* (2002). As discussed all events of the ovarian cycle are controlled by a complex interaction of both local and systemic feedback mechanisms involving hormones secreted by the hypothalamus, anterior pituitary gland, ovaries and uterus (section 1.1). Importantly, the key regulating peptide of reproductive function is GnRH, thus abnormalities in synthesis, storage, release or action of GnRH could potentially result in either partial or complete failure of gonadal function, and on the basis of this knowledge methods which deliberately inhibit GnRH function and thus, reproduction have been and continue (Chapters 3 and 4) to be studied.

1.4 MATURATION OF THE IMMUNE SYSTEM

The ability to respond to vaccination with antibodies is acquired in early gestation (Eskola and Kayhty, 1998; Offit and Hackett, 2003). However, active immunisation of newborns is hampered by an immature immune system and by passively derived maternal immunity (Morein, Abusugra and Blomqvist, 2002).

1.4.1 Defects of the neonatal immune response

Pioneering evidence of an immature neonatal immune system was provided when, newborn mice injected with allogenic hematopoietic cells failed to reject allografts (Billingham, Brent and Medawar, 1956), and the concept of neonatal immune

tolerance was born. A dose-effect relationship between neonatal responsiveness and amount of antigen used in vaccines, provided evidence for Ridge, Fuchs and Matzinger, (1996) to argue that the limited numbers rather than immaturity of lymphocytes might be the cause of neonatal susceptibility to tolerance. Indeed, an under developed neonatal spleen and reduced bone marrow reserve is considered responsible for low numbers of APCs (B cells, dendritic cells (DCs) and macrophages) relative to adults (Kovarik and Siegrist, 1997). This is however, unlikely the sole cause of the limited and altered neonatal responses to vaccination as there are numerous reports of various defects of the neonatal immune system, which stem from the immaturity of a variety of neonatal immune cells (Petty and Hunt, 1998; Suen *et al.*, 1998; Pertmer *et al.*, 2001; see Bot and Bona, 2002) and therefore, it seems likely that both quantitative and qualitative factors are responsible.

1.4.1.1 Immature immune cells

Neonates have reduced capacity to express surface molecules (CD86 and CD40) (Marshall-Clarke *et al.*, 2000), which are required by APCs, most importantly DCs (Petty and Hunt, 1998), to signal to T cells via corresponding ligands (CD86L and CD40L). This signalling allows APC-T cell communication (co-stimulation), which is, in most cases, required for specific B cell (antibody) response. Furthermore, the lack of up-regulation of CD40 and CD40L by neonatal B cells causes impaired expression of IL-12. As a result the response of B cells and their ability to switch to different B cell classes (e.g., IgG, IgA and IgE) and subclasses (e.g., IgG1 and IgG2a) is, along with natural killer (NK) and T cell function, compromised (Durandy *et al.*, 1995). Moreover, the up-regulation of the Major Histocompatability-Class II (MHC-II) antigens on the surface of APCs is also reduced in newborns. MHC-II antigens are required to present the vaccine antigens (after the vaccine antigens have been enzymatically processed in the APC) to the T lymphocytes to induce the specific T cell response (Morein, Abusugra and Blomqvist, 2002). The reduced T cell function and reduced type 1 Helper T cell (Th1) cell stimulation in neonates results in sub-optimal release of 'pro-inflammatory cytokines' such a; IFN-γ and granulocyte-macrophage colony-stimulating factor, which induce macrophage, neutrophil and Th1 activation (Offit and Hackett, 2003). This leads to type 2 Helper T cell (Th2) biased immune responses (Adkins *et al.*, 1993; Suen *et al.*, 1998; Marshall-Clarke *et al.*, 2000). A bias towards Th 1 immunity in infant mice was reported following co-injection of IL-12 or IFN-γ-producing plasmids, an observation that elegantly pinpointed these cytokines as limiting factors during the neonatal period (Pertmer *et al.*, 2001). Opinions in similar neonate and adult T cell comparative human studies

are however, not in complete agreement, and, as exemplified above, accessory cell naivety and numbers (Martinez *et al.*, 1997), particularly DCs, B cells (Marshall-Clarke *et al.*, 2000) and macrophages (Suen *et al.*, 1998) are also responsible for typically reduced humoral immune responses.

1.4.1.2 Immune imbalance-T helper 2 bias

Placental progesterone, prostaglandin E and cytokines (IL-4 and IL-10; Rukavina and Podack, 2000) suppress Th1 inflammatory responses in the mother and foetus, and have evolved to evade immunological rejection of the foetus (Morein, Abusugra and Blomqvist, 2002; Offit and Hackett, 2003). Th2 cells release IL-4, which supports a preferential production of complement-independent IgG1 antibodies, whereas Th1 immune responses are characterised by production of complement-dependent IgG2a antibody (Mosmann *et al.*, 1995; 1997; Pertmer *et al.*, 2001) and cellular immune responses. The Th1-Th2 balance of the immune response may influence the mechanism of action through which, a vaccine to GnRH might work.

1.4.2 Maternally derived antibodies

Passive immunity, acquired entirely through ingestion of colostrum in calves, lambs, piglets and foals, provides protection against disease in early life. However, passive immunity can block the development of serum antibodies when vaccine immunogens are administered to calves with maternally derived antibodies (Husband and Lascelles, 1975; Chappius, 1998). In colostrum fed calves maternally derived antibodies peak 24h after birth, and although their half-lives range from 19-23 days (Brar *et al.*, 1978; Mechor *et al.*, 2001; Kirkpatrick *et al.* 2001; Fulton *et al.*, 2000), a comprehensive study of numerous viral antibody titres, including bovine herpes virus-1 (BHV-1), showed huge variation between individuals in maternally-derived antibodies, and individual differences in the age at which maternal antibodies become undetectable (69-299 days; Kendrick and Franti, 1974; Fulton *et al.*, 2004). Moreover, between species variation in time taken for titres to reach 1-3% of their initial value: 40 days for lambs, 60 days for pigs, 100 days for calves and 115 days for horses (Tizzard, 1977) has been reported, suggesting that some species may be less responsive to neonatal vaccination than others. The time required for cholostral antibodies to drop below detectable concentrations is dependent on amount of antibody ingested and absorbed (Fulton *et al.*, 2004), offspring status i.e., singleton or twin (Keeling and Crighton, 1984), immunoglobulin content, the growth rate of the infant, hence dilution in the circulation and the rate of clearance from the body (Chappuis *et al.*, 1998). In a possibly out-dated study of UK calf rearing, McGuire *et*

al. (1976) estimated that up to 40% of calves might have poor passive transfer of maternal antibodies and might therefore respond to primary vaccination (Fulton *et al.*, 2004). Obviously, the detrimental effects of passively acquired immunity on active immunisation boosters are reduced compared to the primary vaccination earlier in life, indeed a recent review of human infant studies (Glezen, 2003) concluded that maternally dervived antibodies have no effect on booster vaccinations. However, there is some evidence to suggest that even when maternally derived antibodies are undetectable, some blocking of immune responses may remain for a time (Morein, Abusugra and Blomqvist, 2002).

The relative importance of immunocompetence compared to blocking effects of maternally derived antibodies appears to vary with both species and individuals. For example, in contrast to the dog and cat (Chappius, 1998), immunological immaturity has a greater effect in diminishing the response to primary immunisation against viral vaccines in humans, than do maternal derived antibodies (see Glezen, 2003), and failure of low viral antibody titre calves to respond to modified live viral (MLV; Kirkpatrick et al., 2001), or inactivated viral vaccines (Fulton *et al.*, 2004), suggests that this may also be the case in cattle. Furthermore, Fulton *et al.* (2004) after extensive studies recently concluded that, only the transferred of viral antibodies of exceptionally high titre are likely to interfere with antibody response to vaccination in calves. However, most vaccination regimes, regardless of species, should consider the duration of maternal antibodies before the vaccinations of the infants commence. This has not been the case in the past (Morein, Abusugra and Blomqvist, 2002).

1.4.3 Acquisition of immune maturation

At birth the ability to respond is greatest for protein antigens (Glezen, 2003), as used in the studies herein. The ability to exhibit a response to vaccination similar to those seen in adults appears to, be acquired with increased age and body weight, and not surprisingly varies between species and vaccines (Morein, Abusugra and Blomqvist, 2002). In cattle, for example, Adams *et al*, (1996), failed to induce a detectable immune response (>10% at 1:1000) in 47% of 1.5 month old calves immunised against a GnRH-KLH conjugate in FCA. The proportion of responders increased to 80% at 4 months and 100% at 7 months of age. Pollock *et al.* (1991) observed a 2-3 fold increase in immune response to KLH in calves 5 months of age, relative to that noted in calves age 3 weeks. Previous studies showed similar responses (Hauser, Koob and Roth, 1986; Roden *et al.*, 1992). The only exception; showed that a moderate immune response (20% at 1:1,000) to a single GnRH immunisation at 1

month could not be improved upon when primary immunisation was given at 4 or 6 months (Huxsoll, Price and Adams, 1998). This finding is in conflict with the bulk of the literature and earlier reports using the same conjugate and adjuvant by the same research group (Adams *et al.*, 1996). Furthermore, (Huxsoll, Price and Adams, 1998) reported that, GnRH antibody titres were elevated for up to 11 months, after a single primary injection. However, sampling was very infrequent and again this is contrary to other reports (Robertson, Wilson and Fraser, 1979; Robertson et al., 1981; 1982; 1984; Jeffcoate, Lucas and Crighton, 1982; Jago et al., 1995; 1997; Finnerty, Enright and Roche, 1998), as antibody titres in bulls dissipate more rapidly than in rams (Jeffcoate, Lucas and Crighton, 1982). It must be considered that, due to differences between assays, GnRH antibody titre comparisons between studies are unlikely to be accurate. However, titres between studies in heifers and bulls in Chapters 3 and 4 for example, were assayed together and are therefore comparable. Other variables such as genotype (Evans et al., 1991a;b; Evans, McNeiily and Webb, 1994), health status, nutrition (Galyean, Perino and Duff, 1999) and breed also affect immune response.

1.4.4 Recent developments in neonate vaccination

Th2 biased immune responses have prevailed despite the use of a variety of vaccine approaches including, inactivated and live attenuated viral vaccines, live recombinant canary pox viral vaccines (Barrios *et al.*, 1996; Siergrist *et al.*, 1998), and peptides or proteins using conventional adjuvants such as FCA (Singh, Hahn and Sercarz, 1996) or alum (Barrios *et al.*, 1996). As a result, recent attempts to promote more balanced and effective immune responses have included, administration of recombinant cytokines, which caused fever-like side effects (Vogel and Hem, 2003) and DNA vaccination 'genetic adjuvants'. In the latter, a plasmid encoding a gene(s) for an antigenic portion of a virus is administered, resulting in the *in situ* expression of the antigen and antigen-specific immunity. Genetic adjuvants promote Th1 cytokine production and stimulate the structure of the neonatal spleen, where B and T cells and MHC-II cells develop (Pertmer *et al.*, 2001). DNA vaccination against herpes simplex gB antigen (Manickan, Yu and Rouse, 1997) induced a humoral immune response in neonatal mice despite the presence of maternal antibodies and the Th profile elicited by neonatal DNA immunisation comprises an important Th1 component (Bot *et al.*, 1997), resembling the adult response and generally contrasting with the neonatal immunity triggered by conventional antigens. In contrast to standard antigen vaccines, DNA vaccination viral genes are expressed in the host and viral proteins are synthesised before entering the host cells MHC-I pathway (only protens originating inside a cell are processed in this manner). MHC-I molecules then

carry the foreign protein to the cell surface, where they evoke cell-mediated immunity. As the protein encoded by DNA vaccine is produced endogenously and expressed in the context of self MHC-I, there is an increased risk of 'recognition of self', leading to tolerance, or autoimmunity (Mor, 1998).

It is becoming increasingly evident that newborn animals are immune competent albeit with a biased Th2 profile. Indeed, equine Herpes Simplex 2 viral antigen vaccination in 10 days old foals resulted in a potent immune response and protection against the virus (Morein, Abusugra and Blomqvist, 2002). Furthermore, Improvac® claims to be 100% effective after primary vaccination at 3-4 weeks (McCauley *et al.*, 2003). Recently, the view of a low competence of newborns to respond to active immunisation has gradually changed to realise that the competence might be low, but it is there, and can be used if the conditions of the newborn are considered (Chappius, 1998; Morein, Abusugra and Blomqvist, 2002).

1.5 IMMUNOCASTRATION

The rearing of intact animals for meat is associated with numerous problems, which are discussed in more detail in subsequent chapters. Therefore, they will only be briefly mentioned in this literature review. Sexual and aggressive behaviours in bulls (Robertson, Wilson and Fraser, 1979; Jago et al., 1995; 1996; 1997; Finnerty et al., 1996; Huxsoll, Price and Adams, 1998), boar taint and fighting lesions in pigs (Favlo et al., 1986; Awoniyi et al., 1988a;b; Claus and Weiler, 1994; Dunshea et al., 2001; Hohl et al., 2002; Metz et al., 2002; Zeng et al., 2002; Claus, 2003; McCauley et al., 2003; Thun et al., 2003) and unwanted pregnancies in extensively farmed heifers (Hoskinson et al., 1990) result in management and meat quality problems, and increased production costs. Several strategies have been employed to overcome these problems. In males these include, 'bloodless castration', whereby a Burdizzo (instrument) is used to crush the spermatic cord, which carried blood to the testes (Zobell, Goonewardene and Zeigler, 1993), rubber-ring castration (Blowey, 1993), short scrotum castration and intra-testicular lactic acid injections (Fordyce et al., 1989). In females the efficacy of fallopian tube ligation (D'Occhio, 1993) and intra-uterine device implants (Turin *et al.*, 1997; Fordyce *et al.*, 2001) have been investigated. All proved to be ineffective, inefficient and/or unethical (see Mepham and Forbes, 1995). As a result, many producers have continued to resort to surgical castration, despite the associated risks to animal health and life, and the compromise to animal welfare (Meleon et al., 1994; see D'Occhio, 1993; Bonneau and Enright, 1995; Fisher *et al.*, 1996; Thompson Jr, 2000).

1.5.1 Active GnRH Immunisation

The more recent approaches to have included immunisation against GnRH analogues (e.g. octapeptides), synthetic GnRH, GnRH fusion proteins and GnRH constructs (e.g., variations in amino acid sequence, such as tandem repeats; Meleon *et al.*, 1994; Sosa *et al.*, 2000). The use of these GnRH alternatives, have resulted in more prolonged, less varied and greater responses. However, it is difficult to make comparisons between experiments since the new generation of GnRH vaccines have replaced the highly potent Freund's complete and non-complete adjuvant with 'less aggressive' alternatives, which do not interfere with tuberculosis testing in cattle (Goubau *et al.*, 1989). Variations between trials in hapten, conjugate, adjuvant, vaccination schedule (number of boosters etc), dose of vaccine, administration, nutrition, health status, stress, climate, breed and gender, result in obvious limitations to between trial comparisons.

1.5.1.1 Antigen presentation / carrier conjugation

The small molecular weight (1183MW; Fraser, 1980) of GnRH does not induce immunogenicity. To raise antibodies against GnRH the immune system must be fooled into recognising GnRH as foreign (Thompson Jr., 2000; Ferro *et al.*, 2002a;b). Until recently, for the reliable production of GnRH antibodies, the peptide had to first be conjugated to a carrier immunogenic protein molecule (Fraser, 1980; Tables 1.1 and 1.2). However, chemical conjugation can be problematic as, the requirement for a repeatable homogenous chemical preparation from batch to batch (Roche and Crowe, 2004) is virtually impossible to obtain. More recently, a 'second generation' of vaccines has emerged whereby GnRH may be made immunogenic using a recombinant fusion protein into which specific hormone epitopes are incorporated into the sequence of the immunogenic protein (ovalbumin-GnRH; Sosa *et al.*, 2000; Roche and Crowe, 2004).

1.1.5.2 Vaccine formulation / adjuvants

The immune response to a given antigen is enhanced by the inclusion of an adjuvant, (adjuvare, to help or aid) i.e., a substance that non-specifically stimulates an immune response (Alexander and Brewer, 1995). Numerous adjuvants have been used in vaccines, but all work through three basic mechanisms: 1) effects on antigen delivery and presentation, 2) induction of immunomodulatory cytokines, and 3) effects on APCs (Weaver and Unanue, 1990; Vogel and Hem, 2003). As the immune system is antigen-driven, the simplest adjuvants simply slow the release of the antigen. Resulting in a prolonged immune response. To slow the rate of antigen clearance,

the antigen may be mixed with an insoluble antigen to form a "depot". Such adjuvants include, insoluble aluminium salts (e.g., crystalline aluminium oxyhydroxide (ALOOH)). Following the injection of such a compound a macrophage–rich granuloma is formed in the tissues. The antigen within the granuloma is slowly released into the body and so provides a prolonged antigenic stimulus (Tizard, 1982). Evidence of secondary depots forming at draining lymph nodes has been reported (Warren and Chedid, 1988). An alternative method of forming a 'depot' is to incorporate the antigen in a water-in-oil emulsion. The oil stimulates a local inflammatory response and granuloma formation, while the antigen is slowly leached from the aqueous phase of the emulsion. An adjuvant into which, killed mycobacteria are incorporated in a water-in-oil emulsion, is known as Freund's complete adjuvant (FCA). The active fraction of the mycobacteria, which enhances this activity, is known as muramyl dipeptide (n-acetyl-muamyl-L-ananyl-D-isoglutamine). FCA and FA are known to denature antigen, allowing recognition of internal antigen determinants (Kenney *et al.*, 1989). Other compounds with adjuvant activity include, bacterial endotoxins, anaerobic coryneforms, polyribonucleotides, sodium alginate, lanolin, vitamin A, (Tizard, 1982) saponin and purified saponin (Quil A; a complex mixture of triterpine glycosides; Alexander and Brewer, 1995).

The induction of modulatory cytokines by use of cytokines as adjuvants can induce unwanted side effects (Vogel and Hem, 2003). Therefore future focus on co-administration of a 'non-specific immunomodulation vaccine' such as a parapox virus vaccine with a GnRH construct might be advantageous in cattle. The application of poxviruses, are known to induce non-poxvirus specific immune reactions, which protect against non-poxvirus specific diseases. Poxvirus based vaccines possess immunomodulating capacity (formerly called "beneficial side effects") which stimulate production and release of numerous cytokines, chemokines, interleukins and immune cells. Alternatively, CpG oligonucleotide adjuvants are promising and new approaches, which 'redirect' immune responses that have a natural Th2 bias. Short sequences of bacterial DNA containing unmethylated cytosine and guanine dinucleotides, so called CpG motifs, trigger B cell activation and induce cytokine secretion, thus promoting Th1 response (Vogel and Hem, 2003).

1.5.1.3 Vaccine protocols

For both commercial application and animal welfare reasons, the number of vaccinations required should be kept to a minimum, preferably one (Stevens, 1993). Although this has been achieved in anti-disease vaccines, vaccines against "self"

antigens often require more administrations to cause a response of sufficient magnitude to induce the desired physiological effect. Furthermore, at each session, more than 2 injection sites are also agreed as generally unacceptable (Robertson, Wilson and Fraser, 1979). Despite claims of an effective "one shot" vaccine for GnRH in heifers and bulls (see Meloen, 1995), these results have not to our knowledge been confirmed. In contrast to the majority of publications, Adams *et al.* (1996) reported elevated titres and attenuation of testicular growth for up to one year following a single GnRH immunisation in prepubertal bulls.

Data suggests that the number of vaccinations may be reduced, by using a mixture of slow and fast releasing particles, to deliver a primary and booster vaccination at the same time. Fast dissolving particle provide the primary antigenic dose, while slow dissolving particles release their contents when a booster immunisation is required (see Stevens, 1993; Meloen, 1995).

1.5.2 Passive Immunisation Against GnRH

Passive immunisation is not a practical alternative to conventional castration in cattle due to the transient nature of the elevation in antibody titre, high volumes of antiserum required and associated costs. However, passive GnRH immunisation has proved to be a useful tool in scientific research in numerous species such as, the rat (Bercu *et al.*, 1977; Lincoln and Fraser, 1979; Bercu and Jackson, 1980), sheep (Keeling and Crighton, 1984; Millar, Fraser and Brooks, 1998; Parathasarathy *et al.*, 2002) and tammar wallaby (*Macropus eugenii*; Short, Flint and Renfree, 1985). In view of the difficulties encountered by researchers in inducing significant immune responses in a proportion of neonatal and young animals (Bell *et al.*, 1997; Fulton *et al.*, 2004), my opinion is that passive immunisation against GnRH may have an important role in determining when, if at all, the hypothalamic-pituitary gland unit is maximally susceptible to GnRH antibodies.

1.5.3 Physiological Effects of GnRH Immunisation

1.5.3.1 Prepubertal and adult animals

Immunisation of young and adult domestic animals against GnRH, regardless of gender, causes the cessation of reproductive function by preventing gonadotrophin production and release (Tables 1.1 and 1.2; Jeffcoate, Lucas and Crighton, 1982; Robertson, Wilson and Fraser, 1979; Robertson *et al.*, 1981; 1982; Schanbacher, 1982b; Wettermann and Castree, 1988; Adams and Adams, 1990; 1992; Hoskinson *et al.*, 1990; Carson, McCaughey and Steen, 1994, Brown *et al.*, 1994, 1995;

Table 1.1 Summary of previous GnRH vaccination studies in bulls

Author(s) year	Hapten	Carrier protein/ conjugating agent	Adjuvant	Dose / injection site
Robertson *et al.*, 1979; 1981	Synthetic GnRH (2.0mg)	Tetanus toxoid or Thyroglobulin carbodiimide (600mg)	FCA (primary) & FA (boosters)	2 x 2ml s.c.
Robertson *et al.*, 1982; 1984	Synthetic GnRH	HAS (10mg) Carbodiimide (600mg)	FCA & FA	2 x 2ml chest & brisket area
Jeffcoate *et al.*, 1982	Synthetic GnRH (0.1mg)	BSA Carbodiimide	FCA	8 x 0.6ml back
Goubau *et al.*, 1989b	Analogues; C1 & C10 GnRH	OVA (C1) & ESA (C10)	FCA or alternative adjuvants: Havlogen, RAS, DDA, Alhydrogel or Regressin	FCA (6 x 0.5ml; intra-dermal) or 2x 1-1.5ml i.m. neck
Finnerty *et al.*, 1991	GnRH	HSA	Not stated	Not stated
Adams & Adams, 1992; Adams *et al.*, 1993; 1996	GnRH (0.65mg)	KLH (1.0mg)	FCA	2 x 2ml Neck (dorsal)
Lobey *et al.*, 1992	GnRH; Octapeptide (3-10; 1.25mg)	Egg albumin/ carbodiimide	Morris's NUA & (Corybacterium parvum Primary only)	4 x 1ml near iliac nodes
Teague *et al.*, 1992	GnRH	HSA	Not stated	Not stated
Carson *et al.*, 1994	Synthetic GnRH	KLH / Carbodiimide (4.0, 1.0, 0.25, 0.05 and 0mg)	Alum (1.5ml) & Saponin (2mg)	2ml s.c. Brisket
Finnerty et al., 1994; 1995; 1998)	Synthetic analogue: Cys-Gly-GnRH	Has (analogue and HAS dose: 0, 0.1 or 1.0mg)	NUFCA (C. Parvum) or DEAE-D); FA or DEAE-D 1998	2x 1.5ml s.c. neck (brisket)
Jago *et al.*, 1995; 1997b	Carboxyl containing GnRH analogue	OVA	DEAE-D in mineral oil (Vaxstrate®)	2 x 2.5ml s.c. neck
Huxsoll *et al.*, 1998	GnRH (0.65mg)	KLH (1.0mg)	FCA & FA	2 x 1ml neck
Cook *et al.*, 2000	Recombinant fusion protein	Leukotoxin by P. haemolytica	Unknown, but 'commercially acceptable'	Neck
D'Occhio *et al.*, 2001	GnRH	OVA	DEAE-D, Arlocel 80 & Ondena mineral oil (Vaxstrate®)	Neck (dorsal)

Table 1.2 Summary of previous GnRH vaccination studies in heifers

Author(s) year	Hapten	Carrier protein/ conjugating agent	Adjuvant	Dose / injection site
Johnson *et al.*, 1988	GnRH	OVA carbodiimide	FCA (M. paratuberculosis), 6VR-(Quillaha Saponin(QS; 1mg/ml), 5% oil & 95% H_2O. M103-(QS; 63% OIL & 37% H_2O.	2 x 1ms.c. Dewlap
Wetteman & Castree, 1988	GnRH	HSA	FCA & FA	Not stated
Adams & Adams, 1990; Adams *et al.*, 1990	Synthetic GnRH	KLH	FCA & FA	2ml s.c. Neck (dorsal)
Hoskinson *et al.*, 1990	GnRH	OVA (Chicken)	DEAE-D, Arlocel 80 & Ondena mineral oil (Vaxstrate®)	5ml s.c. Neck
D'Occhio *et al.*, 1992	GnRH	OVA	DEAE-D, Arlocel 80 & Ondena mineral oil (Vaxstrate®)	Not stated
Sejrsen *et al.*, 1994	GnRH	HSA	Not stated	Not stated
Vizcarra & Wetteman, 1994	GnRH	OVA	FA & DEAE-D	2 sites Neck
Prendiville *et al.*, 1995a; 1996	Cys-Gly-GnRH	HSA	DEAE-D	2 x 2.5ml s.c. Neck
Prendiville *et al.*, 1995b	Cys-Gly-GnRH	HSA	DEAE-D &/or NUFA (C.parvum:primary)	2 x 2.5ml s.c. Neck
Finnerty et al., 1994; 1995; 1998)	Synthetic analogue: Cys-Gly-GnRH	Has (analogue and HAS dose: 0, 0.1 or 1.0mg)	NUFCA (C. Parvum) or DEAE-D); FA or DEAE-D 1998	2x 1.5ml s.c. neck (brisket)
Jeffrey *et al.*, 1997	GnRH	OVA	DEAE-D, Arlocel 80 & Ondena mineral oil (Vaxstrate®)	5ml s.c. Neck
Bell *et al.*, 1997	GnRH	KLH	FCA	2 sites Neck
Sosa *et al.*, 2000	GnRH-7 (recombinant protein)	GnRH-OVA (recombinant)	Z-Max adjuvant, His-Tag compatible	Intra-mammary
Crowe *et al.*, 2001a;b	Cys-Gly-GnRH	HSA	DEAE-D	2 x 1.5ml s.c. Neck (brisket)

Prendville *et al.*, 1995a, 1995b, 1996; Clarke *et al.*, 1998; Finnerty *et al.*, 1991; Finnerty, Enright and Roche, 1998; Cook *et al.*, 2000; D'Occhio, 1993; 1994; D'Occhio, Aspden and Trigg, 2001). Subsequently, in the post-pubertal male, testicular size is reduced, while prepubertal animals exhibit arrested and/or reduced growth rates (Jeffcoate, Lucas and Crighton, 1982; Lobey *et al.*, 1992; Adams *et al.*, 1993; 1996; Finnerty *et al.*, 1994; Jago *et al.*, 1995; Huxsoll, Price and Adams, 1998; Cook *et al.*, 2000). As a result spermatogenesis is reduced (Robertson, Wilson and Fraser, 1979; Carson, McCaughey and Steen, 1994; Cook *et al.*, 2000), testosterone concentrations fall (Finnerty *et al.*, 1994; Jago *et al.*, 1995; Finnerty, Enright and Roche, 1998; Huxsoll, Price and Adams, 1998; Cook *et al.*, 2000) causing reduced accessory gland growth and/or function. Libido is also inhibited and more docile (steer like) behaviours ensued (Robertson, Wilson and Fraser, 1979; Robertson *et al.*, 1981; 1982; Finnerty *et al.*, 1996; Jago, Bass and Matthews, 1997; Huxsoll, Price and Adams, 1998).

In the female, follicular development is arrested or reduced (Johnson *et al.*, 1988; Adams and Adams, 1990; Prendiville *et al.*, 1995a; 1996; Crowe *et al.*, 2001a; b) and ovaries (Johnson *et al.*, 1988) and uterii are reduced in size (Adams and Adams, 1990). Due to the absence of oestradiol, animals become anoestrus and ovulation is inhibited (Johnson *et al.*, 1988). However, early studies in adult animals indicated that the suppressive effects of GnRH immunisation were reversible, at least to some extent, with time in cattle and sheep (Robertson *et al.*, 1981; 1982; Keeling and Crighton, 1984; Wetteman and Castree, 1988; 1994).

Anabolic effects have also been reported and are discussed in more detail in Chapters 3 and 4. Briefly, the evidence for an anabolic effect of GnRH immunisation is often conflicting in some studies reduced growth rates relative to bulls were found (Lobley *et al.*, 1992; Cook *et al.*, 2000), but not in all trials (Robertson *et al.*, 1982; Huxsoll, Price and Adams, 1998). Furthermore, Adams *et al.* (1993a; 1996) observed improved feedlot gain in bulls immunised against GnRH at 3.6 months of age. As discussed comparisons are difficult between trials due to the myriad of factors, which can cause variations e.g., vaccine response, the extent of immunosuppression, interval from last vaccination to slaughter, nutrition, breed, stress, climate etc. Furthermore, comparisons are made more difficult by variations between research groups in the parameters used to assess carcass characteristics and the fact that some parameters are subjective, and based only on observer opinion. Generally, high responding animals, with suppressed reproductive function, tend to have more

'steer-like' growth rates and carcass compositions, whereas poor responders have bullish characteristics (Lobley *et al.*, 1992; Thompson Jr, 2000). Most immunocastrates show growth and carcass compositions intermediary between bulls and steers (Jago *et al.*, 1995). For example, average daily gains (ADG) for intact bulls, immunocastrates and steers are typically, 0.91, 0.88-0.91 and 0.78-0.81 kg day^{-1}, respectively (Robertson *et al.*, 1982; Bonneau and Enright, 1995).

1.5.3.2 Neonatal immunocastration

Neonatal passive GnRH immunisation studies in rats demonstrated that normal development of pituitary (Bercu and Jackson, 1980) and gonadal function requires the presence of GnRH from day one of life (Bercu *et al.*, 1977). GnRH immunisation led to a permanent impairment of seminiferous tubular size and function, although puberty was not delayed and adult gonadotrophin and testosterone concentrations were similar to control animals. Sexual behaviour was also unaffected, however the authors found in-complete catch-up of penile and vesicular gland growth (Bercu *et al.*, 1977), and probably as a result of the latter, a reduced volume of ejaculate (Bercu and Jackson, 1980). Bercu *et al.* (1977) stated that a decrease in neonatal FSH could be of key importance to gonadal function. Furthermore LH antiserum treatments (Goldman *et al.*, 1972) resulted in adults with reduced penis size and poor mating behaviour, but typical testicular weights. This indicates that neonatal deficiency in LH and/or testosterone does not affect adult testicular size in the rat (Bercu *et al.*, 1977). Although passive immunisation in neonatal rats permanently affected sexual development in males and females (Bercu *et al.*, 1977; Bercu and Jackson, 1980; Vilchez-Martinez and Martin de Lopez, 1980), Keeling and Crighton, (1984), concluded that, similar effects would be unlikely in sheep as growth and sexual development proceeded at the same rate in lambs born to control and GnRH immunised ewes. However, the studies in rats employed high titre antiserum and it remains to be seen whether the levels of antibodies, which were present in colostrum would be sufficient to impair reproductive development in this species (Keeling and Crighton, 1984). It should be considered however that, although intake might vary, colostrum IgG concentrations are four-fold higher than in serum just prior to parturition (Halliday, 1974). The effects observed in the neonatal rat can probably be attributed to the fact that, differentiation of the neural tissues in both sexes occurs during neonatal life (Harris, 1964). Thus, blocking GnRH during this critical period inhibits differentiation of the HPG axis. In contrast, these events occur prenatally in the sheep (Short, 1974; Caldani *et al.*, 1995) and consequently ingestion of GnRH antibodies neonatally is unlikely to affect subsequent patterns of HPG axis

development (Keeling and Crighton, 1984). The situation in other species, such as the cow, appears similar (Wise, Rodgriguez and Kelly, 1987), but remains to be studied in detail.

Keeling and Crighton, (1984) conducted several experiments and found that, some sheep appeared to be particularly responsive to GnRH vaccination, as reproductive function could be inhibited 2 years after immunisation when antibody titres were undetectable, however, the majority of rams returned to fertility between 6-12 months after immunisation, and similar findings were reported in ewes, although the period of in fertility was more variable (Keeling and Crighton, 1984).

More recently, active immunisation against GnRH in male lambs, at 3-4 (neonatal) and 20-21 (peri-pubertal) weeks of age, resulted in long-term suppression of reproductive function. A proportion of rams had juvenile-like reproductive organs and a complete lack of libido 2 years after GnRH vaccination, when antibody titres are undetectable (Brown *et al.*, 1994). However, low gonadotrophin output in most GnRH immunised rams was sufficient to allow exhibition of some sexual behaviour and gonadal function (including spermatogenesis) by 2 years of age (Brown *et al.*, 1994). A follow up study (Brown *et al.*, 1995) published results of an almost identical trial carried out on ewe lambs. Gonadotrophin concentrations in the majority of immunocastrated ewes were insufficient to support reproductive function by two years. Both the degree and longevity of reproductive suppression appeared more severe in GnRH immunised ewes. The fact that the ewes appeared to be more susceptible to the effects of GnRH immunisation than the rams (Brown *et al.*, 1995), may indicate a gender difference in the "window of maximum susceptibility to GnRH antibodies", which corresponds to sex differences in the maturation of the HPG unit. Alternatively, this observation may indicate that basal gonadotrophin output is adequate to maintain reproductive function in males, but not females. More recently still, Clarke *et al*, (1998) studied 3-4 year old sheep that had previously taken part in a neonatal GnRH immunisation programme (Brown *et al.*, 1994, 1995). Following a GnRH challenge at 90 weeks of age by which time anti-GnRH titres had dissipated the pituitary gland was unresponsive to GnRH, producing abnormally low gonadotrophin levels. This suggests that GnRH vaccination induces some degree of impairment to pituitary function, resulting in a reduced capacity of the gland to either respond to GnRH (e.g., down regulation of GnRH receptors) or in reduced synthesis and / or release of LH and FSH (Brown *et al.*, 1994). Many of the sheep displayed a permanent suppression of reproductive function. The authors concluded that

neonatal GnRH immunisation may not be reversible in all treated animals (Brown *et al.*, 1994; 1995; Clarke *et al.*, 1998). A recent GnRH immunisation study in Zebu bulls (D'Occhio, Aspden and Trigg, 2001) found that a similar proportion of animals to the earlier trial in rams (Brown *et al.*, 1994) presented with a long-term suppression of reproductive function. Furthermore, the age (6 vs. 22 months) and status of the bulls (pre- and post-pubertal) had no influence on the proportion of animals, which failed to regain reproductive function.

1.5.4 GnRH immune response: mechanism(s) of action

GnRH only travels a few millimetres in the hypophyseal portal blood vessels before reaching its primary target, the pituitary gland receptors (see McArdle *et al.*, 2002; section 1.3.1.3). Antibodies could therefore act at the level of the gonadotrophin cell membrane, (Fraser, 1980), or they may even gain entry to the median eminence (Hokfelt *et al.*, 1976), as GnRH neurons terminate within the median eminence (ME) and thus, are outside the blood-brain barrier (Molenaar *et al.*, 1993; D'Occhio, Aspden and Trigg, 2001). Furthermore, hypothalamic neurons are capable of taking up macromolecules such as horseradish peroxidase, dextran and latex beads from the blood in to the ME. These substances are then transported retrograde to their perikarya (Van der Krans and Hoogland, 1983; Gross *et al.*, 1986; Leong and Ling, 1990; Molenaar *et al.*, 1993). GnRH antibodies in solution are therefore likely to gain access to GnRH neurones (Molenaar *et al.*, 1993). Studies by Brimijoin and Lennon, (1990) and Tilders *et al.* (1990) demonstrated that direct exposure of neurones to antibodies against their own secretion products, results in the destruction of the neurone or their processes. However, no reports on the effects of GnRH antibodies *in vitro* on ME GnRH neuron terminals have been published. Recent findings strengthen the evidence that the basal hypothalamus- ME is a primary target site for anti-GnRH antibodies. Molenaar *et al.* (1993) studied the hypothalami of 25 boars, selected on the basis of a testes response to GnRH immunisation, using histological and immuno-cytochemical techniques. When compared to control animals, they reported: dystrophy in the perikarya and reduced immunocytochemical activity in the GnRH neuron terminals within the ME, thus indicative of impaired ability of the neurons to release GnRH. Also inflammation (oedema, capillary collapse, fibrosis and fibroblast infiltration), neurosecretum accumulation and hypertrophy of magnocellular neurons were witnessed at the ME, suggesting damage to GnRH neurons resulting from immune cell infiltration. The lesion incidence was correlated to the GnRH and physiological response to vaccination, as indicated by GnRH titres and gonadal size and function (Molenaar *et al.*, 1993). Furthermore, these lesions were restricted to the

ME and did not involve other parts of the hypothalamus, suggesting that GnRH antibodies and immune cells do not cross the blood brain barrier, but were entering neurons at terminals. This observation is surprising in view of a recent publication by Namer and Steibel (2000). These authors reported that Freund's complete adjuvant (FCA), as used by Molenaar *et al.* (1993), contains a compound called mannan, which arises from the cell wall of *Mycobacterium tuberculosis*. Administration of FCA raises anti-mannan antibodies, which induce an immediate, but reversible breakdown of the blood brain barrier in rats (Namer and Steibel, 2000).

Studies on the effects of GnRH immunisation in neonatal cattle are absent and for this reason and the evidence of increased HPU susceptibility to GnRH antibodies during the neonatal period the study in Chapter 3 was conducted. Furthermore the Chapters 3 and 4 trials take a more holistic approach in assessing the effects of vaccination than the majority of publications and investigates the long-term vaccination responses before, during and well beyond puberty simultaneously in both bulls and heifers, which again is seen to be lacking in the current literature. Studies into how GnRH antibodies act to suppress reproductive function, i.e., the mechanism(s) of action and the effects of GnRH antibodies on GnRH neurons are also lacking, despite 25 years of GnRH immunisation in cattle.

1.6 PROLIFICACY

Farmers and scientists have adopted numerous approaches in attempts to increase litter size in sheep and twinning rates in cattle. A thorough review of all these approaches is however, not possible due to the limitations of this thesis. Therefore the approaches will be summarised and the reader referred to relevant review publications where necessary. Although inhibin immunisation will be reviewed in more detail, the review is by no means exhaustive as this approach to increasing prolificacy is discussed further in Chapters 5 and 6.

1.6.1 Nutrition and Metabolic Hormones

The enhanced ovine follicular development and ovulation rates observed after dietary improvements (see Robinson, 1990), i.e., nutritional 'flushing' has been reported to occur by increasing gonadotrophin release (Smith, 1988). However, Downing and Scaramuzzi (1991) found that enhanced follicular development occurred independently of changes in peripheral FSH levels. Therefore it was concluded that the increase in follicular number must be under the control of factors other than those of the hypothalamo-pituitary gland-ovarian axis. Numerous metabolic factors have been implicated in modulating folliculogenesis at the ovarian level (see O'Callaghan

and Boland, 1999; Webb *et al.*, 1999c; Webb *et al.*, 2003 in press). Diets which increase insulin concentrations have resulted in an increased up-take of glucose, which may reduce the capacity of the dominant follicle to suppress the development of subordinate follicles, thus leading to increased ovulation rates (Scaramuzzi, 1994). Insulin like growth factor-I (IGF-I) is synthesised and secreted by a range of cell types, but the liver is acknowledged as the primary producer of this factor. As discussed IGF-I has been shown to stimulate cell proliferation and hormone production in both granulosa and theca cells *in vitro*, and stimulated ovarian follicular development and ovarian androgen and oestradiol secretion *in vivo* (Campbell, 1999). Furthermore, rbGH treatment increased the numbers of 2.0 - 5.0mm and the 2.1-3.0mm follicle populations in heifers (Gong, Bramley and Webb, 1991; 1993; Gong *et al.*, 1996c; 1997) and ewes (Gong *et al.*, 1996b), respectively. Growth hormone (GH) is believed to act by increasing peripheral concentrations of IGF-I and insulin (Gong *et al.*, 1997). Rather than by a direct effect at the ovarian level, since follicle GH receptors have been difficult to detect (Lucy *et al.*, 1999; Lucy, 2000). Species differences in the response to recombinant GH (rGH) have been demonstrated. For example, following rGH treatment of heifers follicle recruitment is enhanced due to an increase in the number of small follicles and the total antral follicle pool increased, whereas in ewes rGH treatment enhanced the development of follicles from the gonadotrophin-sensitive pool into the gonadotrophin dependent stages, resulting in an increase in the number of medium-sized follicles without affecting total antral follicle number (Gong *et al.*, 1996b;c). Multiple mechanisms are involved in the regulation of folliculogenesis and the relative importance of each mechanism may be species specific (see Webb *et al.*, 1992b; Campbell, Scaramuzzi and Webb, 1995).

In a twinning population established at the Meat Animal Research Centre (MARC) in Nebraska the current rate of multiple ovulation is approximately 25% in 12-18 month old heifers, and the current twinning rate for the herd exceeds 35% (Echternkamp and Gregory, 1999a;b). Current results suggest that a gene(s) exists on chromosome 5 that affects ovulation rate in cattle (Kappes *et al.*, 2000), i.e., close to the IGF-I gene (Lien *et al.*, 2000).

Studies of follicular growth in these cattle genetically selected for twinning lend some support to an IGF-I role. Echternkamp *et al*, (1990b) demonstrated that, multiple ovulating or twin bearing cows have increased serum and follicular fluid IGF-I, and it was reported that these cattle have increased follicles ≥4mm (Echternkamp *et al.*,

1990a; Echternkamp, 1992; 1993; Echternkamp and Gregory, 1998), indicative of enhanced recruitment. In contrast, Cushman *et al.* (2000) concluded that, dams predisposed to yielding twin calves do so by keeping more follicles growing during the recruitment and selection processes, rather than by activating more follicles from the primordial or gonadotrophin-sensitive pools.

1.6.2 Hormone Administration

Experiments using exogenous hormone treatments have also been carried out in attempts to increase ovulation rate (see Armstrong, 1993). Ovarian activity has been stimulated using porcine FSH (Hasler *et al.*, 1983; Takedomi *et al.*, 1995b) or follitropin (an FSH preparation with 80% LH activity removed; Wu *et al.*, 1988), equine chorionic gonadotrophin (eCG) with (Wang *et al.*, 1988; Zeitoun *et al.*, 1988; Dieleman *et al.*, 1993; Silva, da Casta and Silva, 2002), or without (Monniaux, Mariana and Gibson, 1984; Boland, Goulding and Roche, 1991; Goulding *et al.*, 1996) anti-eCG antibodies. FSH treatment increases the window of opportunity in which gonadotrophin dependent follicles may develop, while eCG may also prevent atresia and enhance recruitment (see Scaramuzzi *et al.*, 1993). However, variation in response between animals was huge (see Monniaux, Chupin and Saumande, 1983; Boland, Goulding and Roche, 1991) and therefore, such treatments are only useful to induce multiple ovulation for embryo transfer programmes. Interestingly, pre-treatment of heifers with either rbGH enhanced the superovulatory response and quality of embryos to FSH treatments in cattle (Gong *et al.*, 1996c; Nolan *et al.*, 1998; see Armstrong, Gong and Webb, 2003) and sheep (O'Callaghan *et al.*, 2000).

1.6.3 Steroid Immunisation

Scaramuzzi, Davidson and Van Look, published a pioneering report in 1977, stating that active immunisation against androstenedione increased ovulation rate in Welsh mountain ewes. In 1983, a vaccine against androstenedione was released commercially as Fecundin®. The vaccine increased LH secretion, but had little or no affect on FSH (McNatty *et al.*, 1988; Campbell *et al.*, 1991a; see Scaramuzzi *et al.*, 1993). Therefore, androstenedione immunisation only affects large antral follicles (Scaramuzzi *et al.*, 1980; Draincourt, Cahill and Bindon, 1985), possibly, increasing ovulation rate by reducing atresia (Bister *et al.*, 1999), although not proven. However, low concentrations of biologically active androgen may have an effect, which is independent of that of LH (see Scaramuzzi *et al.*, 1993; Terqui *et al.*, 1995). Several other steroids including oestradiol, oestrone and testosterone, or combinations have been targeted in immunomodulation studies (see Scaramuzzi and Hoskinson, 1984;

Smith, 1985; Philipon and Draincourt, 1987; Croker *et al.*, 1988; Scaramuzzi *et al.*, 1993; Terqui *et al.*, 1995).

Attempts to induce twinning in cattle through immunisation against steroids have been less successful than in sheep (see Hillard *et al.*, 1995). For example, the effects of androstenedione immunisation in heifers are conflicting. Wise and Schanbacher (1983) reported increased twinning rates, whereas others (Sreenan, 1984; Walton, 1985; Hoskinson *et al.*, 1986) observed ovarian dysfunction. In addition, active immunisation against testosterone increased ovulation rate, but was associated with a high incidence of anoestrous (Price, Morris and Webb, 1987).

1.6.4 Inhibin Active Immunisation in Sheep and Cattle

Cummings *et al.* (1983) found the ovaries of the prolific Booroola Merino to be deficient in bioactive inhibin. As a result, it was postulated that immunoneutralization of inhibin would lead to elevated levels of FSH, which during the follicular phase, would increase the number of large, ovulatory follicles.

1.6.4.1 Inhibin active immunisation in the ewe

The earliest attempts to immunise animals against inhibin utilised partially purified fractions of follicular fluid as immunogens. Heterologous follicular fluid was used in sheep and cattle, to enhance the immunogenic response relative to homologous follicular fluid (see Findlay *et al.*, 1993). The fluid was stripped of steroids using either charcoal (Henderson *et al.*, 1989) or subjected to chromatography on a Matrex Red A dye column (O'Shea *et al.*, 1984; Henderson *et al.* 1989) or immuno-affinity column (O'Shea *et al.*, 1989b; Findlay *et al.*, 1993) to increase the inhibin activity. Following immunisation in the ewe, researchers reported increased ovulation rates and litter size (O'Shea *et al.*, 1982; 1984; Henderson *et al.* 1984; Cummins *et al.*, 1986). In cattle, ovine follicular fluid preparations also increased ovulation rate (Cummins, O'Shea and Bindon, 1986; Price *et al.*, 1987b; Bindon *et al.*, 1988). However, FSH response was variable and inconsistent (Al-Obaidi *et al.*, 1987a:b) as was ovulation rate. This is important because elevated FSH in some animals, and no increase in FSH in others may be the reason for variations in the number of recruited follicles and ultimately ovulation rate. The relative impurity of the immunogen was held responsible for this variation in response (O'Shea *et al.*, 1989b). As a result some researchers began producing inhibin subunits by recombinant DNA technology.

Fusion proteins expressed by the bacterium Escherichia Coli (E. Coli), comprising either the bovine (Forage *et al.*, 1987a:b) or human α-subunit, have been used as

immunogens in sheep (see Findlay *et al.*, 1993). Ovulation rates of 2-4 (Border Leicester x Merino) and 4-6 (Rambouillet) times those of controls were reported using bovine α_{43}-subunit (Forage *et al.*, 1987a; Tsonis *et al.*, 1989) and human recombinant inhibin (Mizumachi *et al.*, 1990), respectively. The latter study reported elevated FSH (pre- and post-ovulation), but not LH concentrations, and an extended FSH surge. Furthermore, these increases in ovulation rates equated to a 60 - 70% increase in lambs born (Tsonis *et al.*, 1989; Findlay *et al.*, 1993) after only a primary vaccination (Tsonis *et al.*, 1989; see Terqui *et al.*, 1995). Findlay *et al.* (1989) reported increased basal and GnRH stimulated FSH release during the luteal phase, while LH was unaffected. Neither gonadotrophin was affected by immunisation during anoestrus. A positive correlation between inhibin antibody titre and ovulation rate was noted in some studies (Tsonis *et al.*, 1989; Findlay *et al.*, 1989), although Forage *et al.* (1987a;b) found no such relationship.

Other research teams made synthetic porcine (Schanbacher, 1988a; Schanbacher, Schemm and Rhind, 1991), ovine (Meyer *et al.*, 1991; Wheaton, Carlson and Kusina, 1992) and bovine (Wrathall *et al.*, 1990, 1992) α-subunit and peptide fragments (based on N-terminal portion) conjugated to carrier proteins to enhance immunogenicity. Wrathall *et al.* (1990) demonstrated that immunisation against the bovine synthetic peptide resulted in antibodies which bound native (32kD) inhibin, not just the labelled peptide as other authors reported (O'Shea *et al.*, 1989c; Wheaton, Carlson and Kusina, 1992). Following immunisation with synthetic peptide vaccines, 2-4 fold increases in ovulation rate were typical (see Terqui *et al.*, 1995). Puberty was eventually advanced in the Booroola Merino (O'Shea *et al.*, 1989a), despite failure in pubertal advancement in earlier trials (O'Shea *et al.*, 1987; see Findlay *et al*, 1993). However, as with the earlier 'crude' vaccines, changes in serum gonadotrophin concentrations were still inconsistent between studies (see O'Shea *et al.*, 1994). For example, during the breeding season, immunisation (Romney) against an N-terminal (1-29) bovine inhibin α-subunit (B-In-α-N-1-29) tuberculin conjugate, resulted in a doubling of ovulation rate and a 37% increase in FSH, relative to controls, around the time of recruitment (Wrathall *et al.*, 1990). During seasonal anoestrus, the number of pre-ovulatory, oestrogenic antral follicles that ovulated following GnRH treatment increased (McLeod *et al.*, 1992). In Mule ewes, immunised against B-In-α-N-1-29, ovulation rates also increased two fold and FSH levels increased 25% compared with controls, after the second booster. Furthermore, immunisation increased the number of lambs born per ewe mated by 37%. Although immunisation had no adverse affects on conception rate and gestation length, lower birth weight and more 'stillborns' were

presented at lambing, thus no increase in viable lamb crop was attained (Wrathall *et al.*, 1992). In contrast, active immunisation against porcine α-subunit in Suffolk ewes had no affect on FSH levels, although FSH release patterns differed relative to controls. In addition the amplitude of the LH surge was reduced (Schanbacher, Schemm and Rhind, 1991). This may have been due to increased negative feedback of ovarian steroids.

Following inhibin immunisation in Romney ewes, LH pulse amplitude and frequency were unaffected indicating that inhibin does not inhibit LH, although some limited inhibition of basal LH was reduced, as was LH response to GnRH challenge (Wrathall *et al.*, 1990). However, increased ovarian steroid release, due to increased follicle numbers, may be responsible for these changes. For example, plasma progesterone levels are elevated during the luteal phase due to more CLs in some (Wrathall *et al.*, 1990; McLeod *et al.*, 1992; see Terqui *et al.*, 1995), but not all (Campbell *et al.*, 1995) inhibin immunisation experiments.

In long-term inhibin immunised ewes, elevated FSH eventually fell back to control levels (Knight *et al.*, 1991). Fray, Wrathall and Knight (1994) observed that following (B-In-α-N-1-29) immunisation, the ewes from an earlier trial (Wrathall *et al.*, 1992) exhibited detectable antibody titre and increased lambing rates several years after the last booster. Interestingly, however, increased FSH output in treated ewes only remained elevated above that of controls for the first breeding season. This strengthens the case for an intra-ovarian role of inhibin (see Baird and Smith, 1993; Chapters 5 and 6), independent of the established long-loop negative feedback effect on pituitary gland FSH release (Kaneko *et al.*, 1993a;b; 1995; Kusina *et al.*, 1994; 1995; Akagi *et al.*, 1997; Takedomi *et al.*, 1997). Knight *et al.* (1991) suggested that increased ovarian oestradiol output eventually reduces FSH to typical levels, i.e., oestrogens act in a compensatory feedback mechanism (Mann *et al.*, 1989).

1.6.4.2 Active immunisation in the cow

Active immunisation against various synthetic bovine or porcine inhibin α-subunit fragments has resulted in increases (30-100%) in follicle number, ovulation rate, (Glencross *et al.*, 1992; 1994; Morris *et al.*, 1993; Wood *et al.*, 1993; Scanlon *et al.*, 1993; Bleach *et al.*, 1996) and subsequent twinning rates (Morris *et al.*, 1993). Despite a 52% increase in FSH concentrations, Glencross *et al.* (1994) reported no change in LH, oestradiol or progesterone throughout the oestrous cycle. These findings lead Terqui *et al.* (1995) to suggest that inhibin immunisation in cattle increases follicular development and ovulation rate by increasing FSH secretion.

More recently several authors have suggested, and provided evidence, which suggests that pituitary FSH levels are not the sole determinant of ovulation rate, and that an intra-ovarian role of inhibin exists. For example, Bleach *et al.* (1996) studied the response of ovalbumin conjugated to synthetic peptide fragments of the bovine α_c -sub-unit, selected by computer analysis of antigenic prediction methods ('antigenic index'; Jameson and Wolf, 1988). Bleach *et al.* (1996) reported that bovine inhibin α_c (blα_c) fragments (1-29, 63-72 and (1-29 and 63-72 combined)) showed relatively high titres and increased incidence of multiple ovulation (18-65%). However, using (1-16 and 108-123 blα_c) peptides, binding was relatively low and the researchers witnessed little or no increase (0-10%) in multiple ovulations. Although only a small number of animals (n=5) were used in each group, the results were interesting. Significant linear relationships were found ($r=0.42$; $p<0.02$) between antibody titre and plasma FSH concentration and titre and increased ovulation rate ($r=0.89$; $P<0.0001$). However, partial correlation analysis showed a highly significant association between anti-inhibin titre and ovulation response, which was independent of changes in mean plasma FSH concentrations. This provided more evidence of an intra-ovarian role of inhibin. Morris *et al.* (1993), using 107-122 blα_c, reported raised titres and a 16-18% increase in multiple ovulations. Bleach *et al.* (1996) proposed that the use of different carrier proteins, or small differences in peptide sequence were responsible for the reported variations. Much variation between individuals (n=9), with respect to antibody titres and ovulation rates, in animals immunised against 1-29 peptide, were also reported. For example, with 1-29 and 63-72 peptides combined, antibody responses were less than with 1-29 alone, perhaps due to 'antibody competition' (Bleach *et al.*, 1996) where binding of 63-72 directed antibodies to native inhibin sterically hinders the binding of 1-29 directed antibodies (Bleach *et al.*, 1996).

Evidence to date indicates that following immunisation, heifers showing moderate increases in antibody titres (10-25%) and moderate increases in the incidence of multiple ovulation, will have small increases in the number of ovulations per cycle (2 or 3). This could be useful for increasing the twinning rate in commercial situations (Morris *et al.*, 1993; Bleach *et al.*, 1996). However, large variations in both immune and physiological responses to vaccinations and the very undesirable situation of >2 calves means that this technology is unlikely to be successful in this species.

1.6.5 Inhibin Passive Immunisation

Compared with the ovarian response to active immunisation against inhibin, whereby the response is gradual and varies with immunogen, vaccine protocol, breed, species and individual. Passive immunisation can be more precise.

1.6.5.1 Inhibin passive immunisation in the ewe

After passive immunisation of anoestrous (Mule x Suffolk) ewes, an abrupt and dose-dependent increase in plasma FSH has been reported using antiserum to human α-subunit fragment (Wrathall *et al.*, 1990). This indicates that ovarian inhibin production continues during seasonal anoestrus, during which time it inhibits pituitary FSH release. Furthermore, following the administration of antiserum raised against synthetic porcine and ovine α-subunit fragments, plasma FSH was elevated in Scottish Blackface (Mann, 1990; Mann *et al.*, 1989; 1990; 1992; 1993) and Suffolk cross (Wheaton *et al.*, 1992) ewes, respectively. Ovulation rate increased (Mann *et al.*, 1989; Wheaton *et al.*, 1992), while plasma LH levels were unaffected (Mann *et al.*, 1989; 1992; Wrathall *et al.*, 1990). Concomitant passive immunisation against oestradiol and inhibin elicited a considerably greater FSH response than inhibin immunisation alone (Mann *et al.*, 1992), indicating the dual involvement of inhibin and oestradiol in the control of FSH release and follicular development.

1.6.5.2 Inhibin passive immunisation in the cow

Recently passive inhibin immunisation studies have been carried out in cattle with a view to improving MOET responses. Takedomi *et al.* (1997) experimented with passive immunisation in Holstein heifers. Goat anti-bovine 32kDa purified inhibin antiserum was administered (75ml bolus) on day 9-10 of a synchronised luteal phase, with animals receiving PG injections at 48 and 60 hrs after immunisation. Fixed time A.I. at 12 and 24h (post oestrus) was used at the subsequent oestrus. Oestrus occurred earlier in immunised (77.5 ± 3.2h) compared to control heifers (120 ± 1.1h). Treatment did not affect small (<5mm) follicle number, but increased the number of medium-sized (≥5 to <10mm) follicles 48-96h after bolus injection. At oestrus treated heifers had a higher mean number 4.8 ± 2.4 (range 0-16) of large (>10mm) follicles. Furthermore, treated heifers exhibited more ovulations (mean 6.6 ± 2.5) for several weeks after treatment. FSH concentrations increased between 12-60h after treatment, although there was no effect on FSH surge magnitude. A variation in ovulation rate may partly result from a difference in FSH response between heifers, and/or follicles, or to the stage of development of the dominant follicle at the time of antiserum administration (Guilbault *et al.*, 1991; Huhtinen *et al.*, 1992).

Although the total number of antral follicles (≥4mm) was not different, Akagi *et al.* (1997) reported a dose-dependent increase in the number of large (≥10mm) follicles at oestrus with increased anti-inhibin antiserum dose. The mean ovulation rate in 25ml, 37.5ml and 50ml inhibin antiserum dose groups was 1.0, 3.0 ± 0.9 and 3.4 ± 0.9 respectively. Akagi *et al.* (1997) observed a further increase in mean ovulation rate (6.6 ± 2.5) and variability of response with a 75ml inhibin antiserum dose. In these studies the number of large follicles and ovulations were affected by dosage of inhibin antiserum and correlated with persistence of increased FSH levels and circulating antibody titre (Akagi *et al.*, 1997).

1.6.6 Mechanism of Action and Summary

Theoretically, as discussed, inhibin immunisation enhances recruitment of small follicles by increasing the magnitude of the FSH wave and delaying the fall in FSH concentration, which follows recruitment due to increased DF inhibin and oestradiol negative feedback at the pituitary gland level. This mechanism of action has been confirmed in studies in which, active (Glencross *et al.*, 1994) and passive (Kaneko *et al.*, 1993b; 1995; Campbell *et al.*, 1995) immunisation against inhibin increased plasma FSH concentrations. Conversely, in cattle, Price *et al.* (1987); Glencross *et al.* (1992); Morris and Grealy, (1993) and in sheep (Bindon *et al.*, 1994; Anderson *et al.*, 1996), found no significant rise in plasma FSH subsequent to inhibin immunisation. More recently, Bleach *et al.*, (1996) discovered that the FSH rise alone did not account for the ovulatory response observed. In the ewe, Campbell *et al.*, (1995) concluded that, the observed 3–4 fold increases in FSH, were responsible for the initial stimulation of follicle development and steroid secretion. However, over time, due to increased oestradiol, and presumably inhibin production, FSH levels declined yet follicle development was sustained, despite continued high antibody titres. On a long-term basis inhibin immunisation may stimulate ovarian function by interfering with the modulation of follicle development by inhibin at the ovarian level (Campbell *et al.*, 1995). An intra-ovarian effect of inhibin immunisation, in conjunction with elevated FSH is likely to be responsible for significant increases in ovulation rate (Campbell, 1999; personal communication) seen in both sheep (Henderson *et al.*, 1984; Cummins *et al.*, 1986) and cattle (Bleach *et al.*, 1996).

1.7 THESIS OBJECTIVES

As discussed, GnRH immunisation has economic, ethical and environmental advantages over conventional castration (see Bonneau and Enright, 1995; Section 1.7; Chapters 3 and 4). Therefore a successful GnRH vaccine and protocol would be

of enormous benefit to beef farmers in a diverse range of production systems. However, the primary reason for the lack of commercial adoption of this technology is the reversibility of immunocastration effects on reproductive inhibition. Findings in sheep however, suggest that a permanent suppression of reproductive function may be achieved after neonatal GnRH immunisation (Brown *et al.*, 1994; 1995; Clarke *et al.*, 1998). It is apparent from the literature that a study of the long-term effects of neonatal GnRH immunisation in cattle is absent. Therefore, our objective was to determine the effects of neonatal immunisation against GnRH on reproductive function and anabolic response in both the bull and heifer calf.

In addition, as discussed, another method of improving efficiency of beef production is by increasing the twinning rate in beef suckler cattle. The growing body of evidence in the literature indicates that both GH/IGF and inhibin act as intra-ovarian regulators of folliculogenesis. Therefore, the second objective of this thesis was to investigate the interaction between inhibin and GH/IGF. Using ewes, a study was conducted to increase current knowledge of inhibin and GH/IGF affects on precocious follicle development, while simultaneously producing polyclonal inhibin antiserum for our final investigation. As discussed, passive inhibin immunisation may be an optimum method for inducing twin ovulation in cattle. Based on the literature and an earlier preliminary study (Campbell, Gong and Webb unpublished), our final objective was to investigate the effects of a passive inhibin immunisation regime on follicle development and ovulation rate in beef heifers.

CHAPTER TWO
MATERIALS AND METHODS

2.1 EXPERIMENTAL ANIMALS

2.1.1 Sheep

Sixteen ewe lambs (North country mule x Charolais sire) born in March 1999 were obtained from the University of Nottingham's commercial flock. Prior to experimentation, the animals were managed as part of the flock and kept on pasture. Experiments on these animals were carried out between August 1999 and April 2000. During experimental procedures (see Chapter 3) the animals were group housed and fed grass nuts twice daily and had access to *ad-libitum* hay and water.

2.1.2 Cattle

Thirty-six spring born beef x dairy calves (heifers, n=18; bulls, n =18) were obtained from Melton Mowbray Market, Leicestershire, aged between seven and sixteen days. The animals were housed on straw and left to acclimatise for approximately 1 week before experimentation (see Chapters 4, 5 and 6). Until weaning (8-10 weeks of age), the calves were fed Volac milk (via an artificial milk feeder), Volac quicklets and hay *ad libitum*. Thereafter, they were fed a post-weaner diet (19% protein) twice daily and *ad libitum* hay and water (except for 3 weeks in September 2000 when hay was substituted for grass). At 6 months of age animals were fed protein cake (14%) and hay twice daily with *ad libitum* barley straw. The bulls remained housed and were fed on the above diet (minus hay) until slaughter at 16.5 months. The heifers were put out to pasture at 12 months where they remained until October 2001 when they were again housed and fed until slaughter in March / April 2002.

2.2 OESTROUS CYCLE SYNCHRONISATION

2.2.1 Follicular Synchronisation of Anoestrus Ewe Lambs

Follicular waves were synchronised with a single subcutaneous injection (1ml) of a GnRH analogue Receptal® containing 4.2μg ml^{-1} buserelin acetate; equivalent to 0.004 mg ml^{-1} buserelin (Intervet International. www.intervet.com).

2.2.2. Oestrous Cycle Synchronisation of Heifers

Oestrous cycles were synchronised using cloprostenol (526mg) a synthetic prostaglandin F2α analogue (Estrumate™, Scherling-Plough Animal Health). Administration was by intramuscular injection (2ml) into the rump.

2.3 VACCINE PREPARATION

2.3.1 Inhibin Vaccine

Keyhole Limpet Haemocyanin (KLH; Pierce, N°77100, Imject® lyophilized powder form) was reconstituted with 6ml of T. C. grade water (filtered 2µm filter) to give 10mg ml^{-1}. The α-C 1-26 porcine inhibin subunit (2.88mg) was dissolved in 1.2ml of PBS (0.1M) to give 2.4mg of peptide per ml of solution. Subsequently, 1ml of this peptide solution was added to 5ml of the KLH solution and placed on ice for 20 minutes. Thereafter, 1.5mg of 1.5% gluteraldehyde solution (previously chilled) was added; drop wise while shaking on ice, to yield a total volume of 7.5ml, which was stored, overnight at 4°C. For the control group a similar preparation was made which excluded the inhibin peptide.

Using a mixer (Kinematica AG polytron PT 3000) with sterile probe, to both control and treatment group preparations, NUFCA (a water in oil emulsion containing killed mycobacterium) was added (7.5ml) slowly while the probe was mixing at 12, 000 to 17, 000 rpm. Once all the NUFCA was added, the speed was set at 27, 000 for 20 to 30 seconds. The stability of the emulsion was tested in a petri dish of water, and judged fit for administration when a droplet was able to maintain its form. For booster vaccine preparations NUFCA was substituted for NUFA (as NUFCA without mycobacterium). The vaccine was administered to the ewes within 1 hour of preparation as described in Chapter 5.

2.3.2 GnRH Vaccine

GnRH vaccine was provided by Dr. A. R. Peters (andy_peters@sandwich.pfizer.com) from Pfizer Animal Health (www.pfizer.co.uk). Details of the vaccine components and administration are given in Chapters 3.

2.4 GROWTH HORMONE PREPARATION

Recombinant bovine growth hormone (36mg) was dissolved in 36ml of a vehicle containing, (0.9% NaCL, NaHCO3 (0.025M) and Na2CO3 (0.025M) pH9.6). Subsequently, eight 5ml syringes, for treatment group, were loaded with the

rBGH/vehicle solution (>4ml syringe^{-1}), and 4ml injected s.c. The procedure was repeated, minus the rBGH for the eight controls.

2.5 BLOOD SAMPLE COLLECTION

Three methods were used for blood collection: venepuncture or cannulation of live animals and exsanguination ensuing slaughter. Blood samples were processed as serum following venepuncture and exsanguination. This involved leaving the blood over-night at 4°C to allow the clot to retract. Subsequently, samples were centrifuged at 3000 rpm for 15 minutes. Serum was then collected and stored at -20°C. Following collection via cannulae, blood samples were processed as plasma. The samples were transferred to tubes containing spinning granules, to aid separation and pouring off, and heparin (1,000 iu ml^{-1}) to inhibit coagulation, before centrifugation at 3000 rpm for 15 minutes. Plasma was then poured off and stored at -20°C until assayed.

2.5.1 Venepuncture

When blood sampling frequency was once per day, blood was collected by jugular venepuncture using a vacutainer (Precision Glide™, Becton Dickinson, Plymouth PL6 7BP, U.K.), and 21G needle. A chlorohexidine swab was used to clean the sample site, prior to sample collection. For this procedure cattle, greater than 3 months of age, were restrained in a crush with their heads tied up by means of a halter to expose the jugular vein. Sheep and calves, less than three months were manually restrained. Pressure was applied to the jugular vein below the injection site and a 21G vacutainer needle, fitted to a vacutainer tube guide, was inserted into the vein in cranial direction. The required volume of blood was then collected into a pre-vacuumed tube.

2.5.2 Cannulation

Where more frequent sampling was required the jugular vein was chronically cannulated. Two cannulation methods were used. The Braunula method was used in calves 6 months of age or younger, this was substituted for the Seldinger method in cattle older than 6 months, since the cannulae were longer, more robust and easier to repair or replace when required. During both methods of cannulation animals were restrained in a crush, using a calf adapter when required. To minimise the effects of stress in animals younger than 14 months, cannulation was carried out 24 hours prior to sample collection. In animals older than 14 months cannulation was carried out between two to three hours prior to sample collection. In the case of both methods, blood samples were withdrawn through the cannulae using a syringe. The patency of

the cannulae was maintained by an injection of 1.5ml of heparin-saline solution (50 iu ml^{-1}) was withdrawn and discarded prior to the subsequent sample collection.

2.5.2.1 Braunula method

The neck was shaved on one side, washed with warm water containing surgical scrub (HYDREX®, Chlorhexidine gluconate 4%w/v), dried and swabbed (VERNAID®, gauze swabs) with chlorohexidine gluconate 0.5%w/v in 70% w/v Industrial strength mentholated spirit (IMS). Local anaesthetic (Lignavet, 1% lignocaine, C-Vet veterinary products Lancashire PR5 3QN) was injected (2ml; s.c.) at three sites, one on either side of the jugular vein and one above the cannulation entry wound. After several minutes, when the lignocaine had taken effect, the cannula (VYGON® 14G, 80mm) was inserted into the jugular vein. A catheter (VYGON 1.5 – 2.5mm, L50cm), a three way stop cock and a 10ml syringe, containing heparin saline (0.9%) solution 50 $IUml^{-1}$, was attached and patency tested. The cannula was secured with three sutures (MERSILK®, ETHICON®, black silk braided, non-absorbable, 45mm, 3/8 c, reverse cutting, Johnson & Johnson, International), patency was tested again, and a cap with an injectable membrane added (VYGON® Ref. 891.00). The cannula was then flushed with 1,000 IU ml^{-1} heparin saline injected through the cap membrane. Vet rap bandages were used to cover the cannulae and protect against inadvertent removal and antibiotic (Duphapen® L.A.; i.m. 1ml 25kg body $weight^{-1}$containing; Benzathine penicillin/procaine penicillin), was administered (19G needle and 10ml syringe) prior to the animals being released overnight.

2.5.2.2 Seldinger method

The skin over the jugular vein was shaved, washed and swabbed (Chlorohexidine). Local anaesthetic (Lignavet, 1% lignocaine, C-Vet veterinary products Lancashire PR5 3QN) was administered (3-5 ml; s.c.) to the cannulation site. A cannulating needle was then inserted into the jugular vein. A Seldinger guide wire was passed through the cannulating needle to a depth of approximately 25cm. The needle was then withdrawn leaving the guide wire in place. A sterile cannulae (Vygon®: 1.5-2.0mm; 14G; L.30cm), with Elastoplasts tape covering the hub to improve suture grip, was threaded over the guide wire and into the jugular vein. The guide wire was withdrawn and the cannulae tested for patency using heparin saline solution, (50IU ml^{-1}). The cannula was then sutured in place (Mersilk®, Ethicon® black silk braided, non-absorbable, 45mm; 3/8c; reverse cutting, Johnson and Johnson, International). Vet rap bandages were used to cover the cannula and protect against inadvertent

removal. A catheter extension was added to the cannulae and a Vygon® 3-way stop-cock (valve) added to that to act as a tap.

2.5.3 Exsanguination

Details of blood collection at the time of slaughter are given in the specific experimental chapters.

2.6 ULTRASOUND SCANNING OF THE REPRODUCTIVE TRACT

Ultrasound scanning was used to monitor ovarian follicle development (Pierson and Ginther, 1984; 1988) and testes and vesicular glands (Chandolia *et al.*, 1997b). Trans-rectal scanning was conducted using a real-time B-mode, linear array ultrasound scanner fitted with an Aloka prostate probe, which was adapted accordingly (see Figure 2.1) as the animals matured. As the heifers matured the prostate probe was substituted for an adapted (see Figure 2.2) 7.5 MHz rectal transducer (Aloka SSD500; Aloka, Tokyo, Japan), while the prostate probe was adequate for the bulls.

In mature heifers (Chapter 6) the use of the adapted probe (Figure 2.2) was discontinued, as the probe was hand held within the rectum. Animals were restrained in a crush and faeces removed from the rectum. The location of the uterus and ovaries was determined by rectal palpation prior to the introduction of the scanning probe. The presence of all antral follicles on each ovary (Chapter 6) and all follicles with a diameter ≥4mm (Chapter 4) were recorded. The diameters of follicles up to 10mm were estimated against the centimetre scale on the monitor. Follicles >10mm were measured using the electronic calliper feature (see Figure 4.1) on the machine along the longest and shortest axis of the follicle. The mean of these two measurements was the recorded size.

Trans-cutaneous scanning of the testes was initially performed using a Doppler probe ($7.5MH_2$) and Aloka SSD500 scanner. At 28-32 weeks of age a back-fat probe (Kretz Technik® AG Austria; L2-5x120mm) and SA600V scanner (Kretz Technik®) was used.

Figure 2.1 Adapted Aloka prostate probe used to scan vesicular glands in bulls and ovaries in young pre-pubertal heifers. Note the hose extension to the handle (covered by black tape), which improved manoeuvrability and control and enabled deeper rectal penetration. Adapted by Mr. M. Archer.

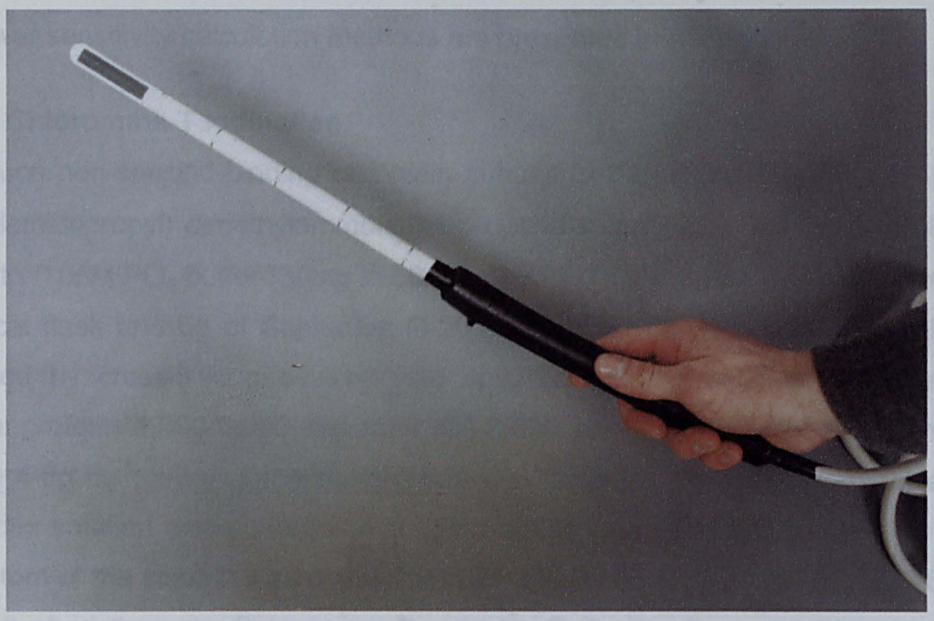
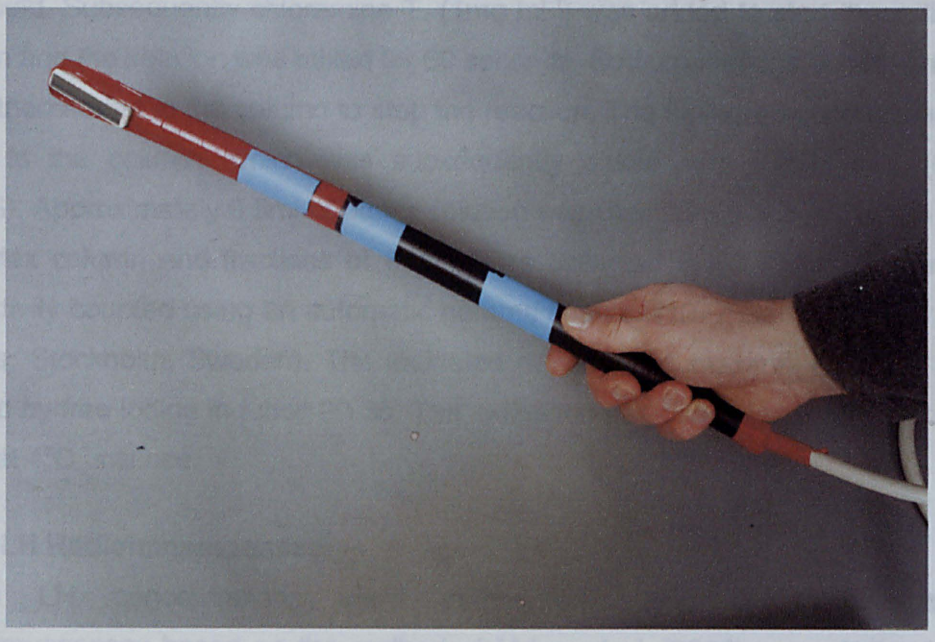


Figure 2.2 Hose adapted rectal transducer probe enabled the ovaries of heifers to be scanned prior to the animals achieving sufficient body size to allow the insertion of the human arm. Adapted by Mr. M. Archer.



2.7 HORMONE RADIOIMMUNOASSAYS

The assays used for the determination of hormone concentrations are described below. The chloromine-T method of iodination was used for all hormone and anti-body assays unless otherwise stated. An experienced Technician carried out all iodinations with the Author's assistance. Intra and inter assay coefficients of variation and lower sensitivity calculation methods are presented in Appendix 2.

2.7.1 Chloromine-T Iodination

To reduce non-specific binding of inhibin subunit to the column, 0.05% CHAPS (3-[(3Cholamidopropyl) dimethylammonio]-1-propane-sulphonate; Sigma® C-5070) was added to 0.05M PO_4 buffer (30mg in 60ml; pH 7.4). This solution was transferred into a conical flask and 6g of Sephadex G-25-80 (fine; SIGMA®; beads of gel filtration produced by cross-linking dextran with epichlorohydrin; fractionation range MW: globular proteins 1,000-5,000, dextrans 100-5,000, dry bead diameter 20-80µm, bead volume 4-6g ml^{-1}; www.sigma-aldrich.com) was added and stored overnight at 4°C. Sephadex solution was poured into a 25cm column and allowed to settle. A tap at the bottom of the column was opened and 25 ml of 0.05M PO_4 (0.05% CHAPS) was slowly flushed through. Chloramine T. and $Na_2S_2O_5$ (sodium metabisulphite; MW 190.13; ICN Biochemicals Inc., Ohio) were dissolved in 20ml 0.5M PO_4 and 0.05M PO_4 buffer solutions, respectively.

In a fume cupboard behind radiation shields, ^{125}I was added to the inhibin peptide and mixed. Subsequently chloramine T. (1mg ml^{-1}) was added to start the iodination reaction and the solution was mixed for 60 seconds. Sodium metabisulphate (1mg ml^{-1}) was then added to the column to stop the reaction. The iodinated peptide was then added to the column, which was subsequently eluted with 0.05M PO_4 (0.05% CHAPS). Approximately 0.5ml of buffer solution was maintained above the top of the Sephadex column and fractions of 0.8ml were collected in 40 LP3 tubes and the radioactivity counted using an automatic gamma counter (1277 Gammamaster, LKB, Wallace, Stockholm, Sweden). The iodinated peptide was maximal in tubes 15-17, followed by free iodine in tubes 30-35. The iodination peak samples were pooled and stored at 4°C until use.

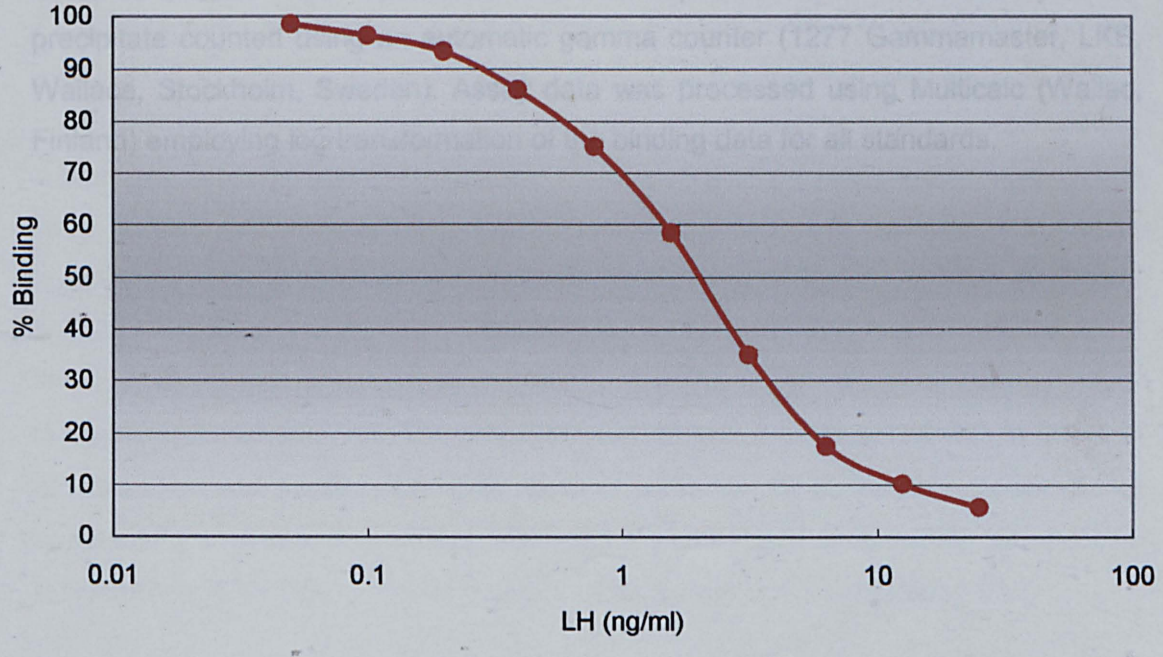
2.7.2 LH Radioimmunoassay

Plasma LH concentrations were determined using a direct iodinated radioimmunoassay based on the method of Mann *et al.* (2000) using NIDDK-rabbit anti-oLH-1, iodinated NIDDK-oLH-I-2 and bovine LH standards (bLH AFP11743B)

supplied by DR AF Parlow (Pituitary Hormones and Antisera Centre, Harbor UCLA Medical Centre, 1000 West Carson Street, Torrance, California 90509, USA).

A set of LH standards, serially diluted from 50ng.ml^{-1} to 0.05ng.ml^{-1}, in 100µl assay buffer, were prepared in triplicate along with duplicate 100µl volumes of sample or quality control plasma. These were made up to a total volume of 300µl with assay buffer (0.05M PO_4 buffered saline with 1% BSA) before the addition of 100µl of the first antibody (NIDDK-anti-oLH-1: 1:200,000 dilution in assay buffer). All tubes were incubated for 48h at 4°C before receiving 100µl of radioactive tracer (NIDDK-oLH-I-2; 15 000 counts per minute (cpm) in buffer) and undergoing further 48h incubation at 4 °C. A volume of 100µl normal rabbit serum (NRS; 1:1 400 dilution), and 100µl donkey anti-rabbit antisera (1:40 dilution) (both obtained from the Scottish Antibody Production Unit, SAPU, Lanarkshire, UK) were added to each tube as a precipitant for the first antibody. The tubes were incubated for 24 hours at 4°C. A 1ml volume of cold phosphate buffered saline (PBS) was added to all tubes prior to centrifugation at 2,000-x gravity (G). Gravity was calculated using the formula 1.12 x 10^{-5} x centrifuge radius (cm) x rpm^2. The supernatant was then aspirated and the radioactivity of the precipitate counted using an automatic gamma counter (1277 Gammamaster, LKB, Wallace, Stockholm, Sweden). Assay data was processed using Multicalc (Wallac OY, Finland) employing log transformation of the binding data for all standards.

Figure 2.3 The composite standard curve from the 32 LH assays. The mean sensitivity (minimum detectable amount) was 0.15 ng.ml^{-1}. The mean inter- and intra-assay coefficients of variation were 9.7% and 7.2%, respectively.

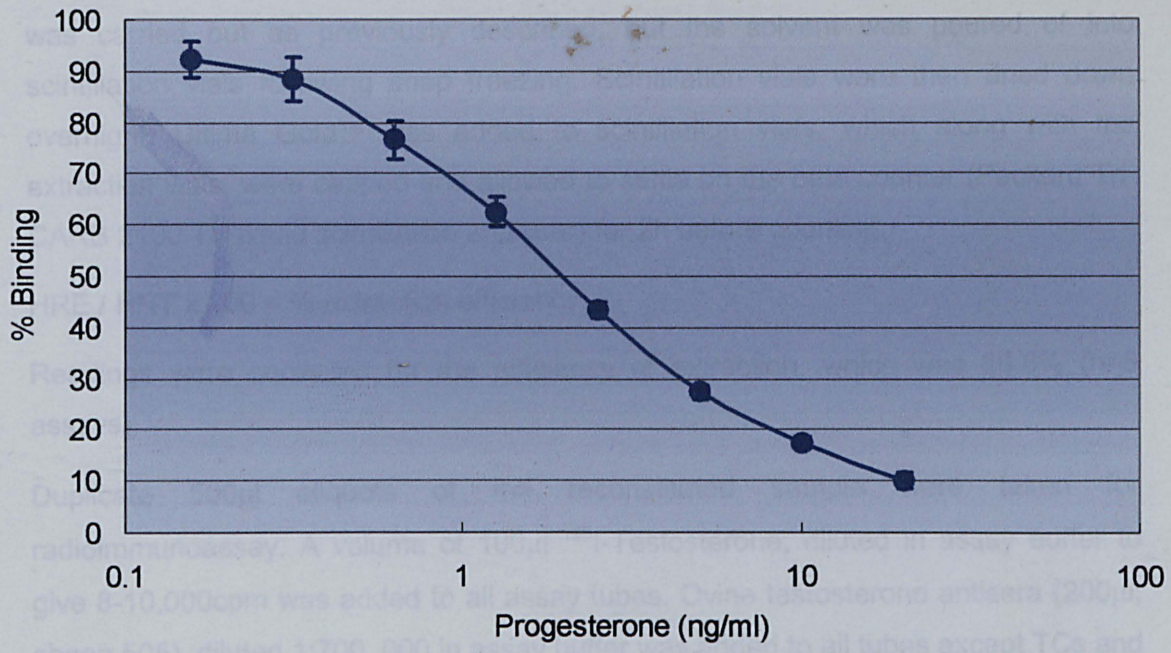


2.7.3 Plasma Progesterone RIA

A direct double antibody [^{125}I] radioimmunoassay, based on the method of Corrie *et al.* (1981) and validated for use on the cow by Law *et al.* (1992), was used. The progesterone (Sigma Chemical company, Poole, UK) standard curve was made using repeated double dilutions from 20.0 to 0.156 ng ml^{-1}. Charcoal Stripped Plasma (CSP; 100 µl) was added to non-specific binding tubes (NSBs) together with 200µl of assay buffer (Phosphate Citrate Buffer with Gelatine (PCGB); pH 7.4), to Reference/Total Binding tubes (TBs) with 100µl assay buffer and to the standards. Duplicate 100µl aliquots of either sample plasma or quality controls (QCs) were diluted with 100µl of assay buffer. ^{125}I-progesterone (Amersham International Plc, Amersham, UK) in tracer assay buffer (ANS 8-Anilio-1-Naphthalene Sulphonic acid (ANS)) was added (100µl; 1mg ml^{-1} ANS; 15,000cpm) to aid the separation of progesterone from its plasma binding protein. The first antibody, rabbit anti-progesterone (Batch 7044x; Scottish Antibody Production Unit (SAPU), Lanarkshire, UK) was diluted to 1:30,000 with assay buffer.

^{125}I-progesterone (100µl; 1mg ml^{-1} ANS; 15,000cpm) was added to all tubes, and 100µl of first antibody was added to all tubes except TC and NSBs. These were incubated for 4h at room temperature (RT). Free and antibody bound tracer were separated by incubation with 200µl of the second antibody, donkey anti-rabbit IgG (DAR; SAPU, Lanarkshire, UK), diluted in assay buffer to 1:240 + 1:900 NRS (SAPU) overnight at 4°C. Total volume of all tubes was diluted and error reduced by the addition of 1ml separation solution (cold assay buffer) before centrifugation at 3,500-x G for 45 minutes. The supernatant was then aspirated and the radioactivity of the precipitate counted using an automatic gamma counter (1277 Gammamaster, LKB, Wallace, Stockholm, Sweden). Assay data was processed using Multicalc (Wallac, Finland) employing log transformation of the binding data for all standards.

Figure 2.4 The composite standard curve from 3 progesterone assays. The assay sensitivity (minimal detectable amount) was 0.31 ng.ml^{-1}. The mean inter- and intra-assay coefficients of variation were 14.9% and 11.0%, respectively.



2.7.4 Testosterone

Serum testosterone concentrations were measured using a solvent extracted radioimmunoassay based on the method and validation by Webb *et al.* (1985).

In 1.5ml eppindorf tubes, 50µl of serum was added to 950µl of assay buffer (Sodium Phosphate with Gelatin; pH 7.0) and vortexed. A 100µl volume of diluted sample was then added to a glass extraction tube, which had previously been baked at 200°C overnight. Subsequently, 2ml of diethyl ether (Romil-SpS™ super purity solvent, Water Beach, Cambridge GB-CB5 9QT) was added to each extraction tube. Tubes were capped and vortexed for 10 minutes, subsequently the aqueous and solvent layers were allowed to separate. The tubes were then placed in a dry ice/diethyl ether bath to a depth of approximately 2cm. When the aqueous layer was frozen, the ether was decanted into glass assay tubes (12 x 75mm; Fisher®) and evaporated to dryness under air at 37°C. The extracted steroid was then reconstituted in 500ul of assay buffer and heated in a water bath at 30°C for 15-20 minutes, before being vortexed for 1 minute. The extraction efficiency was estimated by adding 100µl of 3H-testosterone (5, 000cpm) into 3 glass scintillation vials and 5 extraction tubes.

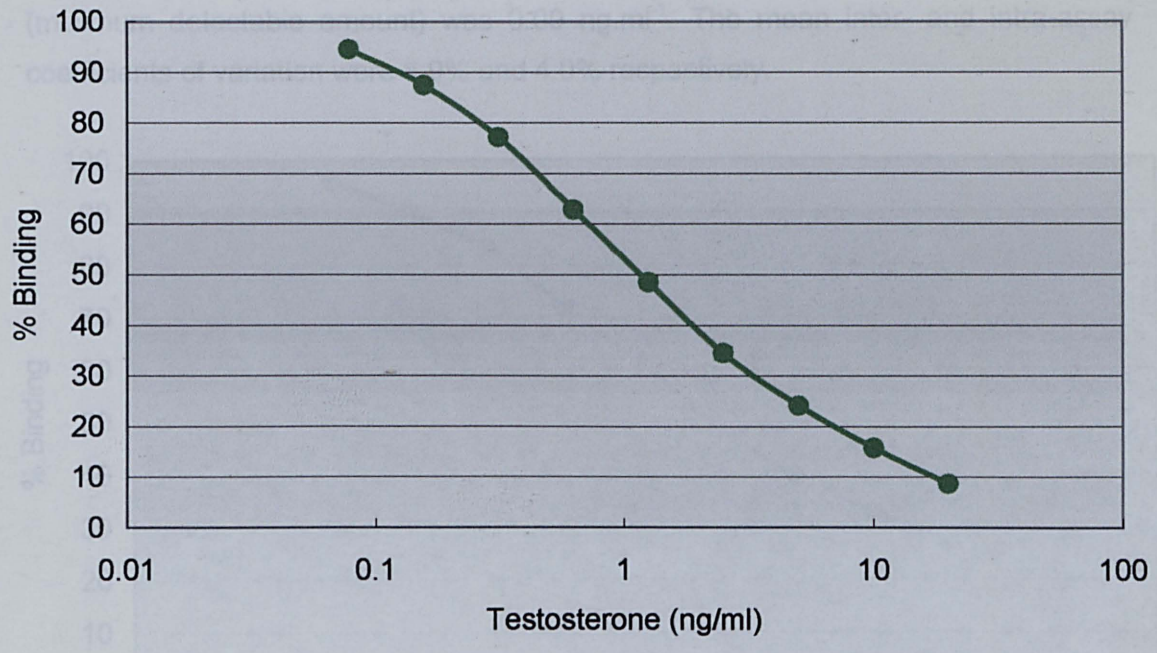
3H Testosterone (Amersham®) in ethanol was dried onto the tube overnight. For hot recovery totals (HRT), to enable the counter to read tritium (3H), Ultima Gold™ scintillation fluid (2ml) was added to scintillation vials. For hot recovery estimates (HRE), the dried hormone was re-suspended by adding blank plasma to the extraction tubes, prior to vortexing and incubating for 20 minutes at 37°C. Extraction was carried out as previously described, but the solvent was poured of into scintillation vials following snap freezing. Scintillation vials were then dried down overnight. Ultima Gold™ was added to scintillation vials, which along with the extraction vials, were capped and allowed to settle on the beta counter (Packard Tri-CARB 2100 TR liquid scintillation analyser) for 2h before counting.

HRE / HRT x 100 = % extraction efficiency.

Readings were corrected for the efficiency of extraction, which was 86.6% (n=8 assays).

Duplicate 500µl aliquots of the reconstituted sample were taken for radioimmunoassay. A volume of 100µl ^{125}I-Testosterone, diluted in assay buffer to give 8-10,000cpm was added to all assay tubes. Ovine testosterone antisera (200µl; sheep 505), diluted 1:700, 000 in assay buffer was added to all tubes except TCs and NSBs. The tubes were then incubated at Room Temperature (RT) for 3-4h. The bound and free fractions were separated by incubation overnight at 4°C, with 200µl of Donkey anti-sheep/goat (DASG; SAPU), diluted 1:25 in EDTA PBS gelatin buffer and Normal Sheep Serum (NSS; 1:1,000; SAPU). The following day, 1ml of cold assay buffer was added to all tubes (except TC's) and all tubes were centrifuged at 3,500-x G for 35 mins at 4°C in a Sorvall® RC 3B centrifuge.

Figure 2.5 The composite standard curve for 8 assays of testosterone. The sensitivity (minimal detectable amount) for the testosterone assay was 0.11 ng.ml^{-1}. The mean inter-assay and intra-assay coefficients of variation were 14.7% and 3.8%, respectively.

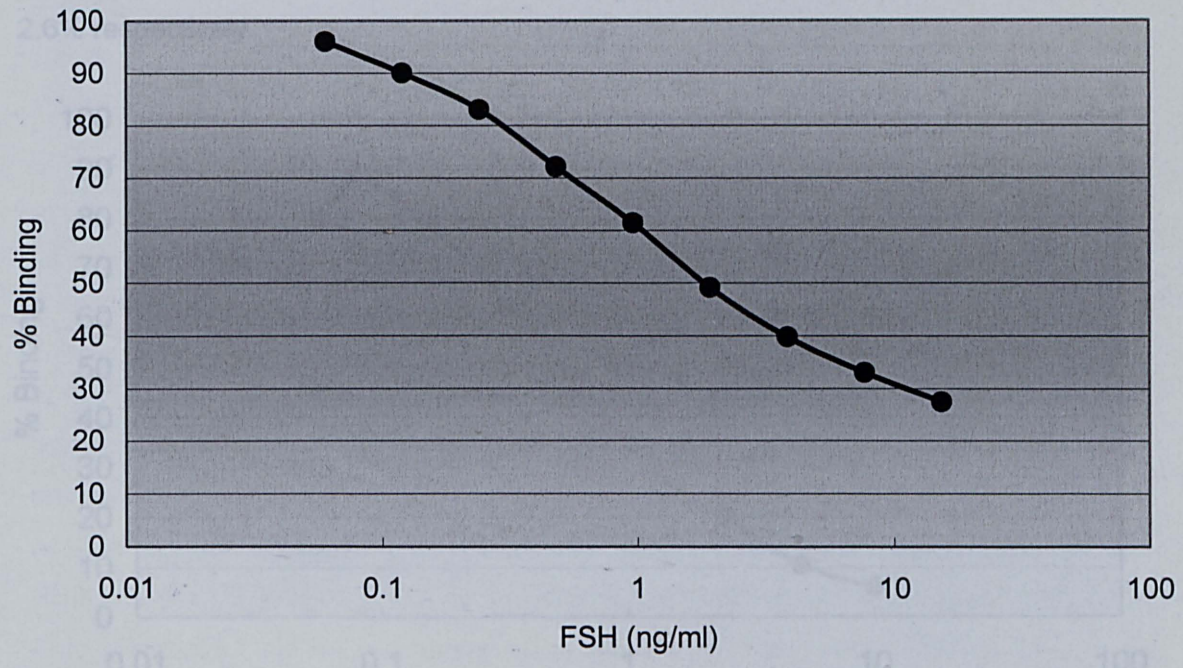


2.7.5 FSH

Peripheral FSH concentrations were determined using a direct iodinated radio-immuno assay based on the method of Campbell *et al.* (1990) using rabbit NIDDK anti-oFSH-1, NIDDK-oFSH-I-SIAFP-21 (for iodination) and USDA-bFSH-I-2 (standards). A set of FSH standards serially diluted from 15ng ml^{-1} to 0.06ng.ml^{-1} in 200µl assay buffer, was prepared in triplicate along with duplicate 200µl volumes of either serum samples or QC plasma. These were all made up to a total volume of 400µl with assay buffer (0.1M PBS with 0.1% BSA and 0.1% sodium azide; pH 7.4) before the simultaneous addition of 50µl of ^{125}I FSH (NIDDK-oFSH-I-SIAFP-21; 15,000cpm in assay buffer) and 50µl of primary antibody (NIDDK-anti-oFSH-1; rabbit) at a 1:16,000 dilution in assay buffer. Note, before pooling the two solutions, 50µl of ^{125}I FSH (NIDDK-oFSH-I-SIAFP-21) was added to NSB and TC tubes. All tubes were incubated at RT for 24h. A volume of 200µl of DAR (SAPU; 1:60 dilution) and NRS (SAPU; 1:1200 dilution) was added to all tubes except TC and incubated for 1-3 days at 4°C. Cold 0.9% saline (500µl) was added to all tubes prior to centrifugation at 2,000-xG for 35 minutes (4°C). The supernatant was aspirated and the radioactivity in the precipitate counted using an automatic gamma counter (1277 Gammamaster,

LKB, Wallace, Stockholm, Sweden). Assay data was processed using Multicalc (Wallac OY, Finland) employing log transformation of the binding data for all standards.

Figure 2.6 A composite standard curve from 14 FSH assays. Assay sensitivity (minimum detectable amount) was 0.09 ng.ml^{-1}. The mean inter- and intra-assay coefficients of variation were 6.9% and 4.0% respectively.



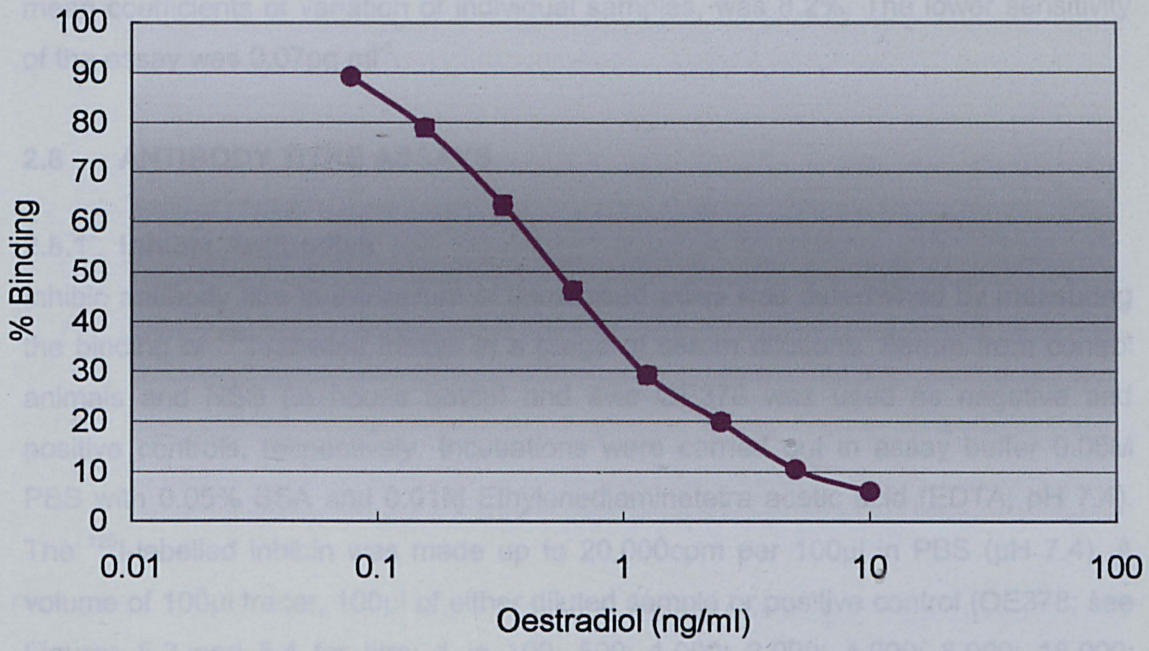
2.7.6 Oestradiol in Culture Media

A set of oestradiol standards, were serially diluted from 78 pg.ml^{-1} to 10,000 pg.ml^{-1} in 100µl assay buffer (0.05M PBS (pH 7.4) with 0.1% gelatin, 0.1% sodium azide). Standards were prepared in triplicate along with duplicate 100µl volumes of sample and QC culture media (150, 750 and 3,000 pg ml^{-1}). These were all made up to a total volume of 300µl with assay buffer before the addition of 100µl of the first antibody (in house, Webb; 1:10,000 in assay buffer) to all tubes except TCs and NSBs. Subsequently, iodinated tracer 12,000cpm in assay buffer was added to all tubes prior to vortexing. Tubes were then left at RT overnight at 4°C.

A volume of 250µl of NRS (SAPU; 1:400) and 250µl of donkey anti-sheep/goat/rabbit (DASGR; SAPU; 1:40) was added to all tubes except TCs. Tubes were vortexed and stored at 4°C overnight. Subsequently, 250µl of assay buffer containing 1% Tween 20 (polyoxyethylene-sorbitan monolaurate; Sigma®, USA) was added to all tubes except the TCs prior to centrifugation at 2,000-xG for 40 minutes at 4°C. The

supernatant was decanted and tubes were left inverted to drain. Pellets were then counted on the automatic gamma counter (1277 Gammamaster, LKB, Wallace, Stockholm, Sweden). Assay data was processed using Multicalc (Wallac OY, Finland) employing log transformation of the binding data for all standards.

Figure 2.7 The composite standard curve (n=4 assays) for the measurement of oestradiol in culture media. The mean sensitivity (minimum detectable amount) was 0.15 ng ml^{-1}. The mean inter- and intra-assay coefficients of variation were 9.1% and 2.6% respectively.



2.7.7 Oestradiol in plasma

The assay used to determine the plasma oestradiol concentration was a solvent extracted radioimmunoassay (Mann, Lamming and Fray, 1995), supplied in an assay kit produced by Serono Diagnostics (E2 MAIA, Serono Diagnostics Ltd., Woking, UK).

Duplicate 500µl aliquots of either sample or reference plasma was extracted by vortexing for 5 minutes in 3ml diethyl ether, in glass tubes previously washed with 3ml ether. The plasma phase was then frozen and the ether phase decanted, dried down and then reconstituted in 250µl of assay buffer (0.1M PBS (pH 7.4) with 0.1% w /v gelatine, 0.2% sodium azide (NaN_3) and 0.3% EDTA, pH 7.6). Oestradiol-17β standard (Sigma Chemicals Ltd., Poole, UK) was used in the range 0.0625 to 16pg per tube made up to 250µl in assay buffer. The first antibody supplied in the kit was

diluted 1:6 with assay buffer before use and the lyophilized [^{125}I]-E2 tracer diluted to 10,000cpm/50µl in assay buffer. Aliquots of 50µl of antibody and tracer were then added to sample and standard tubes, giving a final reaction volume of 350µl. The assay was incubated for 2h at RT and then 100µl of goat anti-rabbit 2nd antibody provided in the kit and diluted 120µl 20ml^{-1} buffer (without gelatine). Tubes were incubated at RT for a further 10 minutes and then 1ml of assay buffer (without gelatine) was added before centrifugation at 2,000-xG at 4°C for 10 minutes. The supernatant was then aspirated and the bound precipitate counted. Extraction efficiency was 93% and the intra-assay coefficient of variation, calculated from the mean coefficients of variation of individual samples, was 8.2%. The lower sensitivity of the assay was 0.07pg ml^{-1}.

2.8 ANTIBODY TITRE ASSAYS

2.8.1 Inhibin Antibodies

Inhibin antibody titre in the serum of immunised ewes was determined by measuring the binding of ^{125}I-labelled inhibin in a range of serum dilutions. Serum from control animals and NSS (in house batch) and ewe OE378 was used as negative and positive controls, respectively. Incubations were carried out in assay buffer 0.05M PBS with 0.05% BSA and 0.01M Ethylenediaminetetra acetic acid (EDTA; pH 7.4). The ^{125}I-labelled inhibin was made up to 20,000cpm per 100µl in PBS (pH 7.4). A volume of 100µl tracer, 100µl of either diluted sample or positive control (OE378; see Figures 5.3 and 5.4 for titre; 1 in 100; 500; 1,000; 2,000; 4,000; 8,000; 16,000; 32,000) and 100µl buffer (final volume 300µl) was vortexed and incubated overnight at 4°C.

A volume of 100µl of NSS (University of Nottingham; 1:1,000 in assay buffer) and 100µl of DASG serum (SAPU; 1:25 in assay buffer) was added to each tube (except TCs) as a precipitant for the first antibody. The tubes were vortexed and incubated for 24 hours at 4°C. Thereafter, 1ml of assay buffer was added to all tubes (except TCs) prior to centrifugation at 2,000-xG for 40 minutes. The supernatant from all tubes was aspirated and the radioactivity of the precipitate counted on an automatic Gamma counter (1277 Gammamaster; LKB, Wallace, Stockholm, Sweden). Assay count data was read using Ultraterm and the titres were calculated by expressing binding of labelled inhibin as the percentage of total labelled inhibin available after subtracting NSBs.

2.8.2 GnRH Antibody Titres

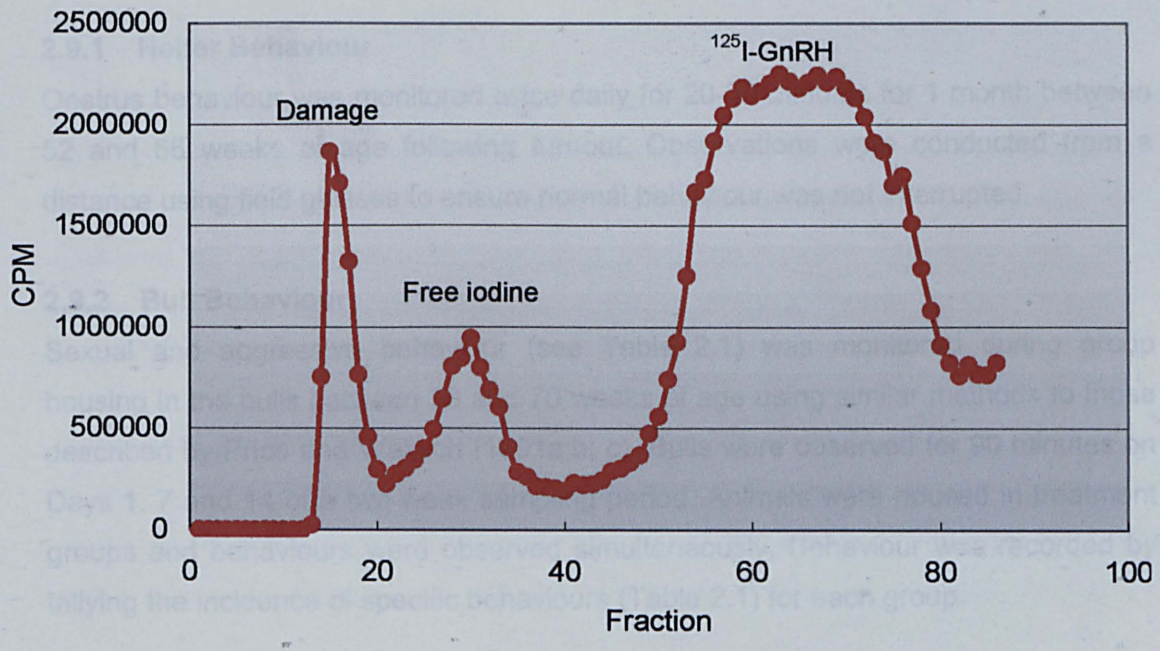
GnRH antibody titre in the serum of immunised cattle was determined by measuring the binding of ^{125}Iodine labelled GnRH.

2.8.2.1 GnRH Iodination

Dr. Tony Bramley, University of Edinburgh, provided the iodination method for GnRH iodination, which was developed by Dr. H. Fraser.

Superfine sephadex (G25; SIGMA™) was soaked in acetic acid (0.01M). Sephadex was poured into a 30cm clamped column, pre-washed with assay buffer (0.1% BSA in 0.01M acetic acid; pH7.4), and containing glass wool to prevent the Sephadex solution leaving the column. This was allowed to settle before priming with 10 ml of 5% BSA in acetic acid (0.01M; pH7.4) and eluted with 40-50 ml of assay buffer. For the iodination 5µg GnRH (1mg ml^{-1} in assay buffer), 40µl of 0.05M PBS (pH 7.4), 5µl lactoperoxidase (100µg ml^{-1} 0.1M Sodium Acetate buffer (pH 5.6)) and 5µl of glucose oxidase (1000µl ml^{-1} PBS) was added to an LP3 tube, together with 20µl of ^{125}I (74 Mega Becquerels) and 20µl of 0.125% glucose solution (100mg 80ml^{-1} H_20). The final solution was mixed and left for 4 minutes, before 300µl of 0.01M acetic acid was added Thereafter, the mixture was added to the pre-primed column. The column was eluted with assay buffer and fractions were counted. Peak ^{125}I-GnRH fractions from the 3rd peak (Figure 2.8) were pooled and stored at 4°C until required.

Figure 2.8 A GnRH iodination profile example. Damaged peptide fragments are first to emerge from the column, then free iodine and finally iodinated GnRH after ~2h.



2.8.3 GnRH Antibody RIA

Serum incubations were carried out in assay buffer (0.05M PBS containing 1% BSA, 0.2% gelatin and 0.01M EDTA (pH 7.4)). ^{125}I-labelled GnRH was made up to 30,000cpm in assay buffer. A volume of 100µl tracer, together with either 100µl of diluted sample (1 in 10, 100, 500,1,000, 2,000, 4,000) or positive control (courtesy of Dr. H. Fraser) was vortexed and incubated overnight at 4°C. A volume of 100µl of Normal Calf Serum (University of Nottingham; 1:1,000 in assay buffer) and 100µl of donkey anti-bovine IgG (Sigma™; 1:25 in assay buffer) was added to each tube (except TCs and the positive controls) as a precipitant for the first antibody. The positive controls received 100µl of NSS (University of Nottingham; 1: 1,000 in assay buffer) and 100µl of DASG serum (SAPU; 1:25 in assay buffer). The tubes were vortexed and incubated for 24h at 4°C. A 1ml volume of ice cold assay buffer containing 12.5% PEG and 2% porcine γ-globulin, was added to all tubes (except TCs) prior to centrifugation at 2,000-xG for 40 minutes. The supernatant from all tubes (except TCs) was aspirated and the radioactivity of the precipitate counted on an automatic Gamma counter (1277 Gammamaster; LKB, Wallace, Stockholm, Sweden). The use of PEG and porcine γ-globulin was on the advice of Dr. Bramley, and successfully increased the pellet size, making aspiration easier and more efficient. Assay data was counted using Ultraterm. The antibody titres were calculated by expressing binding of labelled GnRH as the percentage of TCs after subtracting non-specific binding.

2.9 BEHAVIOUR OBSERVATION

2.9.1 Heifer Behaviour

Oestrus behaviour was monitored twice daily for 20-30 minutes for 1 month between 52 and 56 weeks of age following turnout. Observations were conducted from a distance using field glasses to ensure normal behaviour was not interrupted.

2.9.2 Bull Behaviour

Sexual and aggressive behaviour (see Table 2.1) was monitored during group housing in the bulls between 38 and 70 weeks of age using similar methods to those described by Price and Wallach (1991a;b; c). Bulls were observed for 90 minutes on Days 1, 7 and 14 of a two-week sampling period. Animals were housed in treatment groups and behaviours were observed simultaneously. Behaviour was recorded by tallying the incidence of specific behaviours (Table 2.1) for each group.

Table 2.1 Definitions of behaviours as previously used by Price and Wallach, (1991a; b; c; d) and Jago, Bass and Matthews, (1997).

Behavioural Term	Definition
Flehman (FL)	Upward and backward curling of the upper lip to aid, and in response to, pheromone investigation
Head throw (HT)	Relatively sudden movement of the head and neck in the direction of a target animal, with little or no movement of the body. This behaviour may be perceived as a weak MI by the target stimulus animal
Mount intention (MI)	Head and shoulders are raised and weight is shifted to the rear as if the animal is about to mount, but at least one front foot remains on the ground. Intense chin resting accompanied by vocalising are also indicative of MI
Mount attempt (MA)	Both front feet simultaneously leave the ground, but the animal does not become firmly planted on the mountee's rump
Mount (M)	Animal becomes firmly planted on the mountee's rump. (With or without ejaculation)
Bunt / butts (B)	Bunts and bunt attempts directed at another animal
Sparring (SP)	Head-to-head butting and pushing
Threats	Pawing the ground, rubbing chin and / or head on ground or object. Snorting. Any or all of the above directed towards human handlers.

2.10 SPERM ANALYSES

Mr. I. Sfontouris, an experience and skilled technician, with no previous knowledge of the experimental objectives, carried out tests described in sections 2.10.2-2.10.4.

2.10.1 Sperm Collection

Immediately post-mortem, the testes of each bull were removed from the scrotum and the remnants of the parietal tunica vaginalis and the visceral tunica vaginalis removed by dissection. Following an incision into the caudal epididymis, sperm were aspirated from each testis using a glass pipette. The aspirate was placed into 1ml of warm (34.6°C) Earle's balanced salt solution (EBSS; Sigma™; Cat.No.E2888), supplemented with1g $litre^{-1}$ BSA.

2.10.2 Sperm Motility

A volume of 5µl of sperm suspension was placed on a clean, pre-warmed slide and covered with a cover slip and observed at 100-x magnification. Sperm were graded by Mr. Sfontouris and placed into 1 of 4 motility classification categories:

Grade A: fast progressive (swimming >5 times a head length second^{-1})
Grade B: slow progressive (swimming 1 to 5 times a head length second^{-1})
Grade C: twitching on the spot, but not progressive.
Grade D: immotile sperm
At least 200 sperm were counted from each testis and a percentage of sperm in each classification calculated.

2.10.3 Sperm Viability

The two tests are widely used to assess sperm viability (World Health Organisation, 1999).

2.10.3.1 Eosin-nigrosin Staining

A volume of 5µl of sperm suspension was mixed with 5µl of eosin-negrosin stain on a slide. Using a second slide, the mixture was smeared (spread thinly) over the first slide and left at RT to dry. The slides were mounted with DPX mounting medium (Nustain, Dept. Pathology, University Hospital Nottingham) and a cover slip, and observed at 400-x magnification. Live sperm are known to have a functional membrane, enabling them to exclude the dye and remained white. Dead sperm are unable to exclude the dye and therefore exhibit dark-stained heads.

2.10.3.2 Hypo-osmotic Swelling (HOS) Test.

A volume of 5µl of sperm suspension was diluted with 100µl of EBSS in a 1ml eppendorf tube. The suspension was incubated at 37°C for 1h. Thereafter 5µl of HOS suspension was placed on a slide, a cover slip was added and the sperm were observed at 400-x magnification. Swelling of sperm was identified as changes in the shape of the tail, and the number of swollen tails in a total of 200 sperm was counted and a percentage calculated. The HOS test sample is considered normal if >60% of sperm undergo tail swelling. Before exposure to the HOS solution it is essential that the ejaculate is observed, and sperm which already possess curled and swollen tails be counted and the percentage of sperm these sperm should then be subtracted from the percentage obtained after treatment to obtain the true percentage of sperm that reacted in the HOS test.

2.10.4 Sperm Morphology

A volume of 5µl of the original suspension was placed on a slide, smeared and allowed to dry. Once dry, the slide was stained using the Diff-Quik® staining set (DADE® Switzerland; Cat.No.130832). The staining procedure consisted of a series

of submersions: firstly, the slide was submersed for 15 seconds (s) in fixative (0.002g l^{-1} Fast green in methanol); secondly, 5 dips (1s each) in Eosin buffer (Eosin G; 1.22g l^{-1} in PBS pH6.6); and finally, 5 dips (1s each) in Thiazine buffer (Thiazine dye 1.1g l^{-1} in PBS pH6.6). The slide was then mounted with DPX and a cover slip, before observing sperm at 1,000-x magnification using a phase contrast microscope. Sperm were then categorised according to morphological appearance. Categories included: normal form, pyriform heads, elongated heads, round heads, amorphous heads (i.e., abnormal heads that do not fall in one of the previous categories), coiled tails, no tails, multiple tails etc. At least 200 sperm were counted per testis, and percentages for each category calculated (WHO, 1999).

Due to low numbers of sperm exhibiting some abnormalities, and for statistical analysis, the results from individual animals were pooled and the category number reduced to three: normal, pyriform head abnormalities and other abnormalities.

2.11 FOLLICLE CULTURE

In the abattoir, immediately post mortem, left ovaries were prepared for molecular studies (see 5.2.7.1), and right ovaries were placed in a flask containing warm (37°C) M199 culture media (Sigma®; M2154). Subsequently, ovaries were transported to the laboratory where they were rinsed with fresh warmed (37°C) saline, trimmed of extraneous tissue, and placed in a Petri dish containing M199 media (37°C). Experienced Technicians then manually dissected all antral follicles estimated to be ≥2mm in diameter using 22g syringe needles, under cover of a Laminar flow cabinet. After dissection, follicles counts and diameter measurements were recorded. Burst follicles were put in separate labelled eppendorf tubes, before being snap frozen and stored at -80°C to await molecular studies. Intact antral (≥2mm) follicles were placed into 24 well culture plates (Nunclon Life Technologies; 1 follicle per well). Each culture-well contained 2ml of equilibrated M199 media. Follicles were incubated (Forma Scientific Inc. USA) for 2h at 37°C in an atmosphere of 95% O_2 and 5% CO_2. After incubation follicles from each well was placed in separate labelled eppendorf tubes and stored at -80°C (as described above). The culture-well media was stored at -20°C until RIA for oestradiol content (Section 2.7.6).

CHAPTER THREE

THE EFFECTS OF ACTIVE IMMUNISATION AGAINST GnRH ON REPRODUCTIVE FUNCTION IN BULL CALVES

3.1 INTRODUCTION

The rearing of gonad-intact male cattle in beef production systems is associated with management problems. As a result of gonadal steroid influences on behaviour and sexual competence post-puberty (Price and Wallach, 1991a), bulls are a danger to humans, cause pasture and fencing damage through increased activity and must be separated from heifers at around 7-8 months of age, before the attainment of puberty (Price and Tennesson, 1981; Gregory and Ford, 1983; Bonneau and Enright, 1995).

In contrast to the adverse effects of gonadal steroids on behaviour, these hormones are economically valuable to the beef industry. For example, as discussed by Field (1971), androgens promote lean tissue growth and reduce fat deposition, increase growth rates in bulls by ~17% relative to steers (Harte, Curran and Vial, 1965; Seideman *et al.*, 1982) and increase feed conversion efficiency (FCE) by ~13%. This coupled with a reduction in feed costs and environmental pollutants (nitrogen excreta and methane), because the animals reach their mature slaughter weight sooner.

Surgical castration has long been the solution to these problems. However, this results in a loss of the beneficial effects of androgens and a post-operative growth check. Furthermore, animal welfare and ethical questions are raised as castration is traumatic and stressful (Fisher *et al.*, 1996; Carragher *et al.*, 1997), it increases the risks of morbidity and mortality and is widely perceived as mutilation (see Bonneau and Enright, 1995).

Active immunisation against GnRH inhibits reproductive function by inducing a hypo-gonadotrophic condition associated with gonadal atrophy (Adams and Adams, 1992; Teague *et al.*, 1992; Carson, McCaughley and Steen, 1994; Finnerty *et al.*, 1994; Finnerty *et al.*, 1995; Jago *et al.*, 1995; Jago, Bass and Matthew, 1997; Jago *et al.*, 1997; Finnerty, Enright and Roche, 1998; Huxsoll, Price and Adams, 1998; Cook *et al.*, 2000; D'Occhio, 1993; 1994; D'Occhio, Aspden and Trigg, 2001). Whilst unwanted behaviour and fertility are suppressed, FCE and growth rates are typically intermediate between bulls and steers. Hence GnRH immunocastration is generally

accepted as the most promising alternative to conventional castration and is more favourably perceived by consumers than surgical castration (see Bonneau and Enright, 1995). However, one major reason for the limited commercial application of this technology is that with active GnRH immunisation suppression of reproductive function is only temporary in most individuals, lasting approximately 6 to 8 months (Robertson, Wilson and Fraser, 1979; Keeling and Crighton, 1984; Wetteman and Castree, 1988; 1994). After this time animals return to normal reproductive function (D'Occhio 1993, 1994), subsequent to a decline in circulating anti-GnRH antibodies below a threshold required to neutralise GnRH in the hypophysial portal blood (D'Occhio, Aspden and Trigg, 2001). However, despite numerous studies on immunocastration in cattle, most have been of too short a duration to report on the incidence of permanent suppression. Moreover, the earliest administration of a GnRH vaccine was at 4 weeks (Jeffcoate, Lucas and Crighton, 1982).

An alternative approach to immunocastration is pre-pubertal immunisation. Brown *et al.* (1994) reported that in 20-25% of ram lambs immunised against GnRH, testicular atrophy was evident at 2 years of age. In similar studies with ewe lambs, 80% failed to display oestrus behaviour at 2 years of age (Brown *et al.,* 1995). Despite the absence of GnRH antibodies in the circulation, ewes immunised before 2 months of age showed reduced GnRH secretion at 3-4 years of age (Clarke *et al.,* 1998). Furthermore, morphological studies in boars immunised against GnRH around 4 months of age, demonstrated abnormalities of the median eminence at 6 months of age (Molenaar *et al.,* 1993). D'Occhio *et al.* (2001) investigated the effects of transient GnRH immunisation protocols in cattle at 6 and 22 months of age, on the longevity of reproductive inhibition and reported that, like neonatal rams (Brown *et al.*, 1994), between 16 and 35% of pre-pubertal bulls displayed long-term suppression of LH and testosterone and reduced testes size and function. However, mature bulls had similar levels of long-term reproductive inhibition to calves. The explanation for the apparent reversibility of GnRH immunisation in pre-pubertal calves in contrast to findings in sheep may lie in the different types of vaccine, and vaccine mechanisms of action, or immunisation schedules used. However, by 6 months the hypothalamic pituitary unit would be more mature than that of neonatal sheep. It has been suggested that a gender (Clarke *et al.*, 1998), or species specific time period, may exist during which the exposure of the hypothalamic-pituitary unit to GnRH antibodies may result in permanent impairment of reproductive function.

The hypothesis for the current study was that the relatively immature hypothalamic-pituitary unit of the neonatal bull, may be more susceptible to GnRH antibodies than that of older calves or adults. Therefore, with an adequate antibody response to GnRH vaccination in the neonate, a permanent impairment of reproductive function may result. Hence, the objective of the present study was to investigate the effects of neonatal immunisation against GnRH in bull calves on either the permanent or long-term suppression of reproductive function over a period of 16 months. Using a new and reputedly improved GnRH construct vaccine developed using recombinant technology, combined with an early immunisation (14-20 days of age) protocol we investigated the effects on: 1) pituitary gland function (LH and FSH); 2) testosterone output and sexual behaviour; 3) potential fertility (sperm quality), testicular and seminal vesicle development and 4) growth rate.

3.2 MATERIALS AND METHODS

A detailed description of assay statistics, animals and management and experimental protocols are given in Chapter 2.

3.2.1 Experimental Animals

The experimental animals used in the current study consisted of eighteen beef cross bull calves monitored from 2 to 72 weeks of age.

3.2.2 Experimental Protocol

The experimental period ran from May 2000 to October 2001. On February 19th 2001 all planned procedures were postponed for approximately 1 month because of the outbreak of foot and mouth disease in the UK.

Responses to vaccinations in groups of infants are generally low relative to those in adults (Section 1.4), with higher proportions of poor and non-responding animals (Hauser, Koob and Roth, 1986; Pollock *et al.*, 1991; Roden *et al.*, 1992; Adams *et al.*, 1996). However, despite the immaturity of the neonatal immune system, the decision to actively immunise calves was taken for several reasons. Firstly, studies in sheep suggested that it was during the neonatal period that the HPU was maximally susceptible to GnRH antibodies, and during this period the hypothalamus is still maturing in both cattle and sheep (as discussed 1.2.1.1). For our studies to be more comparable to those in sheep (Brown *et al.*, 1994; 1995), it was necessary to vaccinate at this time. Secondly, publications at the time of study stated that, newborns were immunocompetent (Chappuis, 1998), and therefore, inducing a

neonatal immune response was possible (Kovarick and Siegrist, 1997). Thirdly, our commercial sponsor (Pfizer®) provided a newly developed vaccine, which had been designed to enhance the immune response to vaccination, and Pfizer® supported our experimental protocol. Fourthly, due to the limitations of the PhD Studentship, we could only keep the animals for a maximum of 18 months i.e., the typical period of time taken to fatten beef animals. However, as we intended to investigate the long-term effects of immunisation after GnRH antibody titres had fallen, it was necessary to carry out all the immunisations relatively early in life as based on the literature, titres can take up to 8 months to dissipate after the final booster vaccination (Robertson, Wilson and Fraser, 1979; Robertson *et al.*, 1981; 1984). A final reason for early immunisation was that, we originally intended to compare hypothalamic/pituitary gland morphological examinations between groups after titres had dissipated, however, this was not possible due to BSE restrictions.

Pfizer® did not provide a "control vaccine" i.e., one containing all components of the GnRH vaccine, minus the GnRH-construct and on the sponsor's advice, saline was administered to control animals. Furthermore, a group of steers to compare their performance with those of immunocastrates and bulls under the same experimental conditions, was not included, because of limited funding and because this was not in the interests of our commercial sponsor.

3.2.2.1 Immunisation

Nine calves were immunised (s.c.; brisket) against a GnRH construct conjugated to a 42KD gD carrier protein, subunit of recombinant bovine herpes virus 1 (BHV1) in adjuvant consisting of cholesterol, quil A (saponin), amphigen (refined mineral oil) and cholesterol. A primary injection, first and second booster were administered (brisket; s.c.) at 16 ± 0.6 (2ml), 44 ± 0.6 (2ml) and 94.8 ± 0.1 (1.7ml) days of age respectively. Following injection, pressure was applied to the injection site for 15 seconds using a sterile swab to prevent leakage of vaccine from the skin.

3.2.2.2.1 Response site assessment

The granuloma (spherical shaped lump at injection site) diameter was measured (length, width and breadth; using callipers) on day: 1, 3, 7, 10 and 14 following each administration on vaccine. Subsequently the volume of the granuloma was calculated by taking the mean of the three diameters and employing the formula: $^4/_3 \pi r^3$.

3.2.2.2 Blood sampling

Blood samples were collected by venepunture every 10-14 days to measure circulating GnRH antibody titres, and FSH and testosterone concentrations. In addition, to characterise peripheral pulsatile LH concentrations between the neonatal and post-pubertal, a series of 5 serial sampling periods (every 15 min for 8h) were conducted at the following ages (mean ± s.e.): 0.8 ± 0.02, 3.7 ± 0.03, 6.5 ± 0.03, 10.9 ± 0.03, and 15.3 ± 0.03 months. These ages were rounded up to the nearest week, for ease of presentation, to give: 4, 16, 28, 47 and 66 weeks of age. During serial bleeds blood samples were collected via an indwelling jugular cannula (see Chapter 2). Table 3.1 defines the 4 LH parameters measured from the serial bleeds using MUNRO© (Taylor, 1987).

Table 3.1 A description of all LH parameters analysed in each 8-hour serial bleed.

LH parameter	**Definition**
Basal LH (nadir)	Lowest point within a 35-minute (2 data point) time window. A 60 min (4 samples animal^{-1}) smoothing window was used to calculate the moving average in the baseline calculation.
Pulse frequency	Number of LH pulses. A pulse was measured when LH level was elevated ≥2 s.d. above the mean baseline (nadir) with a minimum pulse interval of 30 min.
Pulse amplitude	Size of the pulse (ng ml^{-1}) above the nadir.
Mean LH	Mean LH concentration in all samples throughout the sampling period.

3.2.2.3 Real-time ultrasound scanning

Testes circumference (Figure 3.1) and vesicular gland dimensions (Figure 3.2) were measured at approximately 4 weekly intervals throughout the experiment from 10-72 weeks. For each testis the scanner calculated circumference, area and volume automatically after measuring the horizontal, vertical and diagonal diameters as shown in Figure 3.1. Vesicular gland width was displayed as the distance between markers (+; see Figure 3.2.), while the length of each gland was measured using the scale on the top of the screen.

Figure 3.1 Testis measurement using ultra-sound scanning (transverse plane scanned cranially). Results are displayed to the right of the image. Note the mediastinum (rete testis), which appears as a white spot at the centre of each gonad.

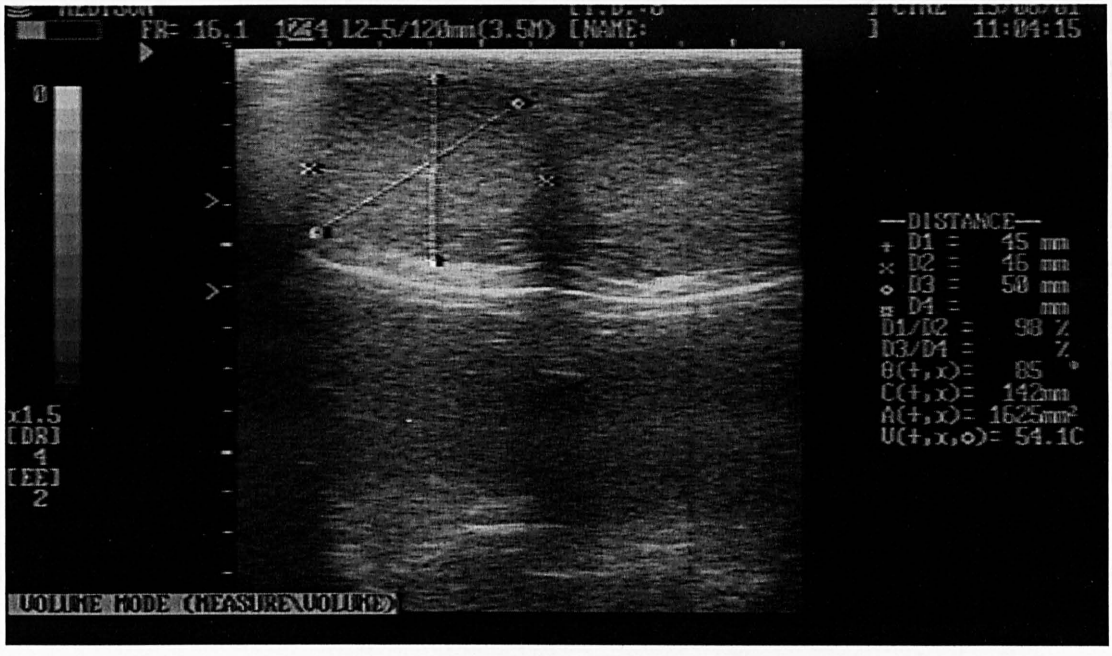
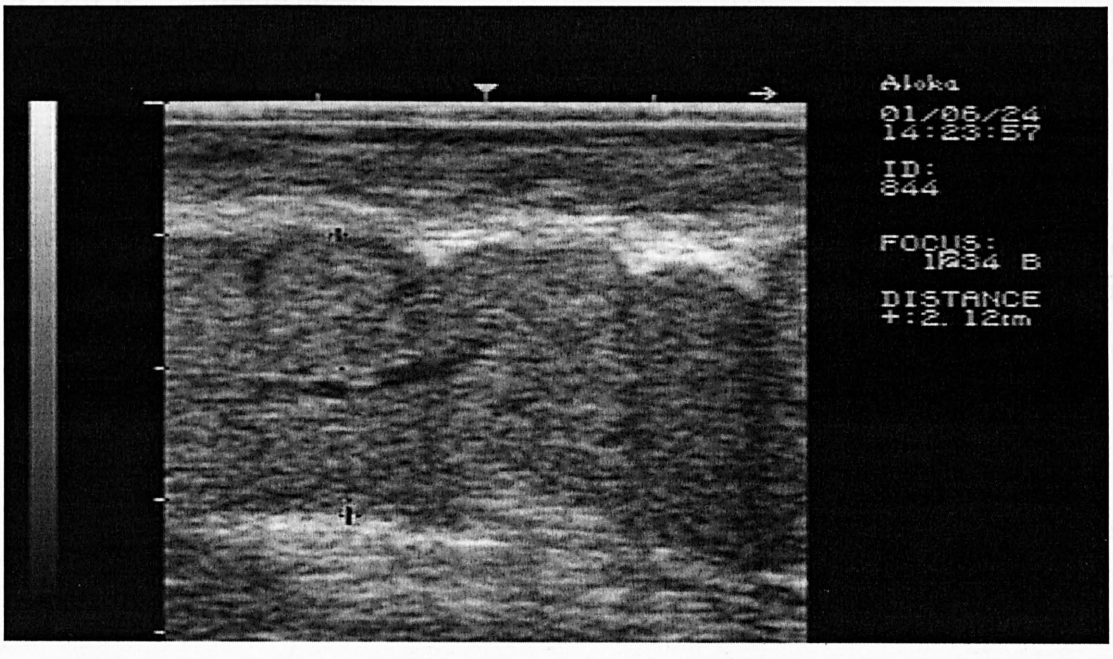


Figure 3.2 Ultra-sound image of a vesicular gland with diameter measurement (median plane, scanned ventrally). Note the lobular structure of the gland and part of the fluid filled duct, which appears as a dark medial line.



3.2.2.4 Live weight

Animals were weighed at 10-14 day intervals, (typically at time of blood sample collection).

3.2.2.5 Behaviour assessment

Sexual and aggressive behaviours, as defined in Chapter 2, were scored during group housing of the bulls between 38-70 weeks of age. Three observation sessions of 90 min were conducted during each study period. Study periods lasted two weeks, with observations sessions of 90 minutes carried out on Days, 1, 7 and 14. After each study period an interval of 14 days followed in which animals were not observed for behaviours. Treated and control bulls were observed during housing in two separate pens (each approx. 8m x 20m), respectively. Observed behaviours were categorised and tallied for each group. For statistical analysis, categories were pooled into either sexual or aggressive behaviour, or total "bullish" behaviours. Individual behaviour scores were not collected due to practical difficulties (animal identification) and time constraints. The study period at 42-44 weeks of age was abandoned due to the FMD outbreak.

3.2.2.6 Post-mortem assessment

Animals were slaughtered at 72 weeks of age and a body weight of 477.33 ± 10.06 kg (mean ± s.e; n=18). Assessments of semen quality, reproductive tract and accessory glands and carcass characteristics are described below.

3.2.2.6.1 Sperm quality

Immediately post-mortem a semen sample was collected from the caudal epididymis. The motility, viability and morphology of the sperm were assessed (see Chapter 2).

3.2.2.6.2 Reproductive tract and accessory glands

Testes and accessory glands (epididymi, seminal vesicles, bulbo-urethral, prostate, penis) were dissected from the carcass, weighed and measured. Tissue samples for future analysis were collected, "snap frozen" in liquid nitrogen and stored at –20°C. Further samples were preserved in Para formaldehyde.

3.2.2.6.3 Carcass characteristics

The carcass (left and right sides) was weighed, killing-out percentage (saleable carcass wt / total dead weight x 100) calculated, and measures of rib-eye muscle and subcutaneous fat taken to determine the carcass quality. The carcass of each bull was given a fat score and conformation grade by an independent Meat and Livestock

Commission (MLC) meat inspector. Conformation grades were based on the established SEUROP classification system (S: superior; E: excellent; U: very good; R: good; O: fair and P: poor). A plus (+) or minus (-) sign is used to provide further information on the quality of the carcass within each grade. Similarly, fat scores are often issued with a high or low status, e.g., 3H, 4L, etc.

3.2.3 Statistical Analyses

MUNRO©, a data analysis program (developed by Dr. P. L. Taylor, Edinburgh 1987, was used to identify LH pulses and calculate LH mean and basal levels, pulse frequency and amplitude (see appendix for calibration details). The resulting statistics were analysed using Genstat 6.1, split-plot analysis of variance (ANOVA) with repeated measures, and degrees of freedom adjusted for the Greenhouse-Geisser epsilon effect.

Repeated measures, reduces the errors in data where data at a point in time may be influenced by the previous time-point data. How dependent a point is, is scored between 0 (extremely dependent) and 1 (independent). The Greenhouse-Geisser adjustment results in degrees of freedom being reduced further, in repeated measures ANOVA. The degrees of freedom in an ANOVA represent the number of independent data that are included in the Sum of Squares for each row of an ANOVA table. The random error in one value may be similar to the error in the next value. Put another way, the closer together in time that one measures something, e.g., hormone concentrations, the more likely one is to measure the same thing twice, rather than separate hormone releases. Thus, each value is obviously not independent, and would only be independent if one waited long enough between each sample. However, the values are not totally dependent either. Indeed, repeated measures data is somewhere in between 0 and 1. The Greenhouse-Geisser approach estimate the degree of non-independence in a time series data set and gives one an adjustment factor to estimate the degrees of freedom, which is somewhere between 1 and the number of times -1(J. Craigon, personal communication).

FSH data was log transformed (ln) before analysis to normalise the distribution. Subsequently, FSH was analysed using split-plot analysis with repeated measures. Degrees of freedom were adjusted for the Greenhouse – Geisser epsilon effect.

Carcass conformation and fat grades were analysed using a Chi squared test in Microsoft Excel. All other ANOVA analyses were carried out using Genstat 6.1 (Lawes Agricultural Trust, 2001, IACR Rothamsted).

3.3 RESULTS

3.3.1 Data Description

Where data have been transformed to a logarithmic scale to normalise the distribution (e.g., FSH), the textual description and statistical significances are based on transformed data. However, for ease of presentation and for presenting standard errors, the tables and figures display the untransformed data unless stated.

Age had a highly significant effect ($P<0.001$) upon live weight. The slope of the regression for live weight on age (Figure 3.3) indicates an average growth rate of all bulls of 6.12kg per week. Growth rates were not significantly different ($P>0.05$), with daily average growth of 0.82kg and 0.85kg for control and immunised bulls, respectively. The live weight (mean ± s.e.) of bulls at 5 time points throughout the trial is presented in Table 3.2.

Figure 3.3 Regression of weight on age in bulls (n=18). The equation of this regression line is; $y= 6.12x + 17.24$ ($r^2=0.92$).

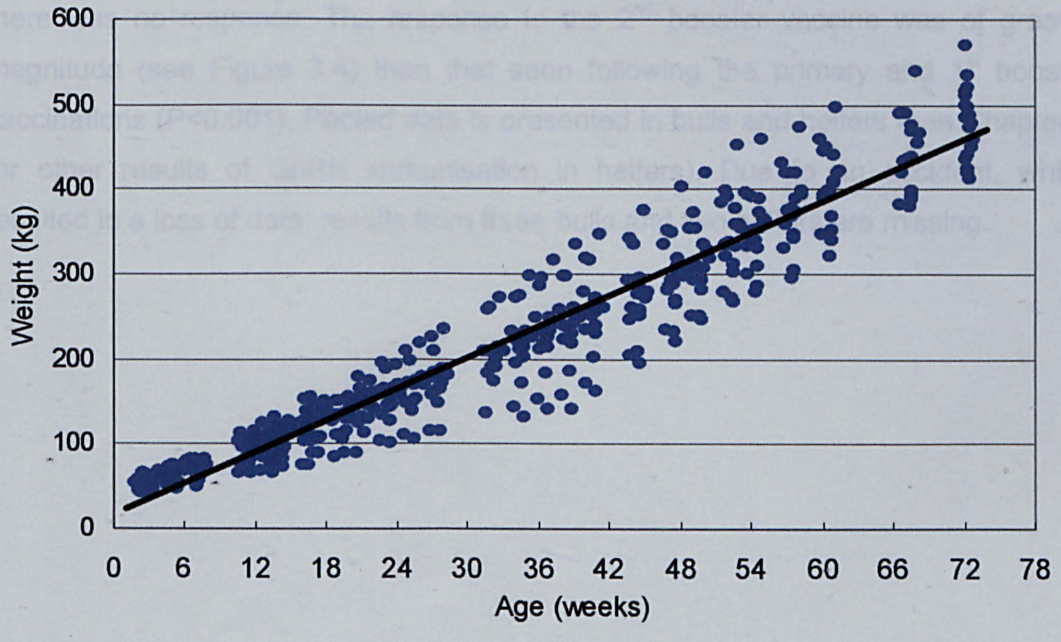


Table 3.2 Mean live weights of control and GnRH immunised bulls at 5 ages (± s.e.). Immunisation against GnRH had no significant (P>0.05) effect on live weight.

Age (weeks)	Control Mean (n=9)	Immunised Mean (n=9)	Overall Mean (n=18)
4	58.3 (2.2)	56.1 (2.2)	57.20 (1.5)
16	118.6 (5.8)	109.3 (7.1)	113.7 (4.3)
28	186.6 (11.2)	176.8 (11.3)	181.7 (7.3)
47	295.1 (13.9)	300.4 (17.2)	297.3 (10.7)
66	435.7 (13.6)	439.1 (16.8)	437.4 (10.5)

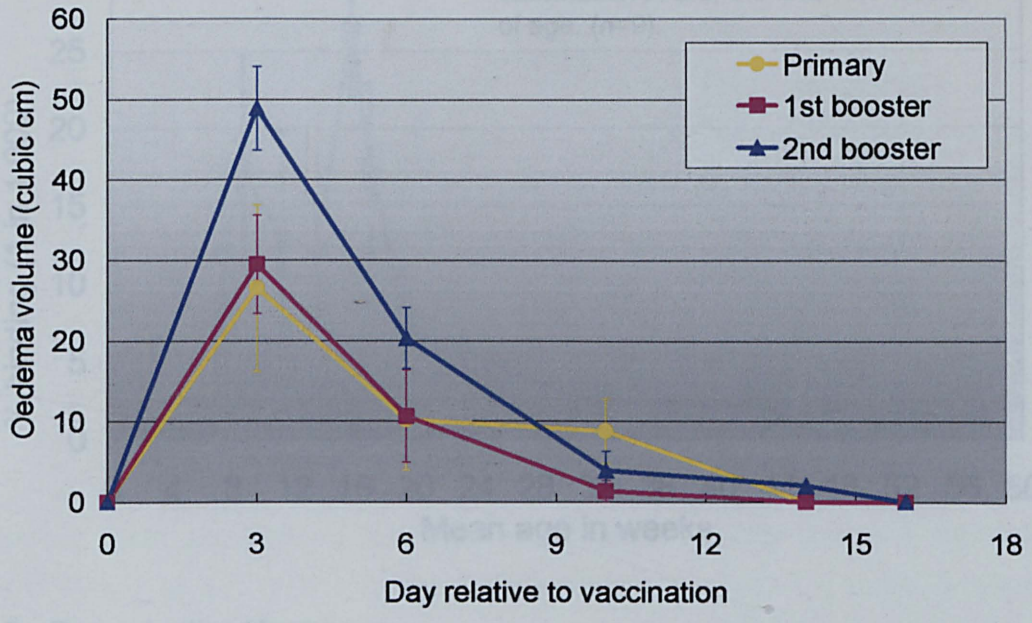
3.3.2 Immune Response to GnRH Immunisation

Inflammation at the site of vaccination and antibody titre, in response to GnRH immunisation, were monitored throughout the study period as described below.

3.3.2.1 Injection site response

The injection site response, measured as the volume of subcutaneous granuloma over time following immunisation (primary vaccination and two boosters), was significant (P<0.001) when compared to the response site in control animals, where there was no response. The response to the 2nd booster vaccine was of greater magnitude (see Figure 3.4) than that seen following the primary and 1st booster vaccinations (P<0.001). Pooled data is presented in bulls and heifers (see Chapter 4 for other results of GnRH immunisation in heifers). Due to an accident, which resulted in a loss of data, results from three bulls and two heifers are missing.

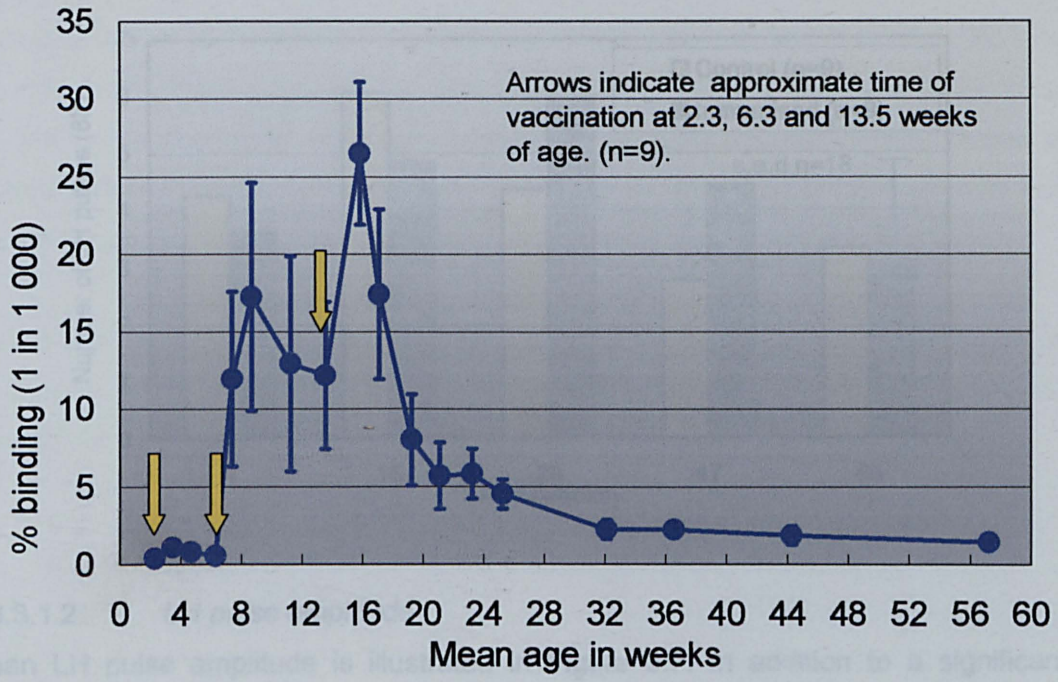
Figure 3.4 Local subcutaneous injection site response to GnRH immunisation in bulls (n=6) and heifers (n=7). After all three vaccinations, granuloma size was largest (P<0.001) on day 3 compared to other monitoring days. Animal gender had no effect (P=0.73) on response site oedema.



3.3.2.2 Antibody titre response

Antibody titre response to primary vaccination was low, and only tended to increase (P=0.14) relative to control animals. Responses to 1st and 2nd booster vaccinations resulted in significant (P<0.001) antibody titres to GnRH (17.2% ± 7.4 and 26.5% ± 4.6% at 1 in 1,000 dilution) and persisted above 15% binding for 2.5 and 4 weeks, respectively (see Figure 3.5). Binding of iodinated GnRH in control bulls was negligible (0.38% ± 0.04) and did not significantly (P>0.05) change over time.

Figure 3.5 Mean (± s.e.) GnRH antibody titre (1 in 1,000 dilution) profiles over time for GnRH immunised bulls. GnRH antibody titres increased (P<0.001) after booster injections.



3.3.3 Reproductive Hormones

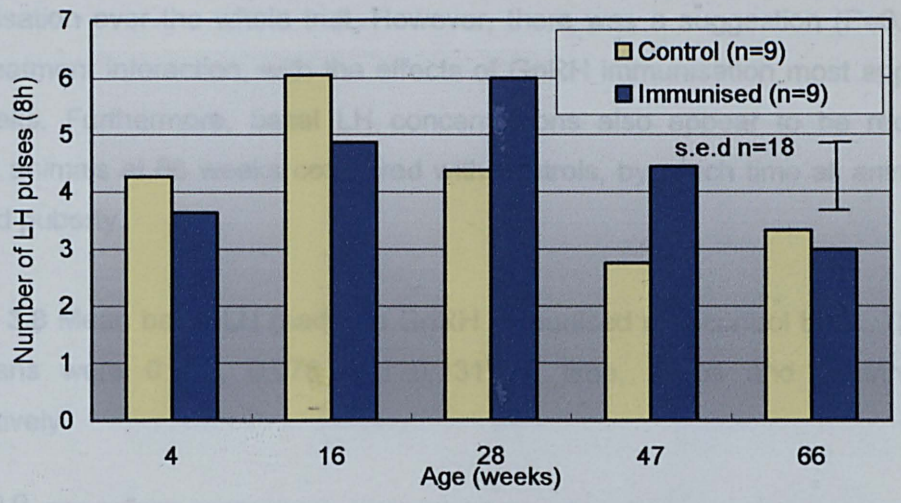
3.3.3.1 LH

Four parameters of LH output were investigated and are defined in Table 3.1.

3.3.3.1.1 *LH pulse frequency*

Mean number of LH pulses at 5 time points, are illustrated in Figure 3.6. Although LH pulse frequency was significantly (P<0.05) affected by age, GnRH immunisation had no significant effect (P=0.67). Although immunised bulls at 4 and 16 weeks of age tended to have reduced pulse frequency, there was no time/treatment interaction (P=0.27).

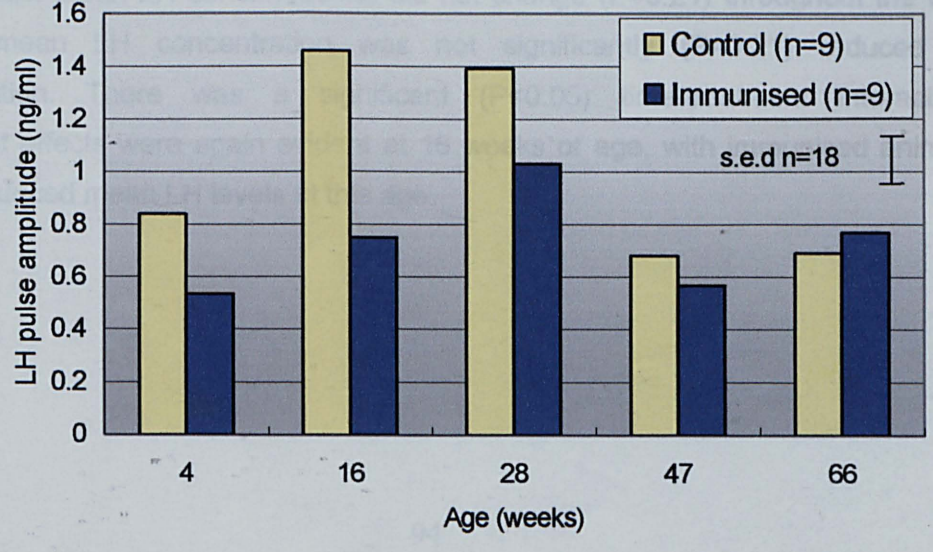
Figure 3.6 A comparison between plasma LH pulse frequency in GnRH immunised and control bulls over time. Standard error of the differences (s.e.d.) of means was 0.804, 0.537 and 1.150 for time, treatment and time/treatment, respectively.



3.3.3.1.2 *LH pulse amplitude*

Mean LH pulse amplitude is illustrated in Figure 3.7. In addition to a significant ($P<0.005$) age effect, GnRH immunisation reduced ($P<0.02$) LH pulse amplitude throughout the course of the experiment. This reduction in pulse amplitude in immunised animals appeared most pronounced at 4, 16 and 28 weeks of age, although there was only a suggestion ($P=0.14$) of a time/treatment interaction. The effect is greatest at 16 weeks, which corresponds with peak GnRH antibody response to immunisation (see Figure 3.5).

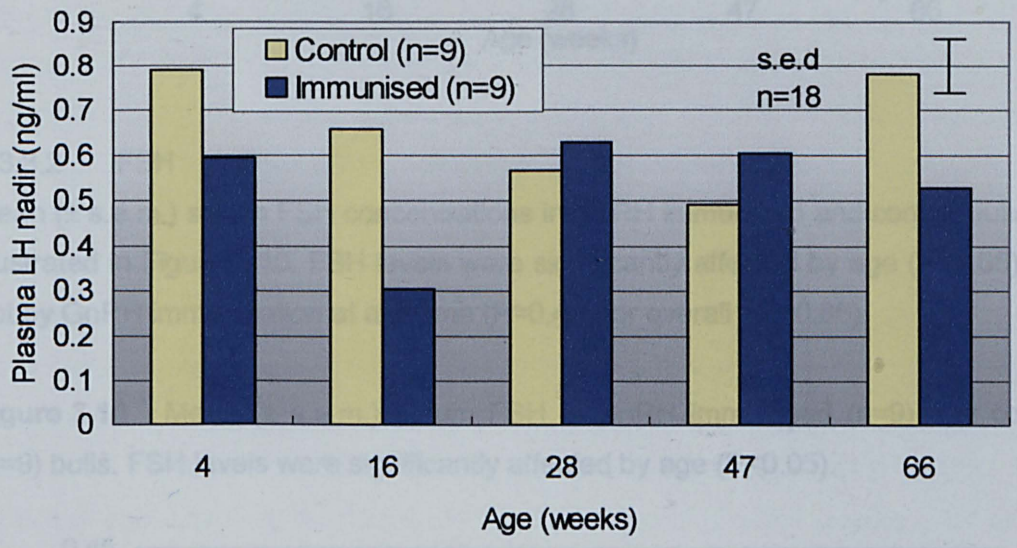
Figure 3.7 Mean LH pulse amplitude in GnRH immunised and control bulls. Standard error of the differences (s.e.d.) of means was 0.144, 0.106 and 0.211 for time, treatment and time/treatment, respectively.



3.3.3.1.3 *Basal (nadir) LH*

Mean basal (nadir) LH concentrations throughout the experimental period are illustrated in Figure 3.8. The age of bulls at the time of sample collection did not significantly (P=0.15) affect basal LH levels. There was no effect (P=0.13) of GnRH immunisation over the whole trial. However, there was a suggestion (P=0.06) of a time/treatment interaction, with the effects of GnRH immunisation most apparent at 16 weeks. Furthermore, basal LH concentrations also appear to be reduced in treated animals at 66 weeks compared with controls, by which time all animals had attained puberty.

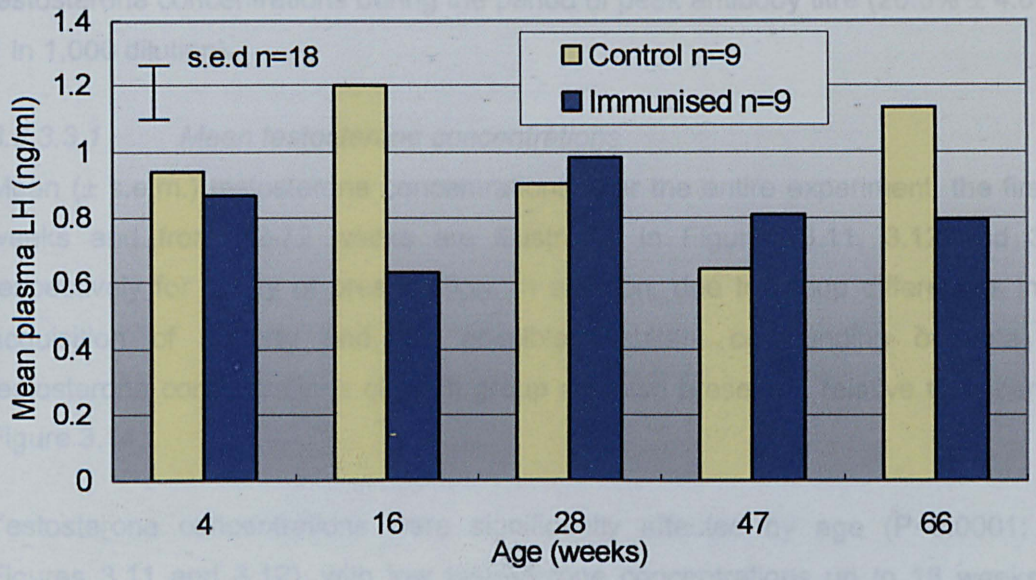
Figure 3.8 Mean basal LH (nadir) in GnRH immunised and control bulls. The s.e.d. of means were 0.083, 0.078 and 0.131 for time, status and time/treatment, respectively.



3.3.3.1.4 *Mean LH concentrations*

Figure 3.9 illustrates the mean plasma LH concentrations in GnRH immunised and control bulls. Mean LH concentrations did not change (P=0.21) throughout the trial. Overall mean LH concentration was not significantly (P=0.21) reduced by immunisation. There was a significant (P<0.05) time/treatment interaction. Treatment effects were again evident at 16 weeks of age, with immunised animals having reduced mean LH levels at this age.

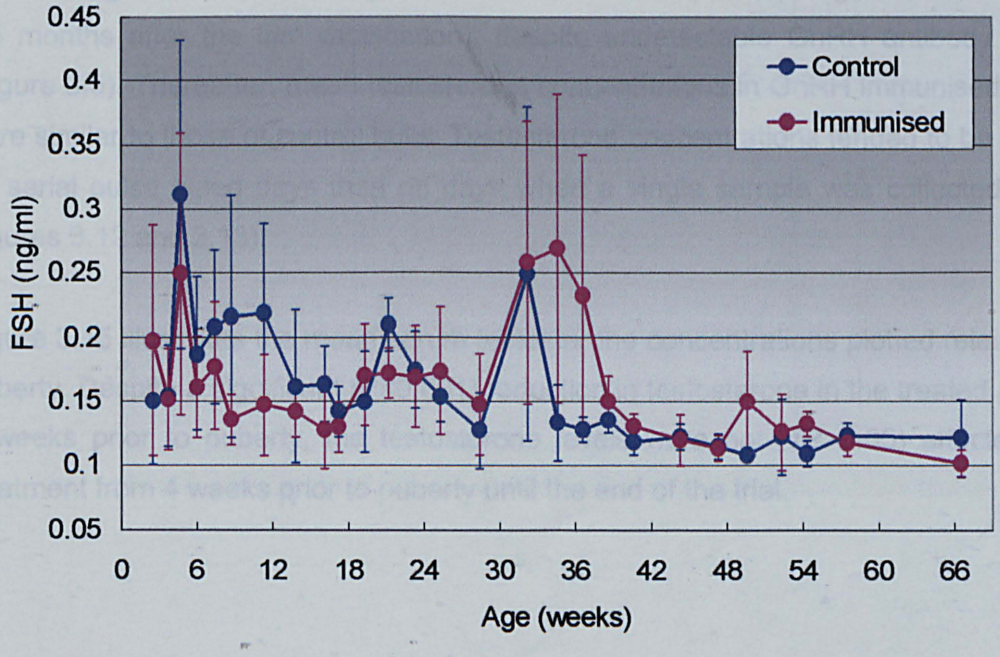
Figure 3.9 Mean plasma LH in GnRH immunised and control bulls. The s.e.d. of means were 0.104, 0.112 and 0.173 for time, status and time/treatment, respectively.



3.3.3.2 FSH

Mean (± s.e.m.) serum FSH concentrations in GnRH immunised and control bulls are illustrated in Figure 3.10. FSH levels were significantly affected by age ($P<0.05$), but not by GnRH immunisation at any time ($P=0.42$), or overall ($P=0.86$).

Figure 3.10 Mean (± s.e.m.) serum FSH in GnRH immunised (n=9) and control (n=9) bulls. FSH levels were significantly affected by age ($P<0.05$).



3.3.3.3 Testosterone

Two testosterone parameters were investigated in the current study: I) mean (± s.e.m.) testosterone concentrations throughout the whole study period and ii) testosterone concentrations during the period of peak antibody titre (26.5% ± 4.6% at 1 in 1,000 dilution).

3.3.3.3.1 Mean testosterone concentrations

Mean (± s.e.m.) testosterone concentrations over the entire experiment, the first 28 weeks and from 32-72 weeks are illustrated in Figures 3.11, 3.12 and 3.13, respectively for clarity of presentation. In addition, due to group differences in the acquisition of puberty and the possible resultant confounding of data, the testosterone concentrations of each group are also presented relative to puberty in Figure 3.14.

Testosterone concentrations were significantly affected by age ($P<0.0001$; see Figures 3.11 and 3.12), with low testosterone concentrations up to 16 weeks, an increase from 16-25 weeks, (see Figure 3.12.), with a further increase in bulls >28 weeks of age (see Figure 3.13.). Although GnRH immunisation did not significantly ($P=0.3$) reduce serum testosterone concentrations over the entire trial, (see Figure 3.11.), or overall during the first 28 weeks of life ($P=0.48$; Figure 3.12), there was a significant ($P<0.001$) time/treatment interaction suggesting that the affects of immunisation altered over time. For example, a reduction in mean testosterone concentrations in GnRH vaccinated bulls occurs briefly ($P<0.05$) following the 1st booster at 7 weeks (for about 5 weeks), following the 2nd booster ($P<0.001$; 15-20 weeks of age; see Figure 3.12.) and from 36-44 weeks ($P<0.01$; Figure 3.13; at least 4.5 months after the last vaccination), despite undetectable GnRH antibody titres (Figure 3.5). Thereafter, mean testosterone concentrations in GnRH immunised bulls were similar to those of control bulls. Testosterone concentrations tended to be lower on serial pulse bleed days than on days when a single sample was collected (see Figures 3.12 and 3.13).

Figure 3.15 illustrates the mean serum testosterone concentrations plotted relative to puberty. Despite a significant ($P<0.001$) reduction in testosterone in the treated group 6 weeks prior to puberty, the testosterone levels were not ($P>0.05$) affected by treatment from 4 weeks prior to puberty until the end of the trial.

Figure 3.11 Mean testosterone (± s.e.m) profiles in GnRH immunised (n=9) and control bulls (n=9) throughout the whole experimental period.

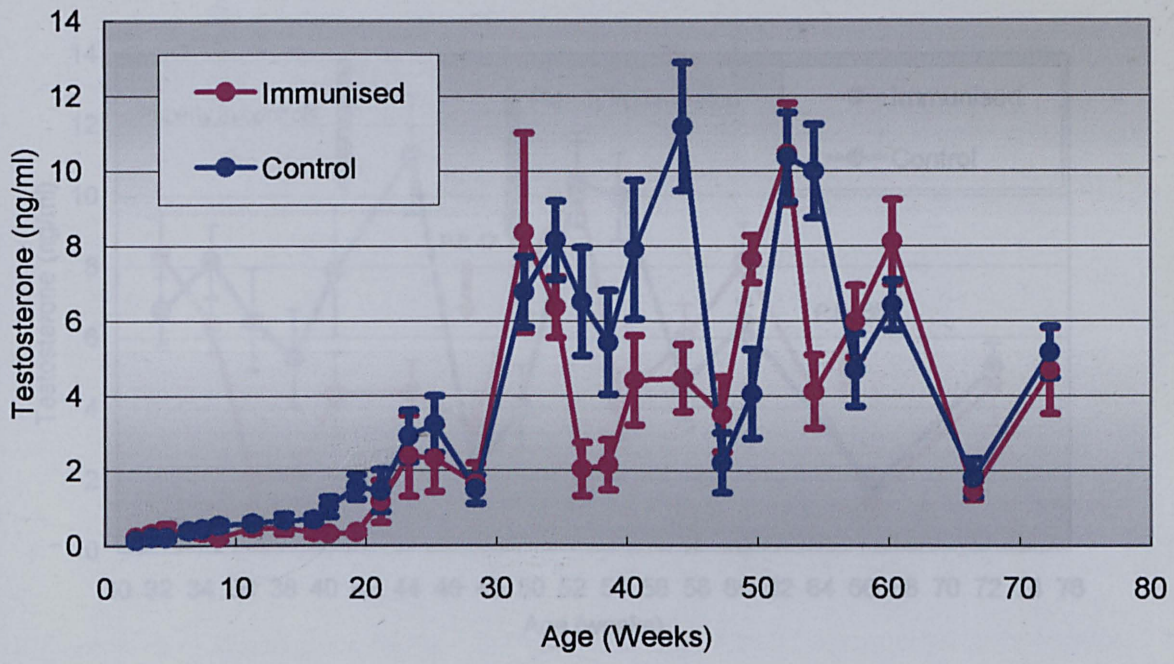


Figure 3.12 Mean testosterone (± s.e.m) concentrations in immunised (n=9) and control bulls (n=9) during the first 28 weeks after birth. Key: Pale blue arrows indicate the approximate time of vaccinations. Red arrows = time of LH pulse bleeds in weeks (P.B.). GnRH antibody titres (1 in 1,000 dilution), are indicated by both pale blue bars (≥ 5% binding) and pale blue bars with red border (≥ 15% binding).

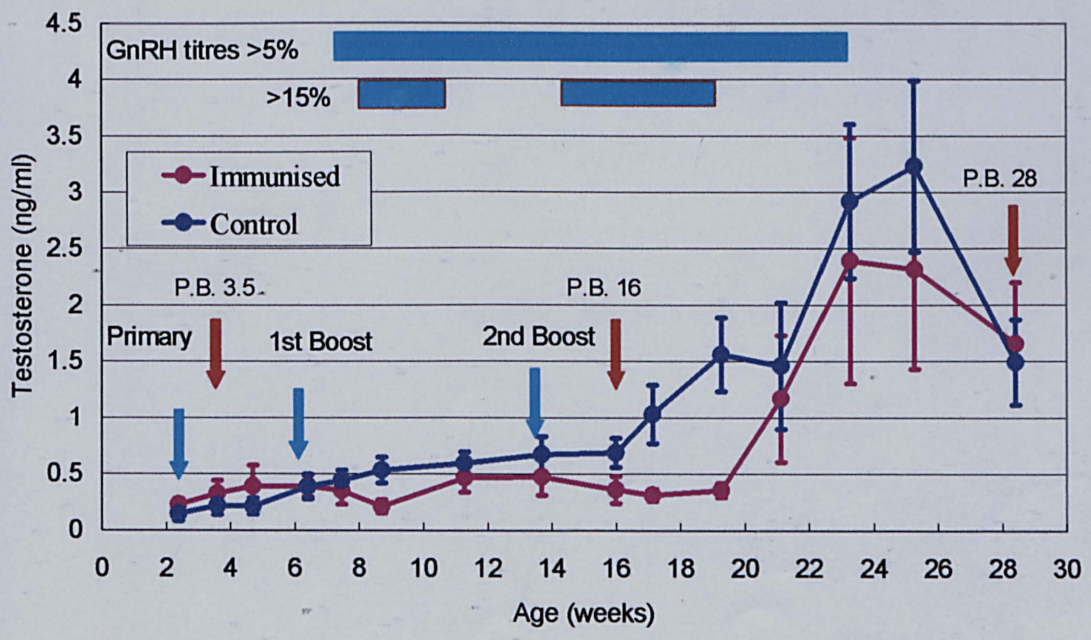
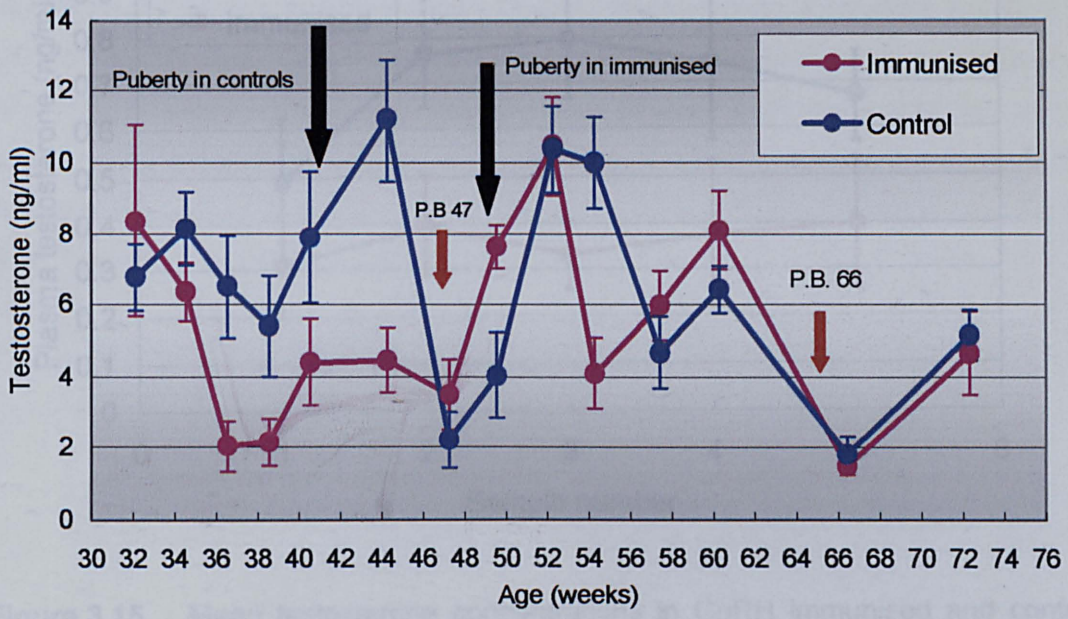


Figure 3.13 Mean testosterone (± s.e.m.) concentrations in GnRH immunised and control bulls from 30-72 weeks. Key: Red arrows = time of LH pulse bleeds at 47 and 66 weeks of age. Black arrows = Puberty (based on testicular measurements).



3.3.3.3.2 Testosterone levels at peak GnRH antibody titre (16 weeks)
Further confirmation of a reduction of plasma testosterone following GnRH immunisation is shown in Figure 3.14, which illustrates a significant ($P<0.05$) reduction in plasma testosterone throughout the 8h serial blood sampling at 16 weeks of age, which was conducted two weeks after the 2nd booster, when anti-GnRH titres peaked. During this period, testosterone levels were unaffected by time ($P=0.17$).

Figure 3.14 Mean plasma testosterone concentrations during pulse bleed in GnRH immunised (n=8) and control (n=9) bulls aged 16 weeks.

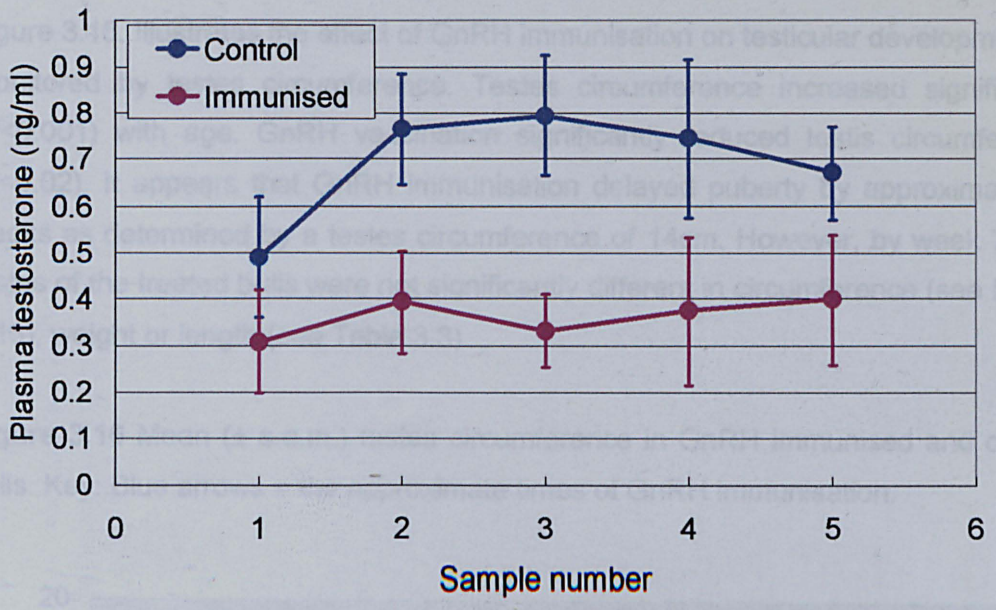
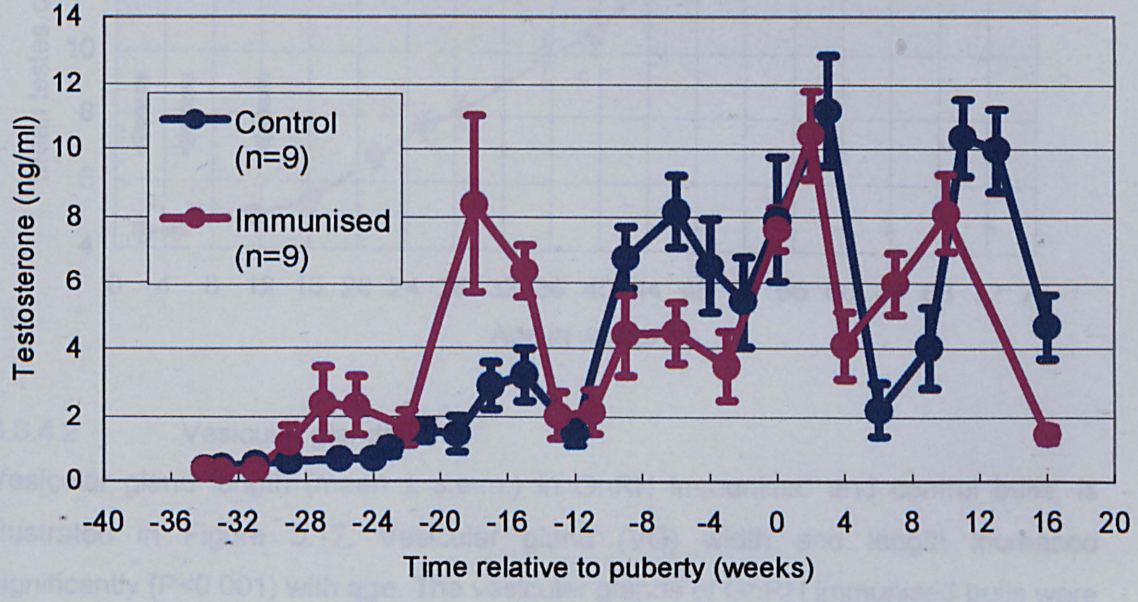


Figure 3.15 Mean testosterone concentrations in GnRH immunised and control bulls presented relative to puberty.

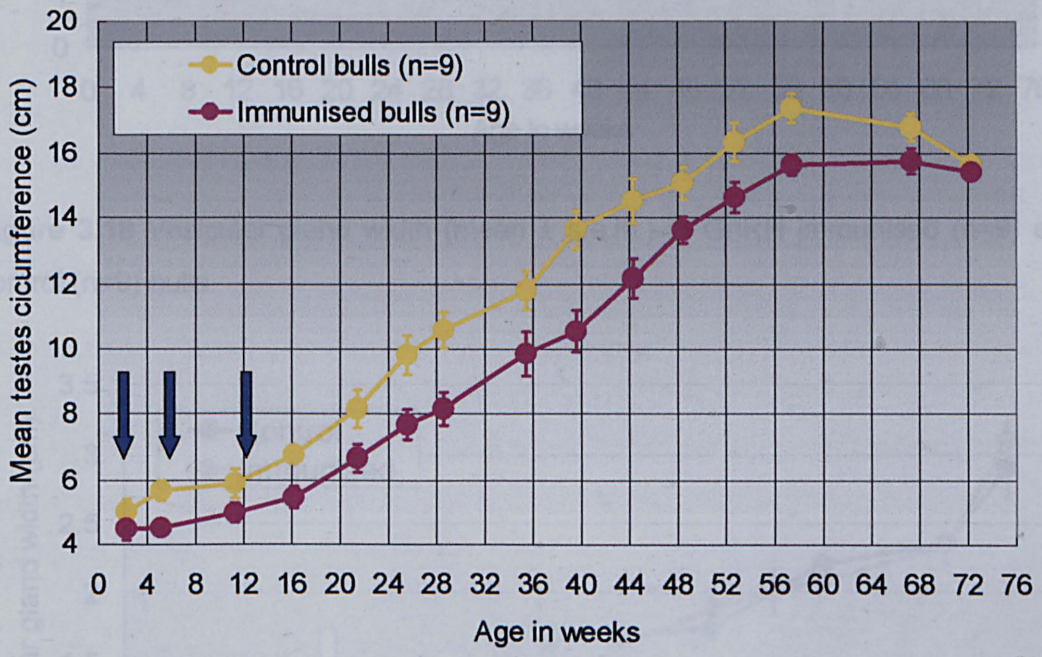


3.3.4 Reproductive Organs and Sperm Analysis

3.3.4.1 Testis circumference

Figure 3.16. Illustrates the effect of GnRH immunisation on testicular development as monitored by testes circumference. Testes circumference increased significantly ($P<0.001$) with age. GnRH vaccination significantly reduced testis circumference ($P<0.02$). It appears that GnRH immunisation delayed puberty by approximately 8 weeks as determined by a testes circumference of 14cm. However, by week 72 the testes of the treated bulls were not significantly different in circumference (see Figure 3.16), weight or length (see Table 3.3).

Figure 3.16 Mean (± s.e.m.) testes circumference in GnRH immunised and control bulls. Key: Blue arrows = the approximate times of GnRH immunisation.



3.3.4.2 Vesicular glands

Vesicular gland length (mean ± s.e.m.) in GnRH immunised and control bulls, is illustrated in Figure 3.17. Vesicular gland (VG) width and length increased significantly ($P<0.001$) with age. The vesicular glands of GnRH immunised bulls were significantly ($P<0.02$) shorter than those of control bulls between 11 and 35 weeks of age. Vesicular gland width did not significantly change after GnRH immunisation ($P>0.05$). but tended ($P=0.07$) to be greater at around 20 weeks of age than those of GnRH immunised bulls.

Figure 3.17 Vesicular gland length (mean ± s.e.m.) in GnRH immunised and control bulls. Key: White arrow = approximate time of peak anti-GnRH titres.

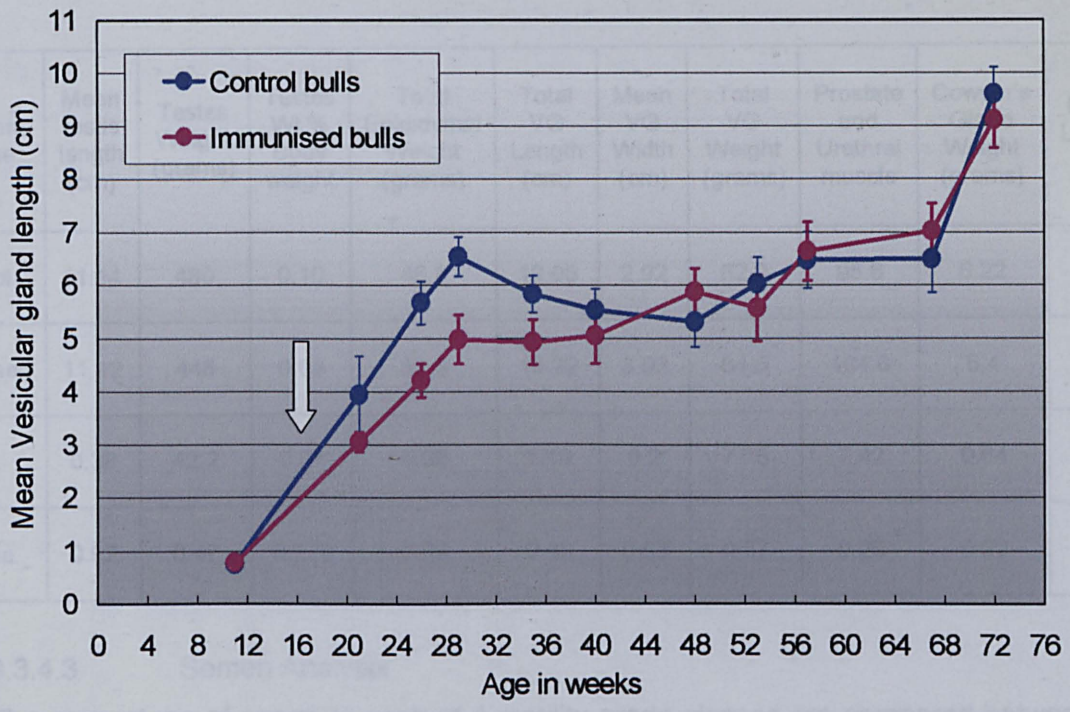


Figure 3.18 Vesicular gland width (mean ± s.e.m.) in GnRH immunised (n=9) and control (n=9) bulls.

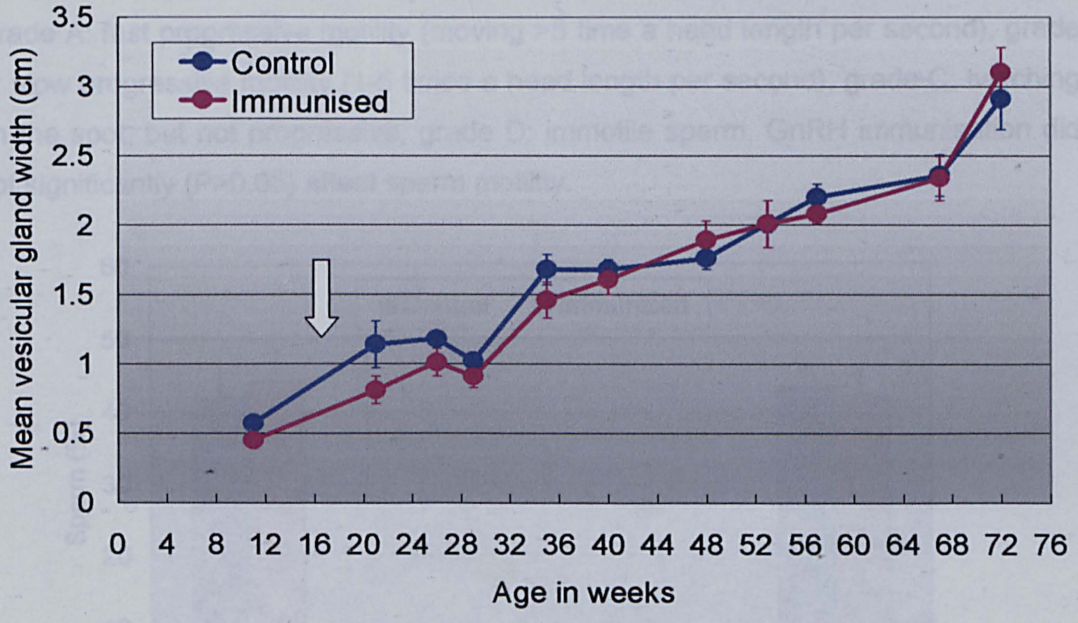


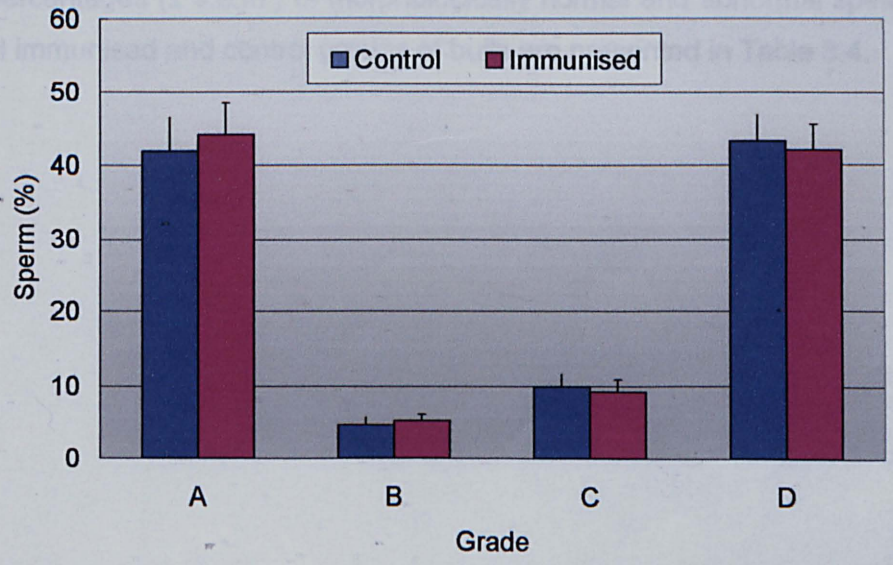
Table 3.3 Comparative weights and measures of gonads and accessory glands in GnRH immunised (n=9) and control (n=9) bulls. GnRH immunisation had no significant (P<0.05) effect on gonads or accessory glands at 72 weeks of age.

Treatment /P value	Mean testis length (cm)	Testes Weight (grams)	Testes Wt % Body weight	Total Epididymal Weight (grams)	Total VG Length (cm)	Mean VG Width (cm)	Total VG Weight (grams)	Prostate and Urethral muscle	Cowper's Gland Weight (grams)	Penis Length (cm)
Control	11.14	480	0.10	46.8	19.06	2.92	62.2	95.6	6.22	75.2
Immunised	11.12	448	0.09	51.3	18.22	3.02	64.5	104.6	5.4	74.2
s.e.d.	0.38	42.2	0.01	5.38	1.19	0.2	7.75	7.42	0.64	2.01
P-value	0.97	0.47	0.278	0.42	0.49	0.63	0.77	0.25	0.22	0.61

3.3.4.3 Semen Analysis

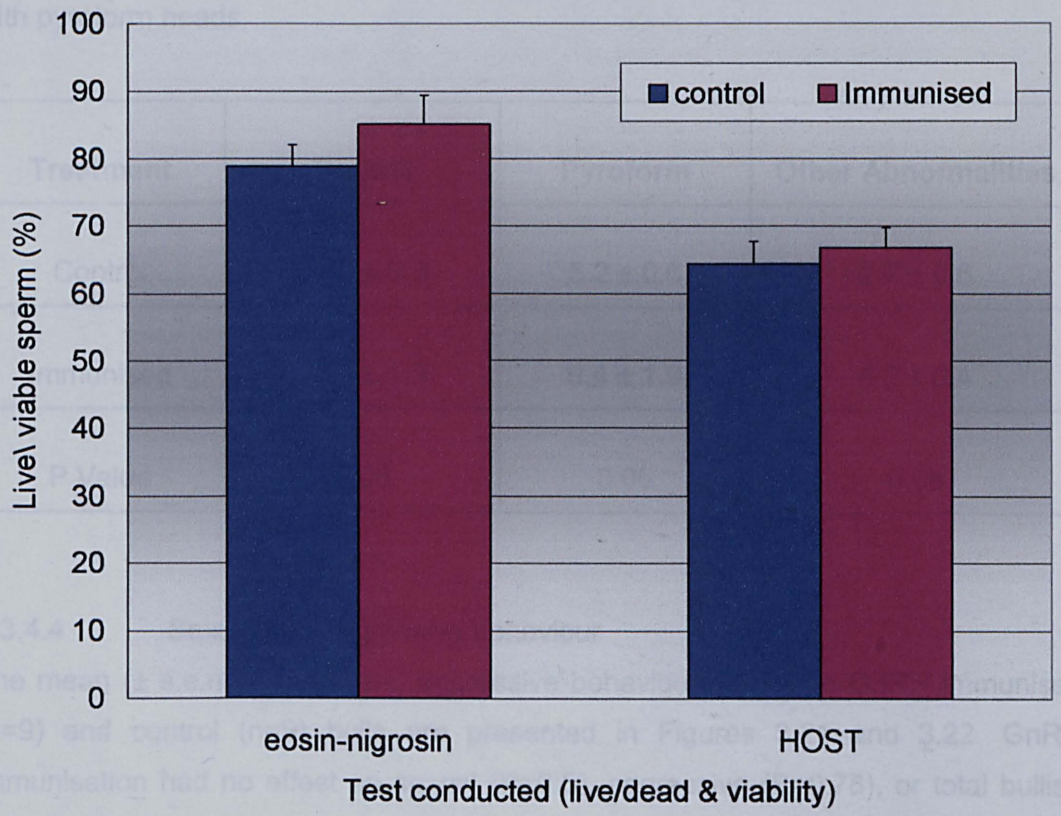
The percentage of sperm in each of 4 motility grade classes are compared between control and immunised bulls in Figure 3.19. GnRH immunisation had no significant affect on sperm motility (P>0.05).

Figure 3.19 Sperm motility in GnRH immunised (n=9) and control (n=9) bulls. Grade A: fast progressive motility (moving >5 time a head length per second), grade B: slow progressive motility (1-5 times a head length per second), grade C: twitching on the spot, but not progressive, grade D: immotile sperm. GnRH immunisation did not significantly (P>0.05) affect sperm motility.



The relative proportions of live and viable sperm in control and GnRH immunised bulls are presented in Figure 3.20. GnRH immunisation had no significant (P>0.05) effect on the number of live and viable sperm.

Figure 3.20 Percentage of live and viable sperm in control (n=9) and GnRH immunised (n=9) bulls, based on results from the eosin-nigrosin stain test and hypo-osmotic swelling test (HOST), respectively.



The percentages (± s.e.m.) of morpholocically normal and abnormal spermatozoa in GnRH immunised and control groups of bulls are presented in Table 3.4.

Table 3.4 Percentage of morphologically normal and abnormal spermatozoa immediately post mortem in GnRH immunised (n=9) and control bulls (n=9). Abnormalities include the tail (coiled tail, multiple tails or no tail) and head, other than pyroform (such as elongated head, round head and amorphous heads i.e., head abnormalities that do not fall into one of the previous catagories). GnRH immunisation resulted in a marginal decrease in the percentage of morphologically normal sperm and a tendency for an increase in morphologically abnormal sperm. The majority of the increased abnormalities result from a higher proportion of sperm with pyroform heads.

Treatment	Normal	Pyroform	Other Abnormalities
Control	92.1 ± 0.8	5.2 ± 0.6	2.7 ± 0.6
Immunised	86.4 ± 1.9	9.4 ± 1.9	4.2 ± 0.4
P Value	0.06	0.06	0.08

3.3.4.4 Sexual and aggressive behaviour

The mean (± s.e.m.) sexual and aggressive behaviours between GnRH immunised (n=9) and control (n=9) bulls are presented in Figures 3.21 and 3.22. GnRH immunisation had no effect on sexual (P=0.5), aggressive (P=0.78), or total bullish behaviours (sexual and aggressive combined; P=1.0), although time significantly (P<0.001) affected all behaviours (P<0.05; P<0.01; P<0.01), respectively. There was no significant time/treatment interaction in sexual (P=0.39), aggressive (P=0.52) and total bullish behaviours (P=0.56).

Figure 3.21 Mean (± s.e.m.) sexual behaviours in control (n=9) and GnRH immunised (n=9) bulls.

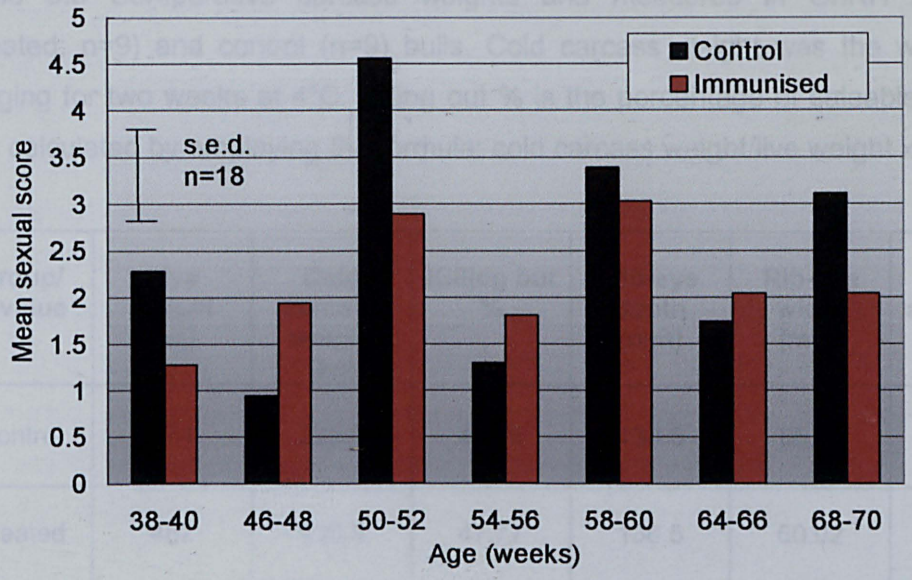
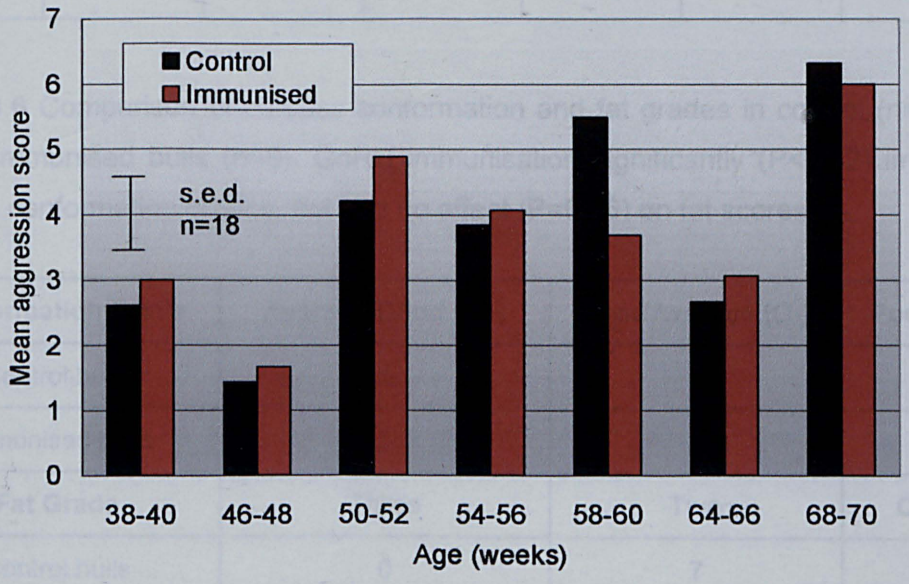


Figure 3.22 Compares mean (± s.e.m) aggressive behaviours in the two treatment groups.



3.3.4 Anabolic Response

Comparative assessment of bull carcasses from GnRH immunised and control animals is presented in Table 3.5. GnRH immunisation significantly increased the rib-eye muscle width ($P<0.05$) at the 10th rib. Tables 3.5 and 3.6 show carcass conformation and fat grades of control and immunised bulls, respectively. GnRH

immunisation significantly (P<0.02) improved carcass conformation grades, but failed to improve fat grades (P=0.16).

Table 3.5 Comparative carcass weights and measures in GnRH immunised (Treated; n=9) and control (n=9) bulls. Cold carcass weight was the weight after hanging for two weeks at 4°C. Killing out % is the percentage of saleable meat and was calculated by employing the formula: cold carcass weight/live weight x 100.

Group/ P-value	Live weight (kg)	Cold carcass weight (kg)	Killing out %	Rib-eye depth (mm)	Rib-eye width (mm)	Rib-eye area (cm^2)
Control	474	223.0	46.96	130.6	55.47	59.7
Treated	482	230.3	47.77	136.5	60.02	62.9
s.e.d.	20.9	10.92	0.84	4.69	1.99	3.51
P-value	0.70	0.52	0.35	0.23	**0.036***	0.373

Table 3.6 Comparison of carcass conformation and fat grades in control (n=9) and GnRH immunised bulls (n=9). GnRH immunisation significantly (P<0.02) improved carcass conformation grades, but had no affect (P=0.16) on fat scores.

Conformation Grade	Average/Good (O+)	Poor/Average (O-)	Poor (P)
Control bulls	1	4	4
Immunised bulls	1	8	0
Fat Grade	**Three**	**Two**	**One**
Control bulls	0	7	2
Immunised bulls	0	1	8

3.4 DISCUSSION

The present study clearly shows that GnRH immunisation in neonatal bulls did not permanently impair reproductive function as was the case in similar studies in sheep (Brown *et al.*, 1994; 1995; Clarke *et al.*, 1998), and a study in bulls (D'Occhio,

Aspden and Trigg, 2001). However, GnRH immunisation at approximately 2 weeks of age did result in a temporary suppression in reproductive function and delayed puberty. This was evident through the disruption of pituitary gland function, as indicated by the reduction of LH pulse amplitude and mean plasma LH concentrations, a reduction in testes circumference, plasma testosterone output, vesicular gland length and a reduction of aggressive behaviours, as based on early subjective behavioural observations. This is in agreement with the findings of Jeffcoate, Lucas and Crighton (1982), Keeling and Crighton (1984) and Prendeville *et al.* (1995a). In addition, some beneficial anabolic effects were observed. These changes all occurred subsequent to increased GnRH antibody titres in immunised bulls. Despite some evidence of prolonged effects after anti-GnRH titres had dissipated, all suppressed parameters, such as LH amplitude, testosterone, testes and vesicular gland growth recovered to levels comparable to those of the control bulls by 16.6 months of age, with the exception of carcass quality.

3.4.1 GnRH Immune Response

The GnRH immunogen successfully induced the production of significant GnRH antibody titres in all vaccinated animals. At a 1:1000 dilution, mean peak titre in bulls was 26.5% (see Figure 3.6). These, as classified by Adams *et al.* (1996), are "good responders" (>10% binding at 1:1000 dilution). Although the titres were higher than those reported by both Johnson *et al.* (1988; >20% binding at 1:1,000) and Prendiville *et al.* (1995a; 15% binding at 1:640) who reported antibody titres adequate to inhibit cyclicity and reproduction function in heifers, they did not reach target titres (>30% at 1:1,000) required for total suppression of reproductive function as suggested by Fraser (1980) and Adams and Adams (1990). However, calculating the exact threshold antibody titre required to suppress reproductive function, based on results from trials with different GnRH immunogens is difficult (Finnerty *et al.*, 1994), because antibody titres per se give little information about the neutralising ability of the antibodies on their target hormone. For example, in the boar following GnRH immunisation fully regressed testicles have been observed in the presence of low antibody binding activity, while also the reverse is seen (Meloen, 1995). Furthermore, despite significant GnRH antibody titres the biological effects of GnRH immunisation on hormone parameters decreased over time in infant bulls (Finnerty, Enright and Roche, 1998). Possibly due to compensatory GnRH output.

Recent studies have reported that the site of GnRH conjugation can influence antibody titre levels, specificity and ability to cause the desired physiological effect

(Silversides *et al.*, 1988; Goubau *et al.*, 1989; Ladd *et al.*, 1990; Ferro *et al.*, 2002a;b). Furthermore, Ghosh and Jackson (1999) suggested that GnRH immunisation induced suppression of fertility is not related to the size of the peak antibody titre, IgG isotypes or affinity of antibodies for GnRH, but is due to the length of time antibodies are in the circulation. However, Ferro *et al.*, (2002a,b) disagreed, stating that antibody avidity for binding free GnRH is more important than antibody titre. These authors found that when immunising rats against modified dimerised GnRH peptides, conjugation with tetanus toxoid at the N-terminal induced antibodies of high affinity for free GnRH (i.e., non-modified decapeptide), which suppressed fertility. C-terminal conjugation, however, had reduced affinity to GnRH, despite high antibody titres to the modified GnRH peptide, but no adverse effect on fertility was observed (Ferro *et al.*, 2002b). This may be the case with more complex modifications of GnRH. As the so called, "second generation" (Sosa *et al.*, 2000) antigens are synthesised using recombinant DNA technologies, the resultant constructs may become more distant from the GnRH decapeptide. Furthermore, in some trials (Ferro *et al.*, 2002a;b) antibodies are measured to the conjugate, rather than to GnRH. Therefore resulting titres may not actually correlate with physiological effects (see Meleon, 1995). However, for the majority of studies there is a significant correlation between anti-GnRH antibody titres and the degree to which reproductive function is inhibited (Lincoln and Fraser, 1979; Molenaar *et al.*, 1993; Wetteman and Castree, 1994; Prendiville *et al.*, 1995a; Parthasarathy *et al.*, 2002).

Bulls in the current study were approximately 8 weeks of age before they produced significant antibodies to GnRH after the 1st booster. This pattern of immune response was in agreement with previous vaccination programmes, which have reported little response to primary vaccination, with the exception of Adams *et al.* (1996), but increased responses to subsequent boosters (Robertson, Wilson and Fraser, 1979; Robertson *et al.*, 1981; 1982; 1984). Although the antibody titre profile in this trial shows a typical immune response, the lack of initial response and generally poor to moderate response overall is more likely to be due to the immaturity of the immune system in younger calves, and possibly blocking effects of maternally derived antibodies (see Sections x and y), than a deficiency in the vaccine. A large individual variation was observed in the current study, with responses to booster vaccinations varying between <5 to >70% binding, as seen in other studies (Prendiville *et al.*, 1995a). However, one bull responded well (>50%) to both booster injections suggesting that age may not be entirely responsible for the generally poor-to-moderate immune response.

3.4.2 Gonadotrophic Drive

A better indication of the effectiveness of a GnRH immunogen may be obtained by investigating physiological parameters such as, gonadotrophic output from the pituitary gland and particularly LH release. As previously discussed in the literature review, GnRH pulses induce the episodic release of LH pulses, promote GnRH receptor expression (Fraser *et al.*, 1982; Popkin and Fraser, 1985) and stimulate gonadotrophin production by the gonadotroph cells of the anterior pituitary gland (Huckle and Conn, 1988; Hawes *et al.*, 1992). LH is immediately responsive to changes in GnRH stimulation, while FSH is largely unresponsive in the short-term, its release being slower and more sustained (Clarke and Cummins, 1982; Levine *et al.*, 1982; Levine and Ramirez, 1982; Fraser and McNeilly, 1983). Furthermore, a GnRH pulse in the portal system always precedes each peripheral LH episode however; every GnRH pulse is not necessarily followed by an LH episode (Clarke and Cummins, 1982; see Clarke, 2002).

In control bulls the observed increase in LH pulse frequency ($P<0.05$) and pulse amplitude ($P<0.005$) between 4 and 16 weeks of age, (peaking at 16 weeks) and the subsequent decrease in pulse frequency at 28 weeks of age, resulting from increased testosterone negative feedback, agrees with previous reports (McCarthy, Convey and Hafs, 1979; McCarthy, Hafs and Convey, 1979; Amann, 1983; Evans, Currie and Rawlings, 1993; Evans *et al.*, 1995; Rawlings and Evans, 1995; Chandolia *et al.*, 1997b; Chandolia, Evans and Rawlings, 1997; Finnerty, Enright and Roche, 1998; Aravindakshan *et al.*, 2000). This pattern of LH release, although reflected in GnRH immunised bulls, was delayed, peaking at 28 weeks of age, rather than at 16 weeks as occurred in the control animals. This finding suggests that due to reduced LH output at around 16 weeks of age, testicular growth and testosterone negative feedback were delayed.

Finnerty, Enright and Roche (1998) reported reduced LH pulse amplitude, as occurred in the current study, and frequency at 16 weeks following GnRH vaccination between 8-10 weeks of age. In the current trial, however, LH pulse frequency was not significantly reduced hence LH pulse amplitude appears to be the more sensitive parameter. The effects of immunocastration on LH pulse amplitude in the present study was significant ($P<0.02$) with the suppression of amplitude evident at the "16 week" serial bleed, 7-9 days after the primary vaccination, despite undetectable GnRH antibody titres in all bulls at this time (1:1,000 dilution). At peak GnRH antibody titre (~16 weeks of age) the suppression of LH pulse amplitude was

greatest. Interestingly, despite antibody titres falling to very low levels at 28 weeks of age, the effects of immunisation on LH pulse amplitude are still evident. This long-term suppression of pulse amplitude may indicate an inability of the pituitary gland to produce adequate LH, following a period of reduced GnRH priming. However, since only 10-20% of LH is released following GnRH stimulation (McNeilly *et al.*, 2003) it may be due to increased sensitivity of the gonadotrophs to steroidal negative feedback, which can modify the responsiveness of gonadotrophs to GnRH stimulation. Thus the amplitude of an LH pulse may not reflect the amplitude of a GnRH pulse (see Clarke, 2002).

Despite the presence of maximal GnRH antibody titres at 16 weeks of age, LH pulse frequency did not alter significantly ($P>0.05$) suggesting that GnRH and LH pulse release was relatively unaffected. In contrast the LH pulse amplitude was reduced. This suggests that: I.) GnRH secretion is inhibited, and smaller pulses induce smaller LH amplitude or that ii.) GnRH secretion is normal, but antibodies sequester/ bind GnRH and inhibit receptor binding, causing a reduction in pulse amplitude, iii.) Both of these effects occur, IV.) gonadotroph sensitivity to testosterone has changed, V.) immune responses between studies may be acting through different mechanisms to suppress LH parameters. In the current study, it appears that titres are not of sufficient magnitude to cause a total cessation of LH release in most animals, whereas it was interesting to note that, in samples from the highest responding bull (GnRH antibody titres >50% binding (1:1000)) at 16 weeks of age no LH was detectable during the entire 8-hour pulse bleed.

As with LH pulse amplitude and frequency, mean LH levels in the GnRH immunisation group were suppressed at peak GnRH antibody titre (~16 weeks), but in addition, mean LH concentrations tended to be reduced at 66 weeks of age, indicating a possible long-term effect. Previous reports have shown that mean LH concentrations are decreased after 4-5 months in bulls (MacMillan and Hafs, 1968; Evans *et al.*, 1993; Finnerty *et al.*, 1998). The reason for the tendency for reduced basal and mean LH concentrations at 66 weeks in immunised bulls is unclear. However, the serial bleed took place in mid-summer on a day during which the temperature peaked at 29°C. It is possible that neonatal GnRH immunisation, made the GnRH immunised bulls more susceptible to heat stress, although we are unaware of any previous publications on heat stress and immunocastration.

The lack of effect of GnRH immunisation on FSH concentrations may be due to the short-term nature of the elevated GnRH antibody titre, as FSH suppression by GnRH immunisation (Miller, Fraser and Brooks, 1998) or GnRH agonist treatment (Gong *et al.*, 1995; 1996a) in cattle takes longer than suppression of LH. As discussed (Chapter 1), FSH release it is not dependent on short term changes in GnRH release. However, with long-term GnRH agonist treatment FSH concentrations did fall after 4 weeks. Despite anti-GnRH titres >10% binding (1:1,000 dilution) lasting for about 10 weeks, no fall in FSH concentrations in GnRH immunised bulls was detected. These results, like many previous studies also report conflicting patterns of FSH release. For example, it has been reported that FSH release increases between 4-16 weeks, before the onset of spermatogenesis (Lee *et al.*, 1976; Amann and Schanbacher, 1983; Evans, Currie and Rawlings, 1993; Evans *et al.*, 1995; 1996) and Evans, Currie and Rawlings (1993), in sheep studies, went on to report that FSH concentrations peaked at 20 weeks. However, McCarthy, Convey and Hafs (1979b) and D'Occhio *et al.* (1987) did not observe a change in FSH concentrations between 4-44 and 16-40 weeks of age, respectively. Due to low frequency of blood sampling in this and other studies (McCarthy, Hafs and Convey, 1979; Schanbacher, 1979), findings may vary. Data from serial blood sampling (Finnerty, Enright and Roche, 1998) showed that FSH concentrations decreased at around 28-32 weeks, in agreement with previous studies (MacMillan and Hafs, 1968; Evans *et al.*, 1995; 1996). The current data are in contrast with these findings, showing that FSH increased between 28-36 weeks, before falling to lower levels thereafter.

The lack of a GnRH immunisation effect on FSH in this trial may be due to a number of factors including a wide variation in FSH concentrations between bulls, or inadequate GnRH antibody titres, and longevity of those titres, which again may be a consequence of an immature immune response (Bot and Bona, 2002). In addition, gonadotroph cells and pathways involved in LH synthesis, storage and release may be more sensitive to a reduction in GnRH stimulation compared with FSH synthesis and release (see McNeilly *et al.*, 2003).

3.4.3 Gonadal and Accessory Gland Responses

3.4.3.1 Testosterone profiles

In control bulls mean plasma testosterone concentrations increased significantly with age (16-28 weeks), as previously reported (McCarthy, Convey and Hafs, 1979), to the lower physiological range seen in adult bulls (Finnerty, Enright and Roche, 1998)

before finally reaching adult concentrations. The oscillating mean testosterone concentrations (1.5 to >11ng/ml) reflect the pulsatile pattern of steroid release. In immunised bulls, mean testosterone concentrations were reduced at around 8 and 16 weeks (following 1st and 2nd boosters respectively) in agreement with those previously published (Finnerty *et al.*, 1996), although in the current study the rise in testosterone at 24-28 weeks was not delayed. Despite the lack of GnRH antibody titre between 36-44 weeks immunised bulls exhibited lower testosterone levels than the control bulls, suggesting that even after antibody titres had fallen significantly there was some degree of long-term impairment in the ability to produce testosterone. Unfortunately, it was not possible to determine whether the testes were capable of responding to LH stimulation during this period since no LH serial bleeds were carried out. Alternatively the observations at 36-44 weeks may simply be due age differences relative to the acquisition of puberty. Indeed, when testosterone concentrations in both groups are plotted relative to age at puberty (Figure 3.15), the duration of the long-term effects of treatment on testosterone was reduced. However, the effect of GnRH immunisation was evident, as between 6-4.5 weeks prior to puberty testosterone was reduced ($P<0.001$).

The fall in testosterone concentrations in the immunised bulls coincides with the previously reported fall in testosterone in control bulls between 36-58 weeks of age (Swanson *et al.*, 1971; Rawlings, Hafs and Swanson, 1972; Sundby and Velle, 1980). This pattern may be influenced by the season in which the bulls reach this age (Foote, Munkenbeck and Green, 1976; Stumpf *et al.*, 1993; Finnerty, Enright and Roche, 1998). It is possible that during this period the testes are particularly sensitive to perturbations in LH output. Indeed, Rawlings *et al.* (1978) noted that the relationship between plasma LH and testosterone was maximal from around 9-11 months of age. Hence this suppression of testosterone may be the result of long-term suppression of LH pulse amplitude at a time when the testes are maximally sensitive to changes in gonadotrophin concentrations. Alternatively, the smaller testes in immunised bulls compared with control bulls may not have been able to maintain testosterone concentrations. The delay in attainment of puberty also coincides with the lower testosterone concentrations between 36-44 weeks in the immunised group.

3.4.3.2 Testicular growth

Onset of rapid testicular growth occurred at around 16-21 weeks, slightly earlier than Amman and Walker (1983) witnessed. The rise in gonadotrophins at around 16 weeks, also observed in the current trial, is believed to initiate testicular growth and

development (McCarthy, Convey and Hafs 1979; McCarthy, Hafs and Convey, 1979; Amman and Walker, 1983). Previously, Evans *et al.* (1995) demonstrated that calves with highest LH level between 6-20 weeks reached puberty earlier than those with lower LH. Furthermore, suppression of LH between 7.5-24 weeks with oestradiol implants reduced testes mass. More recently, using a GnRH agonist to suppress gonadotrophins and testosterone, prior to rapid testicular growth, (Chandolia, Evans and Rawlings, 1997) testes growth rates were suppressed. The testes of treated calves had not caught up with those of controls by the end of the trial at week 50, despite a rebound effect of gonadotrophins by week 24. Thus, early gonadotrophin secretion, by 24 weeks of age is a critical step in the establishment and timing of normal testicular development in bull calves (Chandolia, Evans and Rawlings, 1997). In the current trial GnRH titres were sufficient to reduce LH pulse amplitude and mean LH concentrations causing a delay in the rise in testosterone. In agreement with previous studies, the rate of testicular growth also slowed significantly ($P<0.02$). By week 72, approximately 58.5 weeks after the 2nd booster injection and 48 weeks after the titres fell to <5% binding (1:1000), the GnRH immunised bulls testes circumference was not different to the controls. Thus testes growth and development was significantly reduced, but increased gonadotrophin secretion from 20 weeks onward appears important to sustain testicular growth (see Schanbacher, 1981).

Our results are also similar to those of Adams *et al.* (1993; 1996), Finnerty *et al.* (1996), Finnerty, Enright and Roche, (1998) D'Occhio, (1993), D'Occhio, Aspden and Trigg, (2001) in which testicular growth was reduced after pre-pubertal immunisation. Whereas in peri- and post-pubertal bulls, testicular atrophy following immunisation (Robertson, Wilson and Fraser, 1979; Adams and Adams, 1992; Cook *et al.*, 2000). After a transient suppression of testes growth in earlier studies in bulls (Adams *et al.*, 1993; Finnerty *et al.*, 1996) and rams (Brown *et al.*, 1994), the testes of GnRH immunised animals were eventually able to compensate for the reduction in size and produce normal concentrations of testosterone. The current study agrees with previous reports overall, however as discussed (Section 3.4.3.1), during some stages of development testosterone concentrations were reduced in the treated animals.

3.4.3.3 Vesicular gland response

As with the testes vesicular gland length was significantly affected by age in this and earlier studies (Rocha *et al.*, 1994; Chandolia *et al.*, 1997b). During the first 14 weeks of life, coinciding with increased LH output, fluid accumulates in the gland (Chandolia *et al.*, 1997b). The rapid growth rate from 12-28 weeks in the current trial agrees with

previous reports of increased cellular proliferation from 16-28 weeks (Chandolia *et al.*, 1997b). During this period, and up to week 36, we observed for the first time, a reduction in vesicular gland length in GnRH immunised bulls, which we attribute to the reduced testosterone between 16-20 weeks of age. We speculate that, during this period of transient vesicular gland growth retardation, seminal fluid fructose and citric acid concentrations would have been reduced, as was the case following GnRH immunisation in pubertal bulls (Robertson, Wilson and Fraser, 1979). After week 28 we observed a marginally significant ($P<0.09$) and previously unreported fall in vesicular gland length followed by a plateau in vesicular gland size in control bulls, while vesicular gland growth in immunised bulls plateau. This apparent growth check is probably due to the dramatic increase in vesicular gland secretions (Mann *et al.*, 1967; Chandolia *et al.*, 1997b), which are induced by increased testosterone concentrations at this time (Rawlings *et al.*, 1972; Amann and Walker, 1983). Alternatively, the halt to vesicular gland growth may be due to testosterone negative feedback reducing LH secretion. However, the latter suggestion is unlikely as both vesicular gland weight and fructose and citric acid content are significantly correlated with testosterone (Mann, 1964; Macmillan and Hafs, 1969; Chandolia *et al.*, 1997b). By 40 weeks of age, vesicular gland size was similar in both groups, despite significantly higher testosterone concentrations in control animals from 36-44 weeks of age. Although testosterone concentrations were reduced in immunised bulls, they were still within the typical range for bulls of this age. This suggests that there may be a threshold testosterone level at which the vesicular gland is maximally stimulated. Also this indicates that the vesicular gland was able to recover much more quickly than the testes, following gonadotrophic deprivation. This finding agrees with earlier observations in bulls following GnRH immunisation (Robertson *et al.*, 1982; 1984), in which only small numbers of mostly dead sperm were recovered from each vas deferens and epididymis, but the accessory glands, were observed to be well developed. Vesicular gland width in our study was significantly affected by age however a significant reduction in vesicular gland width following GnRH immunisation was only evident at around 20 weeks of age. The vesicular gland at 11 weeks was 20% of the adult width and only 8 % of the adult length. Throughout the trial the growth phases may be divided into the "proliferation phase" 11-28 weeks and the "secretory growth phase" >28-72 weeks. During these phases the percentage of vesicular gland growth was 66.3% and 33.7% for the length, and 36.7% and 63.3% for the width, respectively.

Several other reproductive organs including the penis, epididymis, prostate, bulbo-urethral and vesicular glands, which are testosterone dependent for normal development (section 1.4.4), were examined at week 72. No effects of GnRH immunisation were observed on any of these accessory organs at this time, thus these organs were either not significantly effected by immunisation, or they were, but the effects were reversible.

3.4.4 Behaviour

The increase in testosterone concentrations at around 6 months is accompanied by a dramatic increase in both sexual and aggressive behaviours (McCarthy, Convey and Hafs, 1979). In the current trial, GnRH immunised bull calves were easier to manage and more docile between 22-30 weeks of age. These observations agree with those published by Robertson, Wilson and Fraser, (1979) and Robertson *et al.* (1981). However, a more objective study (Jago *et al.*, 1995) reported increased mounting behaviour after GnRH immunisation, though these findings are likely due to a "rebound effect" of increased GnRH pulses and gonadotrophic output, which resulted in increased testosterone in the immunised animals following the fall in GnRH antibody titres. In contrast, more recent studies (Finnerty *et al.*, 1996; Jago, Bass and Matthews, 1997; Huxsoll, Price and Adams, 1998) observed reduced sexual and aggressive behaviours following GnRH immunisation in bulls. More objective bull behaviour observations began at 38 weeks of age in the current study, by which time both groups were exhibiting the full array of sexual and aggressive behaviours as defined by Price and Wallach, (1991a;b;c). The fact that the behaviour of both groups was indistinguishable is indicative of the absence of any long-term effect on behaviour after GnRH immunisation. Our findings agree with previous (Jago, Bass and Matthews, 1997) observations on sexual behaviour, however they are in conflict with antagonistic behaviours, which were still reduced at 17 months of age after pre-pubertal GnRH immunisation. This apparent prolonged effect of immunisation (Jago, Bass and Matthews, 1997) may result from a third booster injection and from some animals having reduced testosterone at the time of behavioural assessment. The absence of a suppression in behavioural characteristics in the current study also contrasts with previous work in Ireland (Finnerty *et al.*, 1996), which reported long-term suppressive effects on both sexual and aggressive behaviours 14 months after testosterone concentrations had returned to normal following GnRH immunisation in infant bull calves (8-10 weeks of age). The reason for such contrasting results is unclear, but may involve the fact that, sparring behaviour was not recognised as antagonistic and chin resting and Flehmen were not counted as sexual behaviours

(Finnerty *et al.*, 1996). Furthermore, assessment of behaviours from 8-11 months of age was carried out at pasture were animals had significantly more space than our housed animal study. The aforementioned reasons may also go some way to explaining a further conflicting observation. In the current study, the incidence of sexual and aggressive behaviours was similar overall, with a tendency for increased aggressive behaviours at 68 to 70 weeks. Whereas previously it was observed that 1.4 times more sexual behaviour occurred than aggressive behaviour (Finnerty *et al.*, 1996).

Subsequent to GnRH immunisation, it has previously (Jago, Bass and Matthews, 1997; Jago *et al.*, 1997) been reported that normal HPG function between 2-10 months of age is not necessary for the development or latter expression of these behaviours. Based on the current findings and previous data (Jago, Bass and Matthews, 1997; Jago *et al.*, 1997) we suggest that normal HPG function between 3 weeks to 10 months of age is not necessary for the development or latter expression of these behaviours. This is in contrast to several other species including pigs and sheep, in which the absence of testosterone during specific periods between birth and puberty prevent the development of typical male behaviours (Ford and D'Occhio, 1989). Recently, it has been suggested that exposure of bull calves to testosterone at an early age (neonatal or pre-natal) may be essential for the expression of normal reproductive behaviours in the adult bull (Inwalle and Schillo, 2002). Evidence in other species (Sachs and Meisel, 1988) indicates that testosterone (or it's oestrogenic metabolites) may organise neural substrates critical to the regulation of reproductive behaviour (Inwalle and Schillo, 2002). Indeed, exposure of neonatal male rodents to oestradiol results in prolonged/permanent reproductive suppression (Ulibarri and Yahr, 1996). Research into this area in cattle is lacking and we suggest that passive GnRH immunisation studies in bull calf foetuses would be a good approach to understanding the role of testosterone in early development on bull behaviours.

3.4.3.4 Sperm quality

Caudal epididymal sperm volume appeared to be smaller in some of the immunised bulls, making sperm collection more difficult in these animals. Unfortunately, with our method of collection, it was not possible to obtain an accurate sperm count. Previously however, during periods of increased GnRH antibody titres it was observed that spermatogenesis was arrested or significantly reduced (Robertson *et al.*, 1982). Furthermore, while some degree of behavioural suppression was still

evident, most sperm collected from these bulls were dead (Robertson *et al.*, 1982). In the present trial no significant difference in the numbers of live and motile sperm were observed between control and immunised bulls. However, control bulls tended to have a greater proportion (P=0.055; 92.1 vs. 86.4%) of morphologically normal sperm, with the majority of the abnormal sperm (9.4%) from immunised bulls presenting with pyroform head abnormalities. As sperm quality improves rapidly after puberty (Chicoteau *et al.*, 1990), one could argue that this finding is the result of a delay in the attainment of puberty in GnRH immunised bulls relative to controls. This type of abnormality has also been observed subsequent to an interruption in spermatogenesis (Williams and Savage, 1925; Lagerlof, 1938, Hancock, 1959, Thundathil *et al.*, 1999). However, as the motility and viability of the sperm from immunised bulls was unaffected by treatment and as 86% of sperm were morphologically normal, the immunised bulls at 72 weeks would be fertile (Barth and Oko, 1989; Thundathil *et al.*, 1999). These data, together with previous reports (Robertson, Wilson and Fraser, 1979; Robertson *et al.*, 1981; 1982), suggest that the recovery of spermatogenesis takes place several months after the recovery in testosterone concentration. This is not suppressing as the process of spermatogenesis takes 61 days in bulls (Johnson, 1991). The present study, for the first time, shows that although recovery of spermatogenesis occurs after immunisation, this recovery appears to take about 56 weeks, i.e., 20-22 weeks after re-establishment of typical male behaviours.

3.4.4 Anabolic Response

Despite the transient reductions in peripheral testosterone, there was no difference in growth rates between bulls. Possibly due to long periods of time during which testosterone concentrations in treated animals were similar to those in controls. Despite the fact that the bulls were culled about 4 weeks prior to finishing, there was some evidence of a beneficial effect of GnRH immunisation on carcass conformation in agreement with Cook *et al.* (2000). Rib-eye width was improved ($P<0.05$) in GnRH immunised bulls, as was the carcass conformation grades ($P<0.05$) allocated by an independent meat inspector. These findings are in agreement with Huxsoll, Price and Adams, (1998). Hence future work should either include immunisation with a more responsive adjuvant or at an earlier age, or both.

3.4.5 Future Work

Research in the ovine foetus has shown that the HPG axis undergoes development and maturational changes in response to gonadotrophic stimulation. Elevated

gonadotrophin output during mid-gestation, is critical in normal reproductive development, and may be important in the attainment of puberty in the adult (Brooks, McNeilly and Thomas, 1995). For example, the suppression of foetal gonadotrophins with GnRH agonist implants can lead to reduced testes size and Sertoli cell populations at birth (Thomas *et al.*, 1994). Furthermore, testosterone exposure from day 30-90 in sheep induces the sex specific organisation of the GnRH neural network (see Robinson *et al.*, 2003), which results in sex differences in response to steroid feedback, and may also have a role in behaviour (R. Birch, personal communication). Maternal passive immunisation to GnRH may therefore prove useful as a non-invasive procedure for studying the development of the foetal reproductive axis, as IgGs have been shown to cross the foetal-placental barrier in several species, including sheep (Miller, Fraser and Brooks, 1998). The bovine hypothalamic-pituitary-gonadal axis may be sufficiently mature at birth that the removal of GnRH or exposure to GnRH antibodies has no permanent effect on reproductive development or function. However, this would require further investigation. Immunisation of the pregnant cow may be a useful approach for the study of hypothalamic/pituitary gland maturation and for investigating whether or not a window of susceptibility to GnRH antibodies exists. Alternatively, passive immunisation, or a combination of passive immunisation, followed by GnRH treatment to reduce antibody levels before active immunisation may prove useful future approaches, which may increase the likelihood of exposure of the maturing hypothalamus and pituitary gland to elevated GnRH antibodies, and would reduce the variability of exposure to GnRH antibodies, as occurs after active immunisation.

New innovative vaccines including genetic adjuvants (Pertmer *et al.*, 2001; Bot and Bona, 2002) and antigen beads, with surface-conjugated or liposome containing encapsulated antigen, which stimulate Th1 type responses (Vogel and Hem, 2003), and variable speed antigen delivery (Stevens, 1993) may be required for successful stimulation of the immature immune system. In the short term however, 'prime-boost GnRH vaccination (using different adjuvants or constructs in the primary and booster vaccines) might be advantageous (see Woodland, 2004). Such vaccines could also provide further benefits by reducing diseases in calves. In addition, formulation of diets in neonatal calves should take into account decreased feed intake associated with stress (Galyean, Perino and Duff, 1999). The development of calf diets, which enhance immune function, but protect against immune cytokine-induced catabolism (Cook and Pariza, 1998) may further accelerate the wide-scale adoption of this potentially excellent alternative to conventional castration.

In conclusion, the use of neonatal GnRH immunisation is currently not a practical management tool for the suppression of reproductive function in beef cattle, due to the reversibility of the physiological effects. A more prolonged or permanent suppression of reproductive function may be possible following an earlier, greater and more sustained elevation of antibody titres during the neonatal period, making it a more viable option. Repeating such a trial and giving the 1st booster earlier is problematic as the evidence (Finnerty *et al.*, 1994) suggests that a 56, rather than 28 day interval between primary and 1st booster is optimal. Therefore, further development of GnRH vaccines and protocols, and studies of performance in cattle are required. In the mean time, the vaccine used in this trial may be used to delay puberty in older calves (>28 weeks of age), or transiently suppress reproductive function in cattle to aid management, e.g. during periods of grazing and/or housing, especially with animals of the opposite sex.

CHAPTER 4

THE EFFECTS OF ACTIVE IMMUNISATION AGAINST GnRH ON REPRODUCTIVE FUNCTION IN HEIFER CALVES

4.1 INTRODUCTION

Estimates from feedlots in the United States indicate that at the time of slaughter up to 25% of beef heifers are pregnant (Kreikemeier and Unruh, 1993). Moreover, in extensive range systems of northern Australia, studies report even higher rates of unwanted pregnancies, between 50-80% (Hoskinson *et al.*, 1990). As the calf is of no market value, the slaughter of pregnant cattle for meat represents a major (5-10%) loss of production (Bell *et al.*, 1997). Furthermore, increased activity associated with recurrent oestrus increases energy expenditure (Horstman *et al.*, 1982), heightens the risk of injury, and results in up to 10% reduction in feed conversion efficiency (FCE) and growth relative to steers (Johnson *et al.*, 1988). In addition, oestrus around the time of slaughter also increases the incidence of dark-cutting meat (O'Brian, Bloss and Nicks, 1968; Young, Cundiff and Bradley, 1969; Adams *et al.*, 1990). As a result, heifer calves achieve lower prices at sale than steers of similar age and carcass weight (Todd and Cowell, 1981; Meyer, 1987; Jeffery *et al.*, 1997; Bell *et al.*, 1997).

Traditionally, the only options available to the farmer to prevent unwanted pregnancies have been either surgical spaying or PGF2α injections, to abort pregnant heifers entering the feedlot (Johnson *et al.*, 1988). Flank spaying is effective, but the resulting carcass scaring may further reduce market price (Jeffery *et al.*, 1997). While vaginally castrated heifers escape such scars, the operation is "done blind" and residual ovarian tissue, inadvertently left in the animal, is enough to maintain oestrus and fertility (Adams *et al.*, 1990). Hence these approaches are inefficient, expensive (Johnson *et al.*, 1988) and increase the risk of morbidity and mortality (Johnson *et al.*, 1988; Trigg, 1994). They also compromise animal welfare and good ethical farming practice.

Another method is the separation of heifers from bulls (Rudder and Corlis, 1974; Jeffrey *et al.*, 1997), but this is often impractical. Once pregnant, heifers could be held back and allowed to calve, however, this is not usually an economically viable option as it may take up to a year after calving to recover lost body condition (Jeffrey *et al.*,

1997). In addition to unwanted pregnancies, recurrent oestrus behaviour in feedlot heifers has been blamed for compromising carcass quantity and quality because of reduced growth (Bell *et al.*, 1997) and increased injuries associated with elevated activity.

Numerous attempts have been made to prevent oestrus and pregnancy in the bovine female. For example, feeding synthetic progestagen, to prevent oestrus (Bloss *et al.*, 1966; Bell *et al.*, 1997), immunisation against ovarian steroids such as oestradiol-17β to prevent oestrus (Martin *et al.*, 1978; Martensz, 1980; Wise and Ferrell, 1984; Hoskinson *et al.*, 1990), immunisation against PGF2α (Scaramuzzi and Baird, 1976; Chang, McFarland and Reeves, 1987; Crowe, Enright and Roche, 1995), immunisation against LH (sheep: Schanbacher, 1985; Roberts and Reeves, 1989; cattle: De Silva *et al.*, 1986; Grieger and Reeves, 1988; 1990; Johnson *et al.*, 1988; Grieger *et al.*, 1990; Roberts *et al.*, 1990) and human chorionic gonadotrophin (Johnson *et al.*, 1988). In addition, passive immunisation against eCG (Yadav *et al.*, 1986) has also been investigated. Despite Johnson *et al.* (1988) reporting that, immunisation against an LH-ovalbumin conjugate produced better results than a GnRH-ovalbumin conjugate, using three different adjuvants, GnRH is the preferred approach. This is on account of the role of GnRH in driving reproductive function and its conservation between species. Hence it is widely accepted that GnRH would be the most appropriate hormone to neutralise (Bell *et al.*, 1997).

GnRH immunisation in the post-pubertal bovine female induces a hypogonadotrophic state (Prendiville *et al.*, 1996), which in turn suppresses recurrent ovarian follicular waves (Adams and Adams, 1990; Prendiville *et al.*, 1995a; Crowe *et al.*, 2001a; Roche and Crowe, 2004) and induces ovarian (Johnson *et al.*, 1988; Adams and Adams, 1990) and uterine atrophy (Adams and Adams, 1990). GnRH immunisation in the young heifer delays the onset of puberty (Wetteman and Castree, 1988; D'Occhio, 1993; Sejrsen, Enright and Prendiville, 1994; Prendiville *et al.*, 1995b), while in the post-pubertal animal oestrus and ovulation are inhibited and pregnancy rates reduced (Hoskinson *et al.*, 1990; Vizcarra and Wettemann, 1994; Bell *et al.*, 1997; Jeffery *et al.*, 1997; Sosa *et al.*, 2000). These effects are only temporary, typically lasting 6-8 months (D'Occhio, 1993). Reversibility of immunocastration may be considered advantageous under certain circumstances. However, it is not desirable in production systems where cull females have to be retained for extended periods, often in mixed herds (Hoskinson *et al.*, 1990), before they reach a marketable condition (D'Occhio, 1993). Repeated booster vaccinations may be given

to extend the period of immunocastration, however, this option is seldom viable due to the requirement for increased vaccine costs and mustering. Hence, the reversibility of immunocastration is largely responsible for the lack of wide-scale adoption of this technology.

Recent studies in ewes, however, have reported that following GnRH immunisation in the neonatal or pre-pubertal period, a permanent suppression of reproductive function may be achieved in ~80% of animals (Brown *et al.*, 1995; Clarke *et al.*, 1998). Presumably, this is because exposure of the immature median eminence (ME) /pituitary gland to GnRH antibodies may impair normal maturation of the hypothalamic/pituitary unit during a critical period of development (Clarke *et al.*, 1998). Furthermore, evidence in sheep suggests that these effects and the degree of reproductive suppression may be more pronounced in the female, than in the male (Brown *et al.*, 1995; Clarke *et al.*, 1998).

The objective of this study (which ran concomitantly with the study in bull calves described in Chapter 3) was to investigate the effects of neonatal immunisation against GnRH in female calves and the longevity of these effects. Using a newly developed GnRH vaccine, combined with an early immunisation (14-19 days of age) protocol, we investigated the effects of GnRH vaccination on reproductive function.

4.2 MATERIALS AND METHODS

4.2.1 Experimental Animals

The experimental animals used in the current study consisted of 18 beef cross heifer calves. They were monitored between 2-56 weeks of age; with additional LH pulse bleeds at 63 and 65 weeks of age. Animals were weighed at 10-14 day intervals (typically at the time of blood sample collection). The management of animals was as described in Chapter 2.

4.2.2 Experimental Protocol

The experimental protocol and justification of protocol has been described in section 3.2.2. The experimental period ran from May 2000 to August 2001. The heifers were kept as described in Chapter 2. Where possible, all procedures on heifer calves were carried out concomitantly with those of the bull calves (see Chapter 3). However, in contrast to the experiment described in Chapter 3, the animals in this trial were re-used in an experiment described in Chapter 6, subsequently they were culled in March 2002.

4.2.2.1 Immunisation

Vaccine was administered (single s.c. injection) to the brisket area in nine calves. Primary, first and second booster injections were administered at 16 ± 0.5 (2ml), 44 ± 0.5 (2ml) and 95 ± 0.9 (1.7ml) days of age respectively. Nine control animals received saline on the same days. For vaccine details see Section 3.2.2.1.

4.2.2.1.1 Vaccination site assessment

The granuloma (swelling at the injection site) was measured using callipers on days: 1, 3, 7, 10 and 14 following each vaccination. The vaccination site results are presented in Chapter 3.

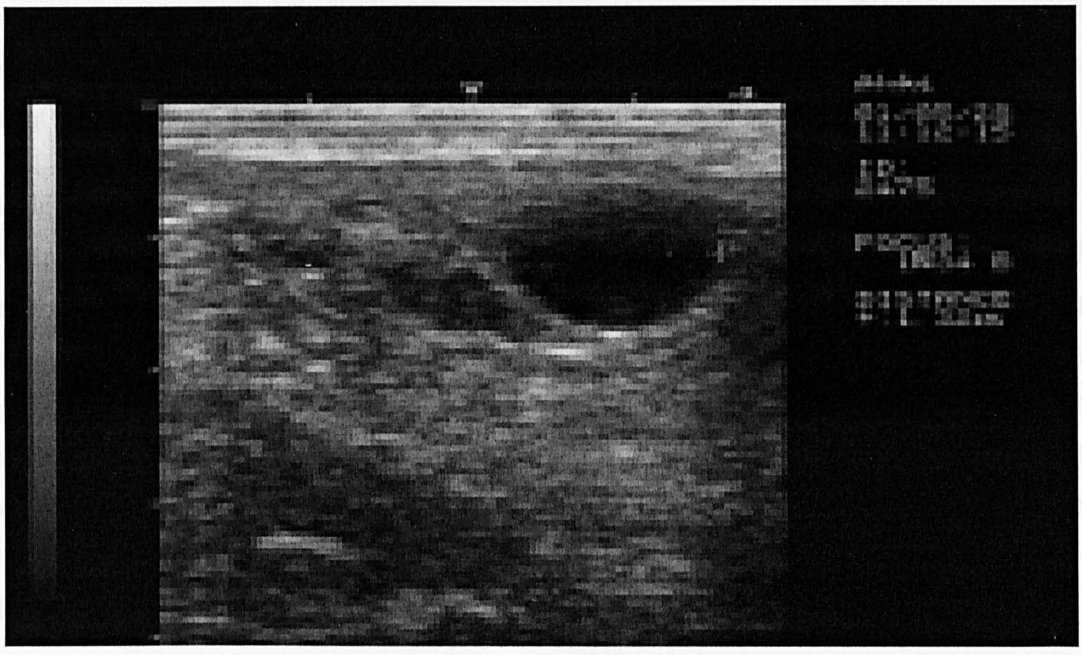
4.2.2.2 Blood sampling

Blood samples were collected by venepuncture every 10-14 days to measure GnRH antibody titres, FSH concentrations and every 7 days from week 35-55 to measure progesterone and the display of oestrous cycles. To characterise peripheral LH concentrations, serial bleeds (every 15 min for 8h; via cannulae) were conducted at the following ages (mean ± s.e.m.): 0.8 ± 0.01, 3.7 ± 0.0, 6.7 ± 0.03, 11.1 ± 0.03, 14.4 ± 0.03 and 14.8 ± 0.03 months. These ages were rounded up or down to the nearest week for ease of presentation to give: 4, 16, 29, 48, 62 and 64 weeks of age. The serial bleeds at 62 and 64 weeks of age were carried out during a synchronised follicular phase and subsequent luteal phase, respectively. Oestrous cycles were synchronised by administering two PG injections, 11 days apart. The follicular and luteal phase bleeds took place 1 and 15 days after the 2nd PG injection, respectively.

4.2.2.3 Real-time ultrasound scanning

Ovarian follicular development was measured monthly from 11.5 to 57 weeks of age using real-time ultrasonography (Figure 5.1). Following rectal palpation of the ovaries, trans rectal scanning was conducted, in multiple planes, in order to count antral follicle numbers. Counts were made of medium-sized (4-7.9mm diameter) and large (≥ 8mm) ovarian follicles.

Figure 4.1 Dominant follicle measurement using ultrasound scanning. The fluid filled antral cavity is non-echogenic and appears black (Pierson and Ginther, 1984). The image below contains one large dominant follicle and several smaller subordinate follicles, however the exact number of smaller follicles is difficult to decipher in this still image. The diameter of the large follicle is displayed as the distance between (+) markers.



4.2.2.4 Behaviour assessment

To support the progesterone (onset of cyclicity) data and to examine heat intensity oestrous behaviour was monitored for 20 min twice daily, between 52-56 weeks of age (following turnout), from a distance using field glasses. Due to practical limitations and foot and mouth disease prevention measures, more thorough monitoring of oestrus was not possible.

4.2.2.5 Post-mortem

The heifers used in this study were culled at 22 months of age, some 82 weeks after the final booster GnRH vaccination and 62 weeks after GnRH antibody titres were last raised above 5% binding at 1 in 1,000. The carcass results are presented in this study, however the post mortem results relating to pregnancy are presented in Chapter 6 following artificial insemination (A.I.), which was carried out as part of the Chapter 6 study protocol.

4.2.2.5.1 Carcass characteristics

The carcass of each heifer was issued with a fat score and conformation grade by an independent MLC meat inspector.

4.2.2.6 Statistical analyses

Prior to any analysis a general ANOVA was performed on the data and the distribution of the data was checked on residual plots. This was carried out to determine the data distribution status, i.e. normal or non-normal distribution. Non-normally distributed data were log transformed. Where possible, log transformation (ln) of the data was avoided.

MUNRO©, a data analysis program (developed by Dr. P. L. Taylor, Edinburgh, 1987), was used to identify LH pulses and calculate LH mean and basal concentrations and pulse frequency and amplitude (see appendix III for calibration details). The resulting statistics were analysed using Genstat 6.1, split-plot analysis of variance with repeated measures. Degrees of freedom were adjusted for the Greenhouse-Geisser epsilon effect (see 3.2.3 for details). In addition, an orthogonal contrast (independent comparison) was carried out on the post-pubertal (follicular and luteal phase) data within the LH analysis. This enabled the comparison of both pre- and post-pubertal follicular and luteal phase LH output.

GnRH antibody titres at 1:1,000 dilutions were also analysed using Genstat 6.1 (ANOVA: Lawes Agricultural Trust, 2001, IACR Rothamsted) split-plot analysis of variance with repeated measures. Degrees of freedom were adjusted for the GGE effect.

Follicle counts were analysed in Genstat 6.1 using generalised linear regression analysis with Poisson distribution and logarithmic link function. Poisson analysis was used because low numbers of large follicles resulted in a skewed distribution of data. This analysis program does not provide standard errors of the differences or means. Poisson analysis of medium-sized follicles from high (n=3) and low (n=3) GnRH titre animals was carried out to compare the physiological response following extremes of immune response to vaccinations. No medium response data is presented as the three remaining animals, which exhibited intermediary GnRH antibody titres were judged too variable in their immune responses, i.e., magnitude and longevity of elevated GnRH antibody titres.

FSH data were log transformed (ln) before analysis, to normalise distribution. Subsequently, FSH was analysed using split-plot analysis with repeated measures. Degrees of freedom were adjusted for the GGE effect. Carcass conformation and fat grades were analysed using Chi squared test in Microsoft Excel. All other analysis was carried out using Genstat 6.1, ANOVA.

Where data are transformed to a logarithmic scale to normalise the distribution (FSH) of data, the textual description and statistical significance are based on transformed data. However, for ease of presentation, especially for presenting standard errors, the tables and figures will display the unadjusted data unless otherwise stated.

4.3 RESULTS

4.3.1 Live weights and Growth Rates

Age had a significant effect ($P<0.001$) upon live weight. The slope of the regression for live weight on age (Figure 4.2) indicates an average overall growth rate of 5.66kg per week i.e., daily growth of 0.79 kg in control heifers and 0.78 kg in immunised heifers (Figure 4.2). These growth rates were not significantly different ($P>0.05$). The mean (± s.e.m.) live weight of both treatment groups of heifers, at 6 ages throughout the trial is presented in Table 4.1.

Figure 4.2. The regression of weight on age in heifers (n=18). The equation of this regression line is $y = 5.66x + 24.30$ ($r^2 = 0.97$). Live weight significantly ($P<0.001$) increased with age.

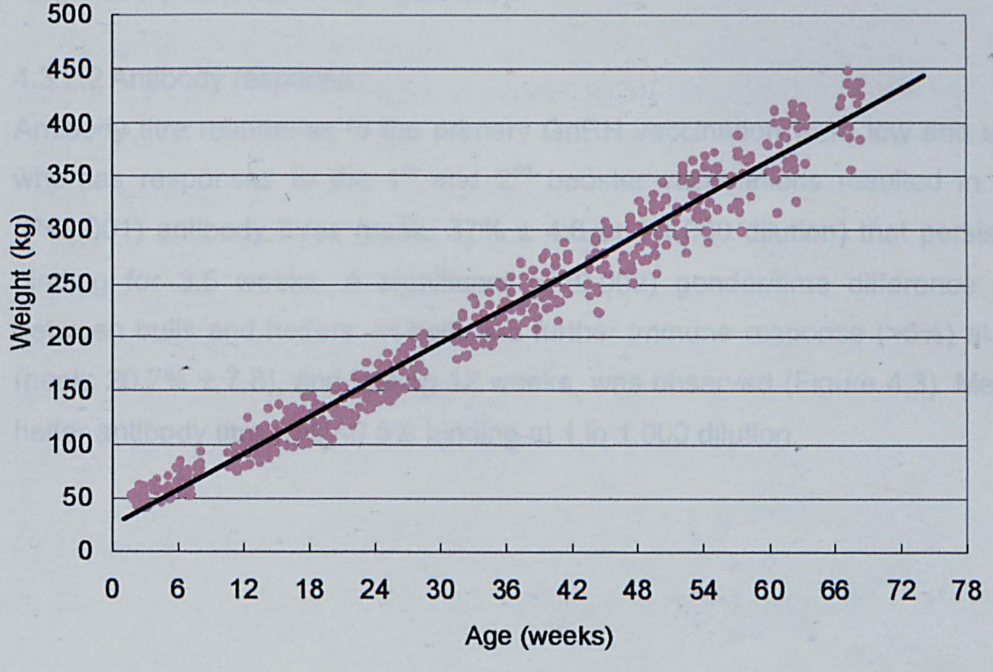


Table 4.1 Live weights (mean ± s.e.m.) of GnRH immunised (n=9) and control (n=9) heifers at six ages taken around the time of LH serial bleeds. Immunisation against GnRH had no significant (P>0.05) effect on live weight.

Age (weeks)	Immunised Mean ± s.e.m. (n=9)	Control Mean ± s.e.m. (n=9)	Overall Mean ± s.e.m. (n=18)
4	55.1 (1.9)	56.2 (2.1)	55.6 (1.4)
16	109.7 (3.4)	115.4 (4.3)	112.6 (2.3)
29	181.4 (5.6)	192.6 (5.2)	187.0 (4.0)
48	292.2 (9.0)	294.0 (6.5)	293.1 (5.4)
63	382.6 (10.7)	395.3 (7.0)	388.9 (6.4)
65	392.7 (10.2)	405.4 (6.3)	399.1 (5.9)

4.3.2 Immune response to GnRH vaccine

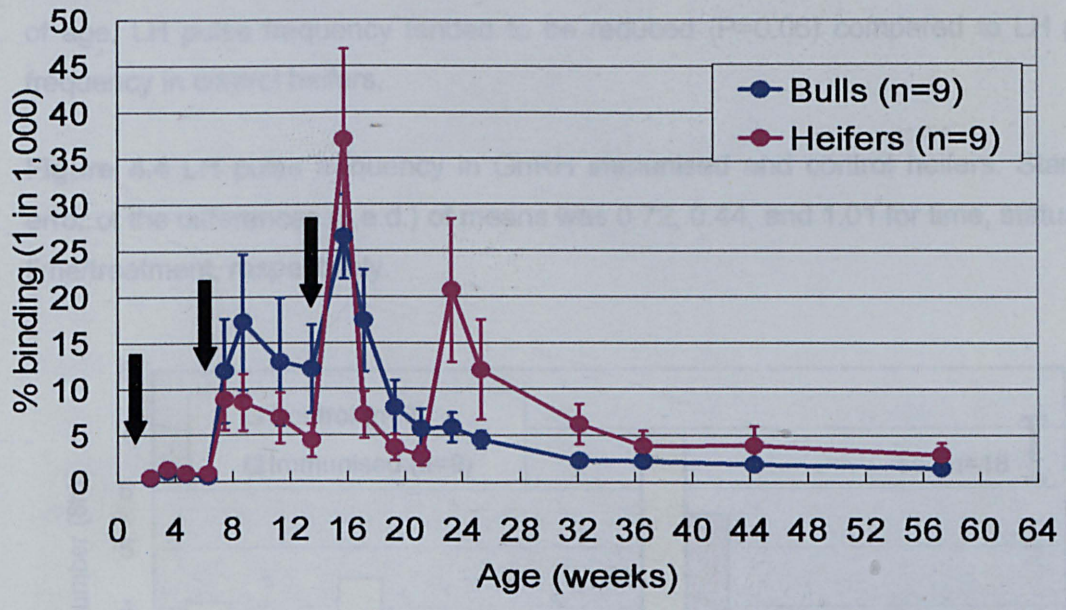
4.3.2.1 Injection site response

The injection site analysis, measured as, the volume of subcutaneous swelling was significant (P<0.001), when compared to the response site in control animals where there was no response. The response to 2nd booster vaccine was of greater magnitude (see Chapter 3; Figure 3.4).

4.3.2.2 Antibody response

Antibody titre responses to the primary GnRH vaccination were low and short-lived, whereas responses to the 1st and 2nd booster vaccinations resulted in significant (P<0.001) antibody titres (peak: 37% ± 4.6 at 1:1,000 dilution) that persisted >15% binding for 3.5 weeks. A significant (P=0.002) gender/time difference was seen between bulls and heifers. In heifers a further immune response (>5%) at 23 weeks (peak: 20.7% ± 7.8), and lasting 12 weeks, was observed (Figure 4.3). Mean control heifer antibody titre was <0.5% binding at 1 in 1,000 dilution.

Figure 4.3 Mean (± s.e.m.) GnRH antibody titre (1:1,000 dilution) profiles over time for GnRH immunised (n=9) heifers and GnRH immunised bulls (n=9). Black arrows indicate approximate time of vaccination at 2, 6 and 13 weeks of age. Although GnRH antibody titres tended to increase (P=0.17) following the primary immunisation, significant (P<0.001) increases occurred after the booster injections. A further significant (P<0.001) increase in GnRH antibody titres was observed in the heifers at ~23 weeks of age. This final increase was significantly (P<0.002) different from the GnRH antibody titres seen in the bulls at this time. Although declining, small, but significant (P<0.05) antibody titres persisted to the end of the sampling period.



4.3.3 Reproductive Hormones

Results of the analysis of reproductive hormones will be presented in 4 sections. The first will describe 4 LH parameters, the second will refer to FSH concentrations and the third describes oestradiol concentrations on LH serial bleed days during the synchronised follicular phase at 63 weeks and the subsequent luteal phase at 65 weeks. Finally, an example of progesterone monitoring with a view to determination of onset of oestrous cyclicity is presented. The decision to measure oestradiol was taken when evidence of long-term disruption of LH pulse frequency was discovered (for details see section 4.3.4.1.1 and Figure 4.4).

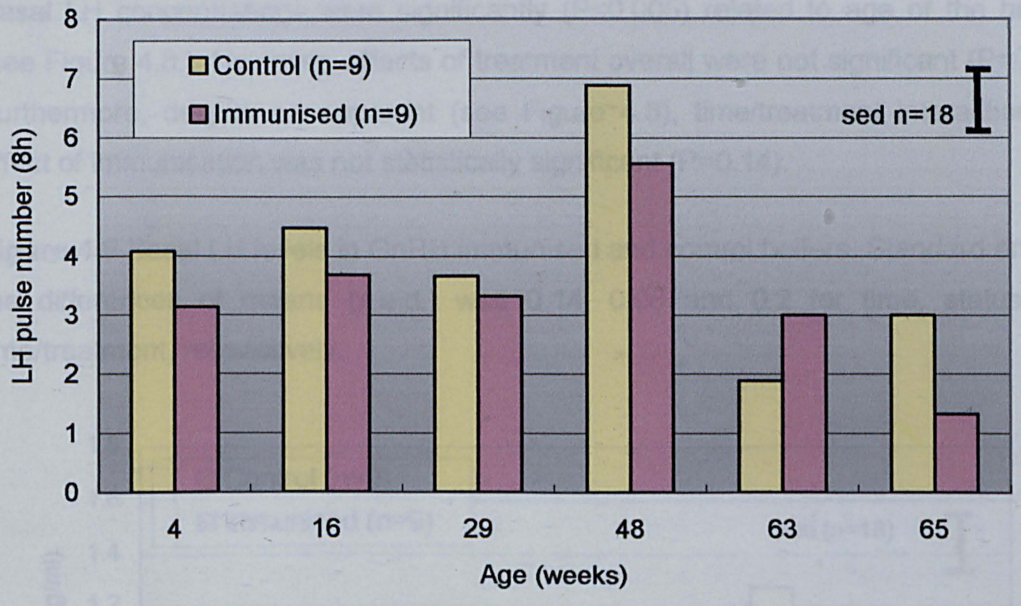
4.3.3.1 Luteinising hormone

Four parameters of LH output were investigated and are defined in Chapter 3; Table 3.1.

4.3.3.1.1 LH pulse frequency

Mean number of LH pulses at 6 time-points is illustrated in Figure 4.6. LH pulse frequency was significantly affected by age (P<0.001). An increase (P<0.001) in LH pulse frequency occurred at 11 months in all heifers (about one month prior to puberty, as identified by progesterone monitoring). More specifically, following puberty (at approximately 12-13 months of age) LH pulse frequency was reduced (P<0.001) relative to that observed prior to puberty (<12 months). Overall, GnRH immunisation did not significantly affect LH pulse frequency (P=0.15). However, post-puberty, during the follicular phase at 63 weeks of age, immunised heifers showed marginally increased pulse frequency (P=0.06), while in the luteal phase, at 65 weeks of age, LH pulse frequency tended to be reduced (P=0.06) compared to LH pulse frequency in control heifers.

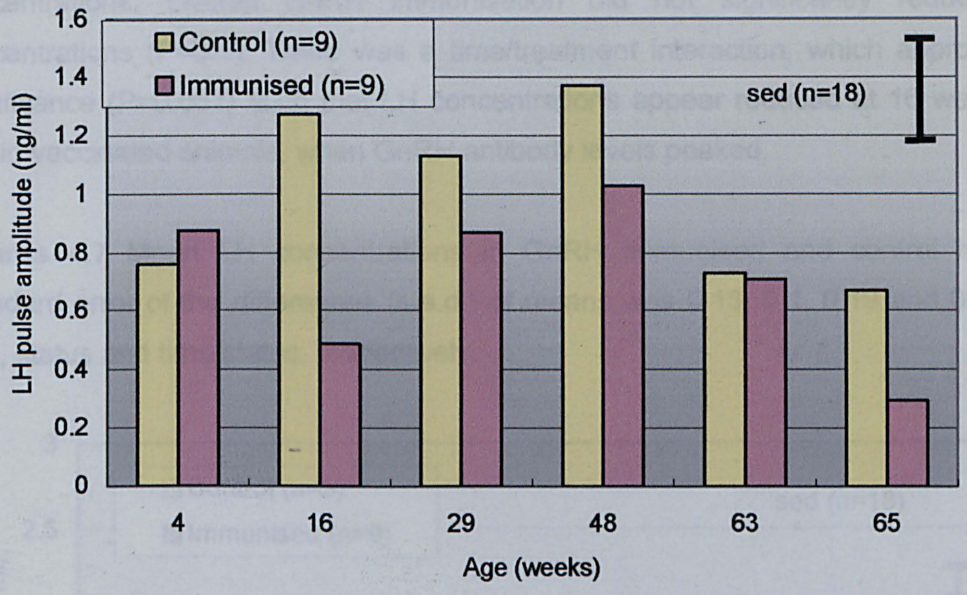
Figure 4.4 LH pulse frequency in GnRH immunised and control heifers. Standard error of the differences (s.e.d.) of means was 0.72, 0.44, and 1.01 for time, status and time/treatment, respectively.



4.3.3.1.2 *LH pulse amplitude*

Mean LH pulse amplitude at six time points are illustrated in Figure 4.7. The effect of age on LH pulse amplitude was significant (P<0.05), with higher LH pulse amplitude at 16, 29 and 48 weeks of age and lower LH pulse amplitude in the neonatal and post-pubertal periods. GnRH immunisation did affect (P<0.05) LH pulse amplitude over the entire trial. Although there was no significant time/treatment interaction (P=0.26), the effects of immunisation are most pronounced at 16 weeks of age.

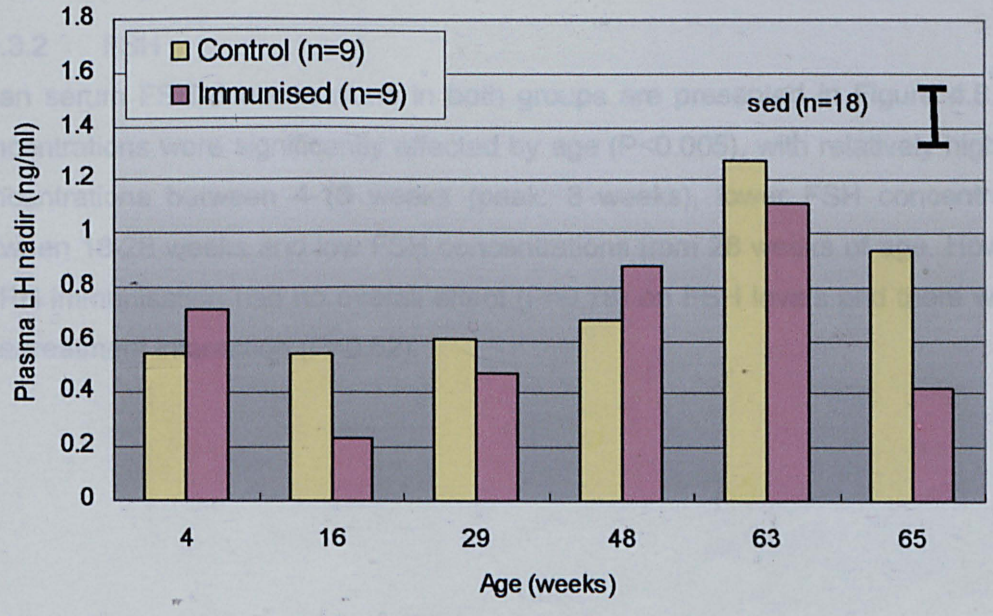
Figure 4.5 Mean LH pulse amplitude in GnRH immunised and control heifers. Standard error of the differences (s.e.d.) of means was 0.19, 0.13, and 0.27 for time, status and time/treatment, respectively.



4.3.3.1.3 Basal LH

Basal LH concentrations were significantly ($P<0.005$) related to age of the heifers (see Figure 4.8.). However, effects of treatment overall were not significant ($P=0.13$). Furthermore, despite an apparent (see Figure 4.6), time/treatment interaction the effect of immunisation was not statistically significant ($P=0.14$).

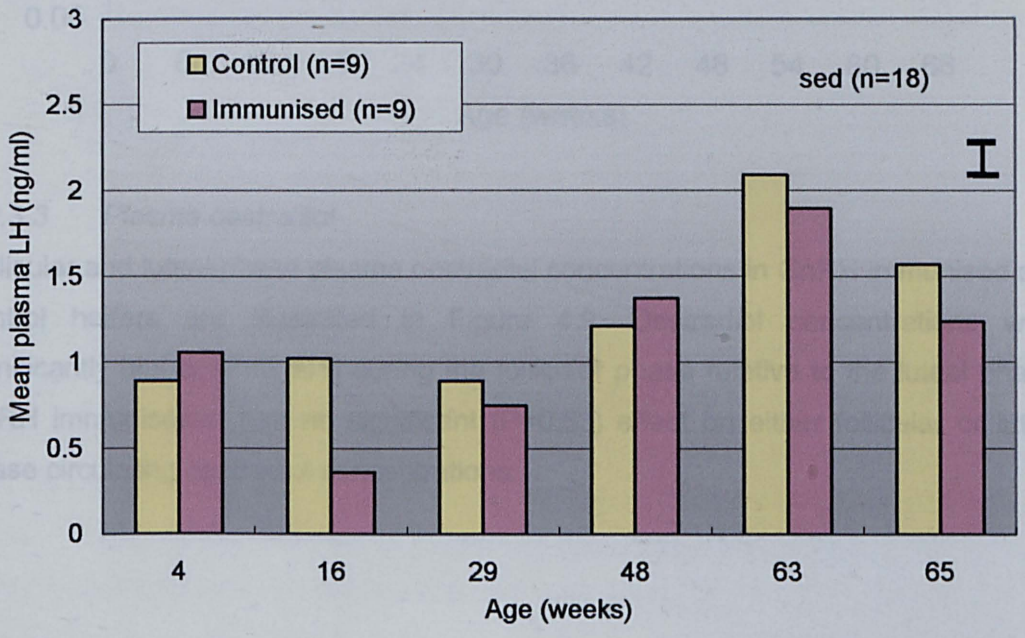
Figure 4.6 Basal LH levels in GnRH immunised and control heifers. Standard error of the differences of means (s.e.d.) was 0.14, 0.08 and 0.2 for time, status and time/treatment, respectively.



4.3.3.1.4 Mean LH concentration

Mean plasma LH concentrations in GnRH immunised and control heifers are illustrated in Figure 4.7. Age had a significant (P<0.0001) effect on mean LH concentrations. Overall GnRH immunisation did not significantly reduce LH concentrations (P=0.1). There was a time/treatment interaction, which approached significance (P=0.067) such that LH concentrations appear reduced at 16 weeks of age in vaccinated animals, when GnRH antibody levels peaked.

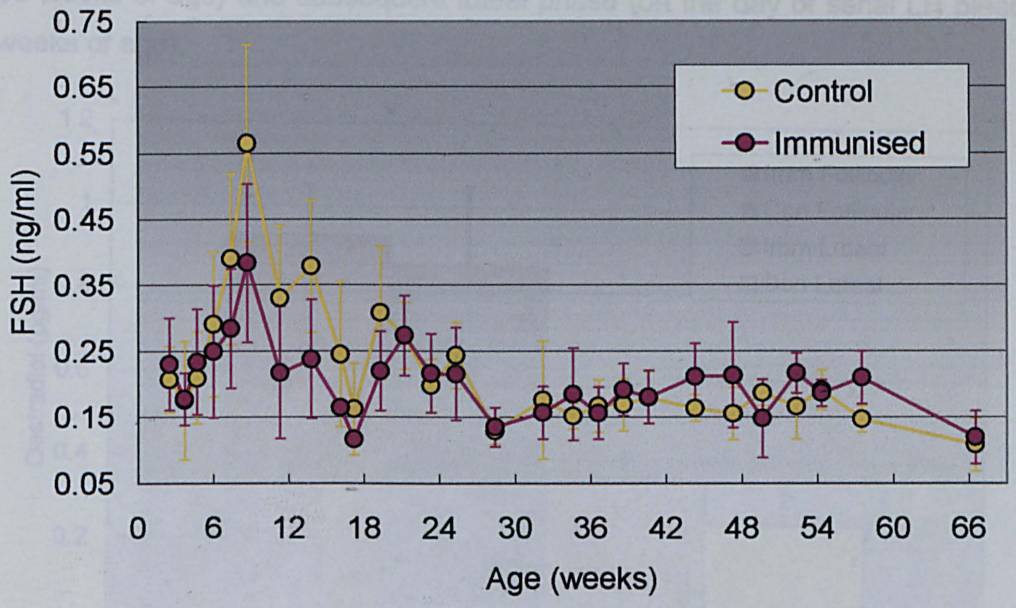
Figures 4.7 Mean LH concentrations in GnRH immunised and control heifers. Standard error of the differences (s.e.d.) of means was 0.13, 0.1, 0.19 and 0.19 for time, status and time/status, respectively.



4.3.3.2 FSH

Mean serum FSH concentrations in both groups are presented in Figure 4.8. FSH concentrations were significantly affected by age (P<0.005), with relatively high FSH concentrations between 4-16 weeks (peak: 8 weeks), lower FSH concentrations between 18-28 weeks and low FSH concentrations from 28 weeks of age. However, GnRH immunisation had no overall effect (P=0.78) on FSH levels and there was no time/treatment interaction (P=0.62).

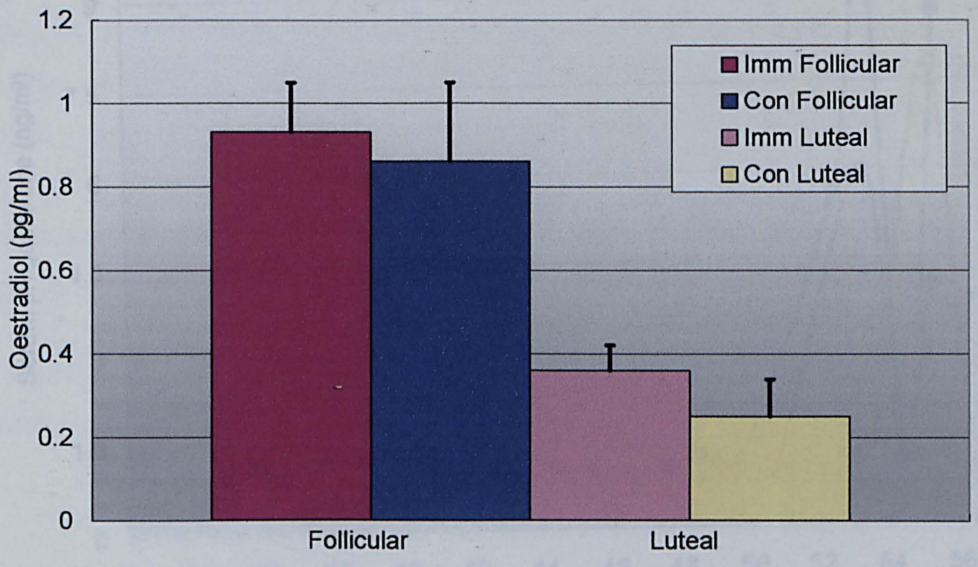
Figure 4.8 Mean (± s.e.m.) serum FSH concentrations in GnRH immunised (n=9) and control (n=9) heifers.



4.3.3.3 Plasma oestradiol

Follicular and luteal phase plasma oestradiol concentrations in GnRH immunised and control heifers are illustrated in Figure 4.9. Oestradiol concentrations were significantly higher ($P<0.001$) during the follicular phase relative to the luteal phase. GnRH immunisation had no significant ($P=0.53$) effect on either follicular or luteal phase circulating oestradiol concentrations.

Figure 4.9 Plasma oestradiol concentrations in GnRH immunised (n=9) and control heifers (n=9) during a synchronised follicular phase (on the day of LH serial bleed at 63 weeks of age) and subsequent luteal phase (on the day of serial LH bleed at 65 weeks of age).



4.3.3.4 Plasma progesterone

Plasma progesterone monitoring was used to determine the onset of oestrous cycles and hence, puberty (see Figure 4.10). Age at puberty was not affected by immunisation, with control (n=9) and immunised heifers (n=9) attaining puberty at 53 ± 0.6 (mean ± s.e.m.) and 50 ± 2.6 weeks of age, respectively. Age at puberty was further confirmed by monitoring oestrous behaviour. Precocious puberty was observed in a single immunised heifer (see Figure 4.11).

Figure 4.10 Typical example of progesterone profiles indicating the onset of oestrous cycles in GnRH immunised (n=2) and control (n=2) heifers.

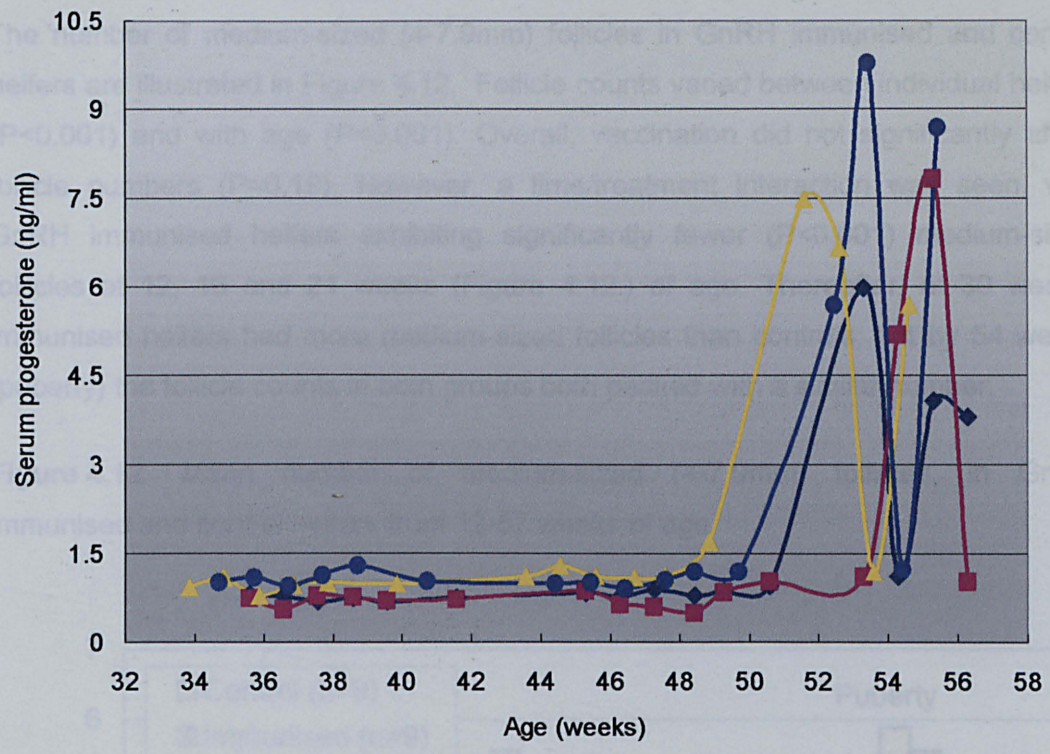
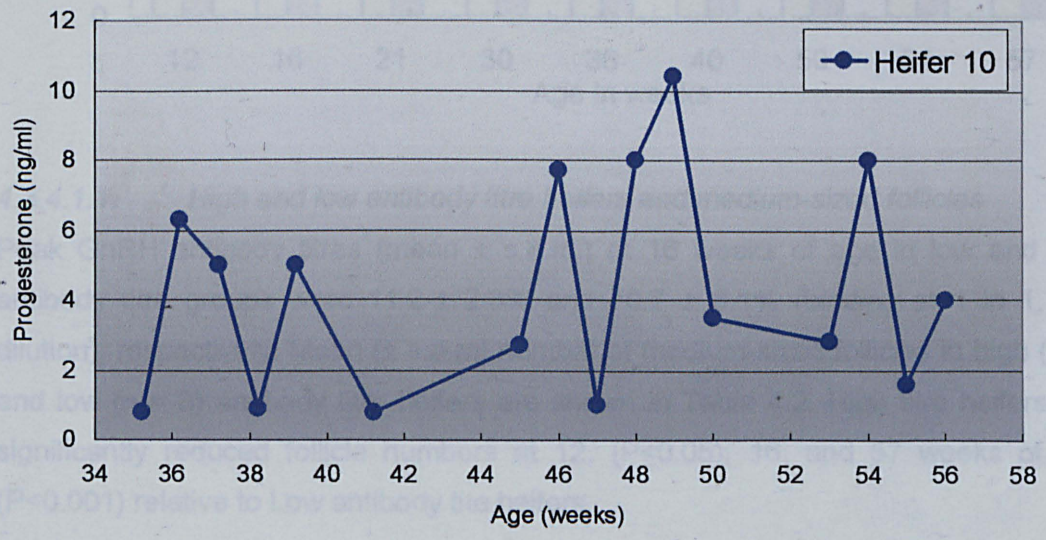


Figure 4.11 Peripheral progesterone concentrations in a heifer exhibiting precocious puberty relative to her 17 herd mates.

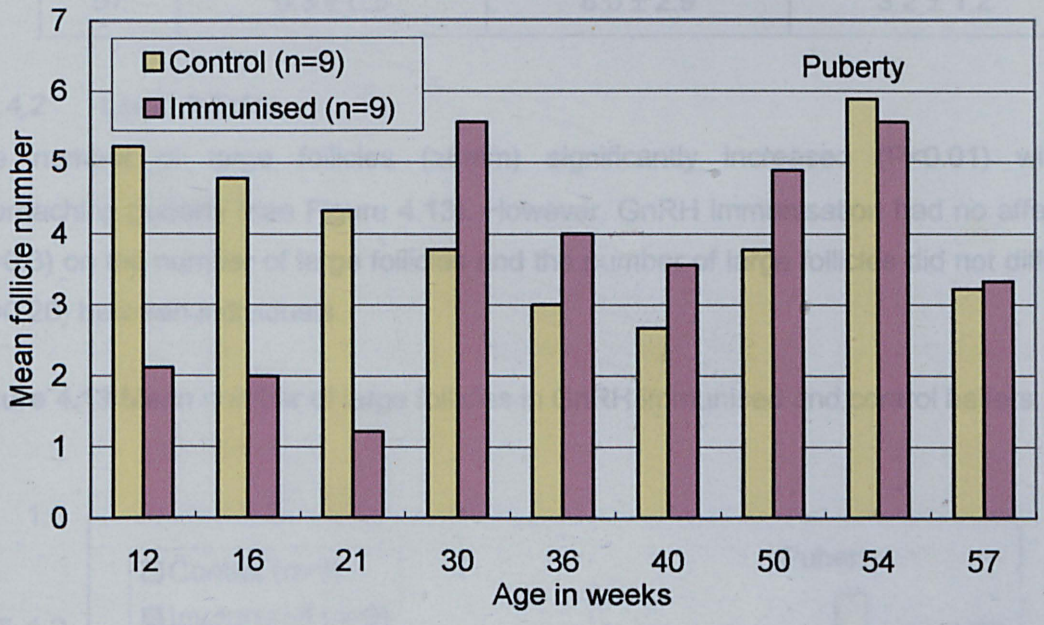


4.3.4 Ovarian follicular development

4.3.4.1 Medium-size follicle counts

The number of medium-sized (4-7.9mm) follicles in GnRH immunised and control heifers are illustrated in Figure 4.12. Follicle counts varied between individual heifers ($P<0.001$) and with age ($P<0.001$). Overall, vaccination did not significantly affect follicle numbers ($P=0.19$). However, a time/treatment interaction was seen, with GnRH immunised heifers exhibiting significantly fewer ($P<0.001$) medium-sized follicles at 12, 16 and 21 weeks (Figure 4.12.) of age. Thereafter, at 30 weeks, immunised heifers had more medium-sized follicles than controls, but by 54 weeks (puberty) the follicle counts in both groups both peaked with a similar number.

Figure 4.12 Mean number of medium-sized (4-7.9mm) follicles, in GnRH immunised and control heifers from 12-57 weeks of age.



4.3.4.1.1 High and low antibody titre heifers and medium-sized follicles

Peak GnRH antibody titres (mean ± s.e.m.) at 16 weeks of age in low and high antibody titre groups were 11.2-± 2.3% and 70.7 ± 8.1% (binding at 1 in 1, 000 dilution), respectively. Mean (± s.e.m) number of medium-sized follicles in high (n=3) and low (n = 3) antibody titre heifers are shown in Table 4.2. High titre heifers had significantly reduced follicle numbers at 12, ($P<0.05$), 16, and 57 weeks of age ($P<0.001$) relative to Low antibody tite heifers.

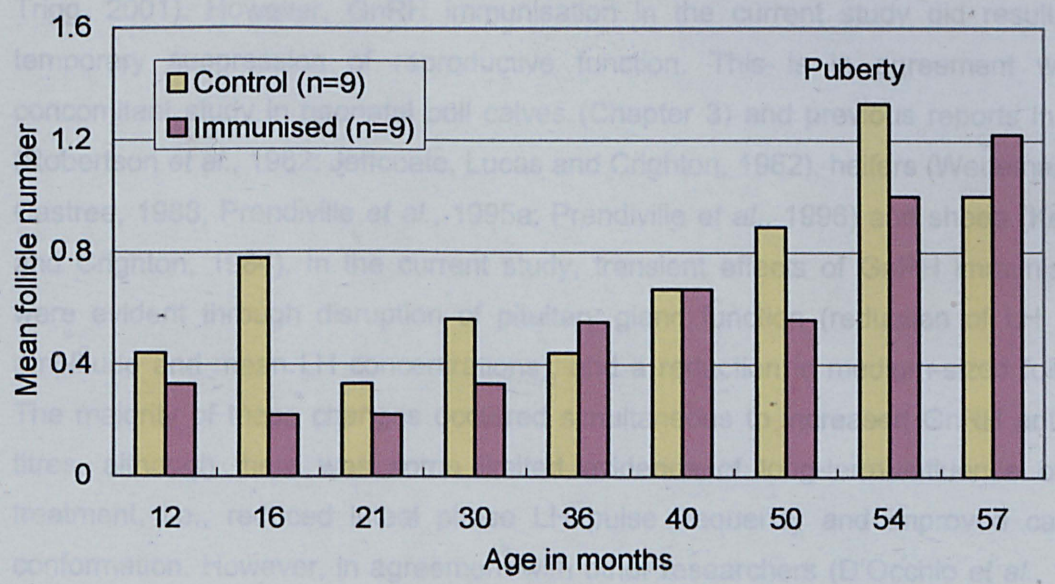
Table 4.2 The Medium-sized follicle number (mean ± s.e.m) in high (n=3) and low (n=3) GnRH antibody titre (AbT) heifers and control (n=9) heifers.

Age weeks	Follicle number (mean ± s.e.m.) High AbT heifers	Follicle number (mean ± s.e.m.) Low AbT heifers	Follicle number (mean ± s.e.m.) Control heifers
12	0.7 ± 0.7	2.0 ± 0.6	5.3 ± 1.1
16	0	3.7 ± 2.0	4.8 ± 1.4
21	0.3 ± 0.3	0.7 ± 0.7	4.3 ± 1.6
30	3.0 ± 1.5	5.7 ± 2.3	3.8 ± 1.4
50	4.3 ± 0.9	4.67 ± 2.33	3.8 ± 1.1
54	5.7 ± 2.2	6.3 ± 1.2	5.9 ± 1.6
57	0.3 ± 0.3	8.0 ± 2.9	3.2 ± 1.2

4.3.4.2 Large follicles

The number of large follicles (≥8mm) significantly increased (P<0.01) with approaching puberty (see Figure 4.13). However, GnRH immunisation had no affect (P=0.3) on the number of large follicles and the number of large follicles did not differ (P=0.26) between individuals.

Figure 4.13 Mean number of large follicles in GnRH immunised and control heifers.



4.3.5 Anabolic Response

4.3.5.1 Carcass conformation

Table 4.3 Shows carcass conformation and fat grades in control (n=9) and GnRH immunised (n=9) heifers, respectively. GnRH immunisation significantly ($P<0.05$) improved carcass classification, but not fat grades ($P>0.05$).

Table 4.3 Carcass and Fat classification in control (n=9) and treated (n=9) heifers.

Conformation grade	Very Good	Good	Average/Good	Average/poor
Control heifers	0	1	8	0
Immunised heifers	0	4	5	0
Fat Grade	**3H**	**4L**	**4H**	**5L**
Control heifers	2	2	3	2
Immunised heifers	0	3	5	1

4.4 DISCUSSION

The present study has provided evidence to show that active immunisation against GnRH in neonatal heifers (at 2-3 weeks of age) did not permanently impair reproductive function. This is in contrast to findings in similar studies in sheep and Zebu bulls (Brown *et al.*, 1994; 1995; Clarke *et al.*, 1998; D'Occhio, Aspden and Trigg, 2001). However, GnRH immunisation in the current study did result in a temporary suppression of reproductive function. This is in agreement with a concomitant study in neonatal bull calves (Chapter 3) and previous reports in bulls (Robertson *et al.*, 1982; Jeffcoate, Lucas and Crighton, 1982), heifers (Wetteman and Castree, 1988; Prendiville *et al.*, 1995a; Prendiville *et al.*, 1996) and sheep (Keeling and Crighton, 1984). In the current study, transient effects of GnRH immunisation were evident through disruption of pituitary gland function (reduction of LH pulse amplitude and mean LH concentrations), and a reduction in medium-sized follicles. The majority of these changes occurred simultaneous to increased GnRH antibody titres, although there was some limited evidence of long-term influence of this treatment, i.e., reduced luteal phase LH pulse frequency and improved carcass conformation. However, in agreement with other researchers (D'Occhio *et al.*, 1992;

Bell *et al.*, 1997), there was no delay in attainment of puberty, no compromise to conception rate and early foetal development (data presented in Chapter 6) following fixed-time insemination.

4.4.1 GnRH Immune Response

The GnRH vaccine successfully induced significant GnRH antibody titres in all vaccinated animals. At a 1:1,000 dilution, mean peak antibody titre in heifers was 37%. This antibody response was similar to those reported by other researchers (Johnson *et al.*, 1988; Adams and Adams, 1990) and based on previous reports (Fraser, 1980; Robertson *et al.*, 1982; Johnson *et al.*, 1988; Adams and Adams, 1990; Prendiville *et al.*, 1995a; Adams, Adams and Sakurai, 1996) should be sufficient to inhibit reproductive function, i.e. 15-20 % binding (1 in 1, 000; Johnson *et al.*, 1988). However, caution should be used when making antibody titre comparisons as due to variations between assays, such comparisons are typically inaccurate and may be misleading. The time at which GnRH antibody titres reached critical threshold levels required for the inhibition of typical reproductive development (14-17 weeks and 24 weeks), may have missed the specific window of time during which the hypothalamic (median eminence; ME))/pituitary gland are maximally susceptible to GnRH antibodies. Sub-threshold antibody titres may indeed be responsible for the limited long-term disruption to LH pulse frequency (or negative feedback response of the pituitary gland). However, this disruption was insufficient to suppress reproductive function, or significantly compromise fertility. Although heifers appeared to achieve higher antibody peak titres, compared with bulls (Chapter 3), mean peak antibody titres tended to show reduced longevity and increased variability, with 4 out of 9 heifer calves showing peak titres <30%. Of these animals, titres peaked after the 2nd booster, at 11.5, 15.0, 4.0 and 7.0% binding (1:1,000). Similar, "poor responders" were reported by Bell *et al.* (1997) after GnRH immunisation in heifers. The response to primary vaccination was small and short-lived, as occurred in the bulls (Robertson, Wilson and Fraser, 1979; Robertson *et al.*, 1981; 1982; 1984) and other studies in heifers (Johnson *et al.*, 1988; Adams and Adams, 1990; Prendiville *et al.*, 1995a; Crowe *et al.*, 2001b). As with the neonatal bulls, and the majority of other studies in heifers (Johnson *et al.*, 1988; Crowe *et al.*, 2001b), the response to subsequent GnRH booster injections (1st and 2nd) was significantly greater.

By 30-34 weeks after the primary vaccination antibodies had fallen to low levels in all vaccinated cattle (Chapters 3 and 4), but remained detectable and significantly

($P<0.05$) elevated, relative to control animals for over one year. Finnerty *et al.* (1996), reported similar findings. There was one exception, which is discussed later.

The large degree of variation in immune response following GnRH immunisation in the newborn calf may have resulted from maternally derived antibodies, which can be very wide ranging and titres in calves can persist for several months after birth (Literature review). Maternally derived antibodies to bovine herpes virus-1 (BHV1) may have blocked or enhanced the immune response to the GnRH-gD subunit BHV1 conjugate in the current experiment even after 2 booster vaccinations. A recent comprehensive study on vaccine development in calves acknowledged that designing and implementing a successful calf vaccination program is difficult (Fulton *et al.*, 2004). Therefore future active immunisation studies should measure maternal antibodies carefully and the affinity of antibodies for the vaccine antigen. However, in the cattle studied herein, qualitative and quantitative information on the dams immune status, colostrum BHV1 antibody content, calf intake and absorption is lacking. As is information on other variables, which are known to suppress immune function such as, calf stress during and after gestation, premature birth and illness prior to arrival at the University farm (Eskola and Kyhty, 1998; Tuchscherer *et al.*, 2002).

1.4.1.1 GnRH antibody titre rebound or autoimmunity

In an earlier study of 3-4 year old ewes, previously immunised against GnRH as neonatal lambs (Brown *et al.*, 1995) and with undetectable antibody titres, Clarke *et al.* (1998) reported that GnRH antibodies returned following attempts to prime the pituitary gland with repeated GnRH injections. Thus exogenous GnRH induced an immune response. We believe that the additional significant elevation ($P<0.002$) in GnRH antibody titre at around 23 weeks (Figure 4.3) in this study, in the absence of an additional immunisation is a novel observation, possibly caused by a similar mechanism. The cause of the additional increase in GnRH titres in the present study is unclear, but it may be a response to increasing endogenous GnRH at around 20-22 weeks of age. However, this speculative suggestion does not explain how endogenous GnRH, which due to size is poorly antigenic (Hoskinson *et al.*, 1990; see Meloen, 1995), induced such a response. One possible explanation is that the cleavage of GnRH from GnRH pro-hormone (Seeburg and Adelman, 1984) is disrupted by neonatal immunisation and the subsequent release of the large precursor molecule into the hypophyseal portal system may have induced significant antibody titre. Damage to the axons of the GnRH neurones following immunisation,

as described in boars (Molenaar *et al.*, 1993), may have resulted in leakage of pro-GnRH into the portal blood. Unfortunately, the occurrence of hypothalamic lesions in the current trial was not confirmed, due to restrictions related to BSE. Furthermore, if increasing GnRH output at around 23 weeks stimulated an immune response it is not clear why a further immune response was not seen at about 48 weeks of age. At this time, < 50 days before puberty, in our study, as with previous studies (see Kinder *et al.*, 1995), LH pulse frequency peaked, indicating increased GnRH pulse frequency (Clarke, 2002). In the previous study in pigs (Molenaar *et al.*, 1993), antibody titres were still elevated at slaughter when the hypothalami were examined and lesions discovered. It is therefore conceivable that ME damage was caused by the peak antibody titres and, through axon leakage and/or infiltration of immune cells (a cellular response) into axons, and the GnRH pro-hormone complex induced an additional immune response. Five months later, at the time of increased GnRH pulse frequency prior to puberty, it is possible that earlier ME damage had been repaired and/or new neural networks established in the maturing hypothalamus. Subsequently, the increased release of the cleaved GnRH decapeptide was not able to induce a further antibody response, which would explain the lack of an "autoimmune response" at 48 weeks. However, against this argument, in ewes, even after years of undetectable titres there was an increase in GnRH antibody titres after GnRH priming. It should be noted that an increase in the immune response to increased GnRH pulse frequency at around 23 weeks occurred after a period of elevated GnRH antibody titres, but did not affect LH pulse frequency. In ewes, however, GnRH release was significantly reduced in the long-term relative to controls (Clarke *et al.*, 1998), subsequently relatively high levels of exogenous GnRH, which itself stimulates several cytokines (Dixit *et al.*, 2003; Tanriverdi *et al.*, 2003), were presented to the immune system (Clarke *et al.*, 1998). In other words, long-term exposure of GnRH to the immune system in heifers may have led to a reduced immune response to the hapten over time.

The autoimmune suggestion is however, speculative and there is no direct evidence presented to support the argument. It is possible that there was no autoimmune response and the titre rebound was caused by other factors. Although, we are unable to explain this phenomenon, an alternative suggestion is that a proliferation in idiotypes and anti-idiotypes, in response to the peak antibody titres, stimulated a rebound in titre at about 23 weeks. Idiotypes are often referred to as 'internal images of the antigen', (Hay and Thanavala, 1984), which can interact with free antibody and lymphocytes to produce anti-idiotypes. Anti-idiotypes are antibodies raised against

the antigen binding V-region (idiotype) of an antibody, and some anti-idiotypes have a 3-dimensional structure similar to the original immunogen (GnRH-BHV1 in this case). Hence, anti-idiotypes are likely to be immunogenic. Idiotype antibodies can be formed in the course of a normal immune response and have been shown to have both stimulatory and suppressive effects on immune responses (Keeling and Cighton, 1984; Wigzel, 1987). Furthermore, this suggestion might also explain the absence of a further response to increased endogenous GnRH prior to puberty, as titres had not recently been elevated at this time.

Interestingly, this suspected autoimmune response or antibody titre rebound resulted in mean (± s.e.m.) antibody titres of 51 ± 2.7% (binding at 1:1,000) in 3/9 immunised heifers. A less dramatic response (>5% binding 1 to 1,000) occurred in 2 of the remaining 6 heifers and only 1 bull in 9. Differential timing of increased endogenous GnRH relative to the 2nd booster vaccination between sexes may influence the apparent sex difference in 'titre rebound'. Indeed, LH output in response to increased pulsatile GnRH, increased at 16-20 weeks of age in bulls (Rodrigez and Wise, 1989; McCarthy, Convey and Hafs, 1979), ~16 weeks in ewes (Claypool and Foster, 1990) and 12-16 weeks of age in heifers (Swanson and McCarthy, 1978; Schillo *et al.*, 1982; Dodson *et al.*, 1988).

Responsiveness of the HPU between sexes to gonadal steroids may also be involved. Claypool and Foster (1990) reported age differences between ewes and rams in hypothalamic responsiveness to exogenous oestradiol. Thus, differences in the magnitude or timing of the GnRH pulse frequency increase may result from gender differential stimulation of the immune system. Alternatively, the lack of titre rebound, or at least its amplitude, in bulls may reflect gender differences in the immune response as occurs in other species (Martin, 2000) following immune challenges. These gender differences may be the result of androgen suppression of the thymus in males, or sex specific differential steroid, or GnRH, enhancement of immune function (Jacobson, 2000).

The individual variation in immune responses in this study may be influenced further by individual maturational changes in the immune system. Alternatively, it may suggest that the female bovine immune system may be more responsive to the GnRH vaccination, and their humoral responses more responsive to changes in GnRH levels than males. Indeed, sexual dimorphism of the immune system has been reported in rodents and humans, with greater immune responses in females (Martin, 2000; Tanriverdi *et al.*, 2003). Therefore, it may be that both bulls and heifers

produce similar degrees of increased GnRH at 16-22 weeks, but due to a more responsive antibody, and possibly also cell mediated, immune system, the greater response is observed in heifers. Furthermore, the reduced titre rebound in bulls may have resulted because prior to this response antibody titres tended to be higher in bulls than in heifer (Figure 4.3). Interestingly, compared to males, females display a striking increased incidence of autoimmune reactions (Tanriverdi *et al.*, 2003).

As with a previous study by Prendiville *et al.* (1996) the animals in our trial were divided into groups of either low (11.2 ± 2.3% binding at 1:1,000; n = 3) or high GnRH antibody titres (70.7 ± 8.1% binding at 1,1000; n = 3). Because of the small animal numbers in these groups, information on effects of antibody titre on gonadotrophin secretion is limited. However, some reference to comparisons between the high and low antibody titre groups will be made throughout the discussion.

4.4.2 Gonadotrophic Drive

4.4.2.1 LH

Reports of an episodic pattern of LH release developing during the first month of life in the heifer (Schams *et al.*, 1981; Dodson *et al.*, 1988), bull (Amann and Walker, 1983) and lamb (Foster *et al.*, 1975) are supported by this study. However, at about 24 days of age, a large degree of between-animal variation in episode frequency was observed with between 0 and 9 pulses per 8-hour sampling period. Dodson *et al.* (1988) reported similar findings and suggested that at this age maturational changes are under way.

As previously reported (Day *et al.*, 1987; Bergfeld *et al*, 1994 see Kinder *et al.*, 1995), LH pulse frequency increased 4-8 weeks prior to puberty, possibly due to reduced sensitivity to oestradiol negative feed back on the hypothalamus (see Kinder *et al.*, 1995). However, the increase in mean LH concentrations before puberty (Schillo, Dierschke and Hauser, 1982), were less obvious in the current study.

As with GnRH immunised bulls, there was no overall effect of treatment on LH pulse frequency. This finding is in contrast with an earlier study (Prendiville *et al.*, 1996), in which LH pulse frequency, but not LH pulse amplitude, was reduced. The exact reason for the lack of effect on amplitude in the earlier study is unclear. However, it is possible that despite GnRH antibody induced anoestrous status, the heifers were subjected to exogenous GnRH agonist, GnRH, and oestradiol treatments. Higher oestradiol concentrations are known to increase pituitary sensitivity to GnRH (Kesner

et al., 1981), possibly through enhancing GnRH receptor expression (Schoenemann, Convey and Anderson, 1985). Furthermore, with reduced LH pulse frequency, pituitary stores would be not so depleted of LH and FSH (Clarke, 2002).

It is now well established that no LH pulses occur without pulsatile GnRH stimulation of the gonadotroph (Clarke and Cummins, 1984; Clarke, 2002). Therefore LH pulses may still occur in GnRH immunised animals, if the antibody titres were not sufficient to neutralise all the GnRH being released (D'Occhio, Aspden and Trigg, 2001). For example, in earlier *in vitro* studies in sheep (Naor *et al.*, 1980), the gonadotrophs were observed to respond with nearly maximal LH release when only 20% of available GnRH receptors were bound to GnRH (see McNeilly *et al.*, 2003). Further studies *in vivo* have shown that when 50% of GnRH receptors are blocked with a GnRH antagonist, ewes could still respond fully to a GnRH challenge (Wise *et al.*, 1984). However, LH pulse amplitude was significantly reduced in both the current trial and in neonatal bulls (Chapter 3). Earlier work (Clarke and Cummins, 1982) has shown that removal of some of the GnRH that reaches the pituitary gland results in a reduction in the amplitude of LH pulses. Furthermore, LH pulse amplitude is a direct reflection of the releasable pool of LH in the pituitary gonadotrophs (Clarke and Cummins, 1985; see Clarke, 2002). Therefore, although GnRH pulses are inducing LH release, the amount of GnRH reaching the pituitary gland appears to have been reduced by GnRH immunisation and as a result, de novo synthesis of LH and hence gonadotroph cell stores of the hormone, may also be reduced (McNeilly *et al.*, 2003). A concomitant reduction in GnRH receptor expression, following a decrease in GnRH stimulation (Fraser *et al.*, 1982; Popkin and Fraser, 1985) is also likely, however such a reduction may not have been sufficient to reduce LH pulse frequency in the current study. This is in contrast to findings in anoestrous heifers, in which LH pulse frequency and mean LH was reduced, while amplitude and basal LH levels were unaffected (Prendiville *et al*, 1996; titres~20% at 1:640).

The fact that LH pulse amplitude was reduced over the entire trial despite GnRH antibody titres falling and remaining low (<5% binding at 1:1,000) by 33 weeks of age, indicates a prolonged suppression of GnRH amplitude following neonatal immunisation in heifer calves. Interestingly, although LH pulse amplitude in GnRH immunised heifers was not different to that of control heifers at 29, 48 and 63 weeks of age, there was an apparent, although non-significant reduction in LH pulse amplitude at 65 weeks, during the luteal phase. This may be the result of reduced GnRH pulse amplitude, or differential sensitivity of the gonadotrophs to progesterone

negative feedback. Steroids are capable of modifying the responsiveness of gonadotrophs to GnRH, and hence reducing LH pulse amplitude (Clarke, 2002).

No affect of GnRH immunisation on LH pulse frequency overall, or during peak antibody titres, was observed during this study. After anti-GnRH titres had fallen to low levels, however, GnRH immunised animals still tended to exhibit increased LH pulse frequency 24 hours prior to oestrus and a marginally significant reduction in pulse frequency during the luteal phase (day 12) relative to control heifers. These effects are unexpected and difficult to explain, however, the effect during the follicular phase may simply reflect variations in the speed of CL regression after PG treatment. The more pronounced reduction in LH pulse frequency, seen in GnRH immunised heifers during the luteal phase, may indicate increased sensitivity of the hypothalamic–pituitary unit to progesterone negative feedback following neonatal immunisation.

Lower LH pulse amplitude post-puberty in all heifers may result from increased baseline LH at this time. In the heifers there was a lack of LH pulse amplitude inhibition after the primary vaccination, whereas in the bulls, there was a marginally significant reduction in amplitude, despite low titres (<2%), in both groups. The effects seen at 65 weeks may indicate a long-term effect on LH amplitude, which may indicate an increased responsiveness to progesterone negative feedback, or that immunisation reduced the ability of the pituitary gland to synthesise LH-β subunit in response to progesterone. Even when antibody titres are low at 65 weeks of age, they may be having some suppressive effect on GnRH drive. Alone, low antibody titre effects on LH output may be insignificant. However, the combined affects of low antibody titres and progesterone negative feedback, at both the hypothalamic and pituitary gland levels, may be sufficient to marginally suppress LH pulse frequency and to a lesser extent LH pulse amplitude during the luteal phase. Alternatively oestradiol, which was not different between the two groups, may have reduced pituitary LH amplitude. Oestradiol has been shown to increase the sensitivity of the hypothalamus to the negative feedback effects of progesterone (Peters, 1985).

Basal LH concentrations increased ($P<0.005$) prior to puberty before peaking at 63 weeks. As with bulls (see Chapter 3) there was a suggestion of reduced basal LH at 16 and 66 weeks. This finding may reflect the tendency for reduced LH pulse amplitude and frequency, which were reduced at this time. Overall GnRH immunisation did not significantly reduce mean LH, but mean LH tended to be reduced ($P=0.067$) at 16 weeks, and to a lesser extent at 65 weeks. The suppression

of mean LH when GnRH antibody titres are elevated has been previously reported in adult heifers (Prendiville *et al.*, 1996), although in other GnRH immunisation (**Ref**) and GnRH agonist studies basal LH is unaffected by treatment (see D'Occhio and Aspden, 1999).

4.4.2.2 FSH

In the current study, FSH was elevated ($P<0.001$) during the first 16 weeks of life in the heifers, peaking at around 8 weeks. In support of this observation, FSH concentrations in the first 16 weeks of life have recently been reported to be higher than in cycling cows (Nakada *et al.*, 2002). The reduction of FSH with increasing age may reflect increased numbers of oestrogenic follicles, and elevated peripheral inhibin concentrations which have been shown to inhibit FSH concentrations from 60 days of age onwards (Kaneko *et al.*, 1993; 1995) in heifers. Interestingly, and in support of our results, the peak of FSH and speed of the FSH rise after a GnRH challenge falls with increasing age up to 36 weeks (Nakada *et al.*, 2002). Previous reports observed reduced FSH levels following GnRH immunisation (titres ~20% 1:640) in post-pubertal heifers (Prendiville *et al.*, 1996). However, it is widely accepted that FSH is less responsive to short-term fluctuations in GnRH output (Gong *et al.*, 1995; 1996a; Prendiville *et al.*, 1996). Therefore, in the current study GnRH antibody titres may have been elevated above a critical threshold for insufficient time to suppress FSH concentrations. Alternatively, titres may need to be higher in neonatally immunised compared to post-pubertal heifers.

4.4.3 Gonadal Response

The medium-sized follicle population in pre-pubertal heifers was maximal at 12 weeks, at the start of the scanning regime. This is slightly earlier than the 16-26 week peak in antral follicle numbers previously reported (Desjardin and Hafs, 1969), However, it is in agreement with recent and ongoing studies in young calves (Campbell, unpublished). In our study, medium-sized follicle numbers were reduced ($P<0.001$) in GnRH immunised heifers at 12 and 16 weeks when titres >20%, but also at 21 weeks, just prior to the detection of the titre rebound. Thus suppression of medium-sized follicles at 21 weeks appears to be due to the immune response to endogenous GnRH and/or a prolonged period of low gonadotrophin release following clearance of GnRH antibodies from the body. The fact that low antibody titre animals exhibited significantly fewer medium-sized follicles at 21 weeks, relative to 16 weeks, supports this conclusion (see Table 4.2). Earlier studies on GnRH immunisation in post-pubertal heifers reported that growth of follicles (>4-5mm) was either reduced, or arrested (Johnson *et al.*, 1988; Adams and Adams, 1990; Prendiville *et al.*, 1995a;

1996; Crowe *et al.*, 2001a;b). Evidence of a 'catch-up effect' is seen in Figure 4.12. After a prolonged period of suppression medium-sized follicle numbers in treated heifers increase, and exceed control values by 30 weeks of age. The explanation for this 'rebound' is that, in treated animals a larger pool of follicles developed up to a stage where gonadotrophins are required for further development. Following adequate gonadotrophin release, a larger number of responsive follicles are recruited. However, there was a lack of correlation between FSH and number of medium-sized follicles, which are known to be FSH dependent (Gong *et al.*, 1995; 1996a). By 54 weeks, medium-sized follicle populations were similar in both treated and control groups. Table 4.2 shows further evidence of adequate immune response following GnRH immunisation since the high antibody titre animals had total cessation of follicle growth at 16 weeks, when antibody titres were maximal. The reason for the suppression of medium-sized follicles, despite no detectable reduction in FSH concentrations following GnRH immunisation is unexpected. Particularly in view of previous GnRH immunisation reports (Crowe *et al.*, 2001a;b), in which both medium-sized follicle populations and FSH were reduced.

The increase ($P<0.01$) in number of large follicles with approaching puberty seen in both control and GnRH immunised heifers in this trial is similar to earlier reports (Draincourt, 2001). However, the reason for the increase in large follicle number in control heifers at 16 weeks of age is unclear, but may reflect the high number of medium-sized follicles and increased LH pulse amplitude at this time. Although generally smaller than dominant follicles in adults, pre-pubertal heifers did have some follicles >8mm (see Draincourt, 2001). There was no detectable affect on the large follicle population following immunisation in this trial. In previous studies in GnRH vaccinated pre- and post-pubertal heifers large follicle numbers (Prendiville *et al.*, 1995a;b; Crowe *et al.*, 2001a;b), the proportion of oestrogenic follicles, and thus peripheral oestradiol concentrations, were reduced (Prendiville *et al.*, 1995a), as was testosterone in the GnRH immunised bulls (Chapter 3). In the current study, some medium-sized follicles were present in the GnRH immunised animals and LH output was not entirely suppressed. It is possible that LH output from the pituitary gland was adequate to enable dominant follicle establishment. The fact that all antral follicles have the potential to become dominant (Draincourt *et al.*, 2001) supports this proposal.

4.4.4 Attainment of puberty

In earlier studies in the pre-pubertal heifer (Wetteman and Castree, 1988; 1994; D'Occhio *et al.*, 1992; Sejrsen, Enright and Prendiville, 1994; Prendiville *et al.*,

1995b), puberty is delayed depending on the protocol and adjuvant used (Prendiville *et al.*, 1995b). In the current study, following neonatal immunisation against GnRH, puberty was not delayed. This is likely due to the extended time between the fall in GnRH antibody titres and typical age at puberty. For example, in other studies pre-pubertal heifers are immunised against GnRH nearer to the age of puberty (Wetteman and Castree, 1994), either at 6 (D'Occhio *et al.*, 1992), 8 (Sejrsen, Enright and Prendiville, 1994; Prendiville *et al.*, 1995b) or 13 months of age (Wetteman and Castree, 1988). Therefore titres are elevated at the time of puberty and hence onset of cyclicity is delayed until titres fall below a critical threshold level to allow resumption of GnRH drive.

Precocious puberty, i.e., transient increases in progesterone before 300 days of age (Kinder *et al.*, 1995) was seen to have occurred in one heifer, by the start of the sampling period at 245 days of age (see Figure 4.13). This was an immunised animal, which responded well (>70% binding at 1:1,000 after 2nd booster and >50% titre rebound). Interestingly, the heifer's titres, unlike those of all other animals, reached a plateau and did not fall below ~13% binding by week 55. Therefore the animal was cycling early with titres of between 13-20% binding. Prendiville *et al.* (1996) witnessed a resumption of cyclicity in heifers when titres fell (to ~14% at 1:640) and Bell *et al.* (1997) reported similar findings.

Progesterone levels were seen to rise, but sampling ended before the full cyclic pattern of progesterone (two weeks high, one week low) was established. However, by 54-56 weeks of age all heifers had exhibited behaviours associated with oestrus. By this time the majority of heifers (6/9 controls and 7/9 immunised) had commenced luteal activity. However, most heifers did not show the characteristic progesterone profiles associated with cyclicity until near the end of the sampling period (~56 weeks of age). Based on ultrasound scanning data from a subsequent trial, all but one heifer had attained puberty before 78 weeks of age. By 82 weeks of age, intense sampling on the subsequent trial showed that all heifers were post-pubertal. There was no evidence to suggest that neonatal GnRH immunisation delayed puberty. However, the evidence from the high and low responding groups and the entire GnRH immunisation group indicates that GnRH suppression was at best absolute in only 3/9 heifers when antibody titres peaked.

After a 15-week break on pasture, heifers in this trial were re-used in a subsequent study (Chapter 6). Following the Chapter 6 study in which heifers were A.I., all animals were culled. Post-mortem examination of the reproductive tracts at 96-98

weeks of age revealed that the ovaries, uteri and attendant mid-gestation foetuses, were not different (~8 months since the final LH serial bleed in this study) between control and immunised heifers (data not shown). This finding agrees with earlier reports (D'Occhio *et al.*, 1992; Bell *et al*, 1997) in which immunisation commenced at 24-36 weeks of age, and supports the previous evidence that suppression of reproductive function, by active immunisation against GnRH, in the neonate is reversible.

4.4.5 Anabolic Responses

In the current and previous studies (Prendiville *et al.*, 1995a; Jeffery *et al.*, 1997) no significant difference in overall growth rates were observed, although in Bos indicus heifers a tendency for reduced overall growth rates following immunisation was suggested (Jeffery *et al.*, 1997). In contrast, following GnRH immunisation some researchers have reported a reduction in both FCE and overall animal growth rates (Adams and Adams, 1990; Adams *et al.*, 1990). Regardless of the overall rate of growth, average daily growth in immunised animals was often reported (Adams and Adams, 1990; Adams *et al.*, 1990; Prendiville *et al.*, 1995a;b) to be lower than in intact heifers during anoestrous in both pre- and post-pubertal animals (Roche and Crowe, 2004). There are several possible explanations. First, a reduction in the anabolic steroid oestradiol, subsequent to elevated antibody titres in GnRH immunised animals, may reduce average daily gains (Moran *et al.*, 1990). However, following a fall in GnRH antibody titres and a presumably (no data shown in current study) subsequent oestradiol rise (Draincourt *et al.*, 2001) the growth rates overall may recover given time (Roche and Crowe, 2004). Second, immunisation per se and the resulting immune response may be responsible for reducing average daily gain in the short-term after booster immunisations (Crowe, Enright and Roche, 1995; Prendiville *et al.*, 1995a;b), possibly by increasing concentrations of various cytokines, which can have catabolic effects (Cook and Pariza, 1998). Unfortunately, serum oestradiol concentrations and immune cytokines were not measured in the current study during the period in which antibody titres were elevated, although testosterone concentrations were reduced at this time in bulls (Chapter 3). It is likely that the differences in heifer performance between studies, was due to the duration of the suppression of reproductive function (Prendiville *et al.*, 1995a), relative to the study length. Furthermore, it has been suggested that a difference in overall growth rate following GnRH immunisation was more likely to be seen when average daily gains were high (Prendiville *et al.*, 1995a), rather than average as in the current study.

Our observation that carcass conformation was improved following GnRH immunisation in the neonatal heifer is novel. This improved carcass conformation in the current trial may have resulted from suppression of oestradiol during elevated GnRH antibody titres, followed by increased response of lean tissue to anabolic effects of steroids, or a rebound in oestradiol at 26-30 weeks of age, as immunised heifers had an increased number of medium-sized follicles at 30 weeks of age, relative to controls. More thorough studies may be required into the effects of neonatal GnRH immunisation on body growth should this approach to inhibiting reproduction be adopted.

4.4.6 **Conclusions and Future Work**

Active GnRH immunisation in neonatal heifers did not permanently impair reproductive function or delay puberty. Further work is needed to investigate the effects of exposure of the neonatal hypothalamus to prolonged high GnRH antibody titres. A total suppression of pituitary stimulation by GnRH would be optimal. However, due to the immaturity of the bovine neonatal immune system, the possible blocking effects of maternally derived antibodies, and general poor response to active primary vaccinations, a passive immunisation approach may be required. Passive immunisation, of the pregnant cow or neonate, or active and passive combination protocols, may help to identify a critical period during which the hypothalamic-pituitary-unit is maximally susceptible to GnRH antibodies. Subsequently, on the basis of this information, the more practical active immunisation regimes may be designed. However, the inclusion of diets, which stimulate the maturation and response of the immune system, immunomodulation vaccines and carrier proteins to which, the dam is unlikely to possess antibodies (e.g., diphtheria toxin as used successfully in Improvac® the commercial GnRH vaccine for pigs), may go some way towards reducing the variability in antibody response between animals (Reeves *et al.*, 1989; Bell *et al.*, 1997), increasing the proportion of animals that respond, and increasing the magnitude, longevity and 'balance' of the immune response to vaccination early in life. However, due to the phenotypic variation in immune response and longevity of physiological affects in active immunisation experiments (Prendiville *et al.*, 1995b), large groups of animals (>24 animals per treatment group) may be necessary in future studies.

Neonatal GnRH immunisation can result in an additional increase in GnRH antibody titres, however both the cause and mechanism through which this response occurred

is unknown. Therefore future study is required in this area, as a predictable GnRH antibody titre increase might be incorporated into an immunisation protocol to act as a "self-booster". However, as no previous reports of such an event have been published, this phenomenon is likely to be the result of neonatal immunisation. When attempting to repeat such a response it should be noted that photoperiod, temperature, nutrition, stress and season of birth might influence the timing and magnitude of this event.

There is a lack of understanding in the mechanism of action, through which GnRH vaccines elicit their effects. For example, Vaxstrate®, has been withdrawn from the market place because prior to suppressing reproductive function animals were exhibiting increased fertility. Thus, the recent opinion is that the GnRH-OVA conjugate is binding GnRH receptors and acting as a GnRH agonist as well as raising GnRH antibodies (Dr. R. Henderson, Pfizer Inc, personal communication). To our knowledge there are no publications of this theory. This is unfortunate, as the GnRH vaccine/agonist action might help explain the long-term inhibition of fertility in sheep (Brown *et al.*, 1994; 1995; Clarke *et al.*, 1998) and bulls (D'Occhio, Aspden and Trigg, 2001) following immunisation, and gender differences between males and females. Furthermore, the mechanism of action may vary between vaccine preparations and we strongly suggest that, experiments into type of immune response (cellular and humoral), the effects of combined GnRH immunisation and GnRH agonist, and the mechanism of action through which Vaxstrate® and other vaccines act should be conducted. In addition, investigation into whether or not MHC-II molecules are expressed on GnRH neuron terminals might contribute to our understanding of the apparent gender and species variation in GnRH vaccine response, as we are unaware of any research at this level to date. Further understanding of immune function and the development of reliable and effective GnRH immunisation methods for young animals, is imperative as without this the field cannot develop.

CHAPTER FIVE
EFFECTS OF EXOGENOUS GROWTH HORMONE AND INHIBIN IMMUNISATION ON OVARIAN FOLLICLES IN ANOESTROUS EWES

5.1 INTRODUCTION

The economic viability of animal production systems such as beef and lamb are closely related to rates of reproduction. In domestic ruminants, ovulation rate is the major factor limiting the number of offspring. As a result, numerous attempts have been made over many years to increase ovulation rates in amounts compatible with uterine capacity (Terqui *et al.*, 1995).

Increased nutrition prior to mating (flushing) can improve fertility and enhance the yield of lambs born by up to 20% (Smith, Jagusch and Farguhar, 1983). The exact mechanisms through which diet influences reproductive function are still under investigation, however, it is thought to be associated with increased sensitivity of the follicles to gonadotrophins due to the effects of a number of trophic factors (e.g. GH, IGF-I, insulin) (Campbell and Scaramuzzi, 1996; Campbell, 1997; Gutierrez *et al.*, 1997) and leptin (Keisler, Daniel and Morrison, 1999). Alternatively, exogenous hormone administration (e.g. eCG, FSH) and steroid and inhibin immunisation (Scaramuzzi, Davidson and Van Look, 1977; Scaramuzzi and Hoskinson, 1984; O'Shea *et al.*, 1984; Wrathall *et al.*, 1992; Boland *et al.*, 1994; Hillard *et al.*, 1995) have been used to modulate folliculogenesis, increase ovulation rates and ultimately birth rates. These techniques have not been commercially successful, due to variations in animal responses (Reeves *et al.*, 1989) and practicalities for on-farm use. However, through these studies and comparative studies on very prolific breeds of sheep such as the Booroola Merino, Finnish Landrace and the Romanov (Boulton *et al.*, 1995; Draincourt, Hermier and Hanrahan, 1996; Gibbons *et al.*, 1999; Mandiki *et al.*, 2000; Dufour *et al.*, 2000), a better understanding has been acquired as to how such breeds of sheep are able to overcome the strong dominant follicle selection mechanism which limits cattle and less prolific sheep breeds (e.g.,Galway) to single ovulations (Draincourt, 2001). In order to develop successful superovulatory procedures and to promote their use by the agricultural industry and medical profession, a clearer and more detailed understanding of folliculogenesis is essential.

Previous research has demonstrated that both the GH/IGF-I (Spicer, Alpizar and Echternkamp, 1993; Campbell, Scaramuzzi and Webb, 1996; Lucy *et al.*, 1999) and gonadotrophin/inhibin (Campbell and Scaramuzzi, 1995; Bleach *et al.*, 1996) axes are involved in folliculogenesis. For example, exogenous GH administration in sheep and cattle results in an increase in the number of small follicles (Gong, Bramley and Webb, 1991; Gong *et al.*, 1993a;b; 1996b;c). GH administration increases peripheral GH, insulin and IGF-I, in addition to increasing IGF-I from ovarian follicles (Gong *et al.*, 1996b). Peripheral IGF-I is synthesised and released from the liver and is believed to be primarily responsible for the physiological effects of GH administration. IGF-I increases the sensitivity of the follicles to gonadotrophins, stimulating cell proliferation and steroidogenesis (Armstrong and Webb, 1997) and enhancing the responsiveness of the granulosa cells to FSH (Spicer, Alpizar and Echternkamp, 1993; Campbell, Scaramuzzi and Webb, 1996). As a result the number of follicles in the 2.1-3.0mm size range increase (Gong *et al.*, 1996b).

Studies in prolific breeds of sheep have shown that two possible strategies may be used in the production of large litters of lambs (Scaramuzzi *et al.*, 1993). The first strategy is by extending the time that FSH is maintained above a threshold. For example, Finnish Landrace and Romanov breeds, despite increased oestrogen levels compared to less prolific sheep, appear to have reduced hypothalamic-pituitary gland sensitivity to the negative and positive feedback effects of oestradiol. Thus, FSH levels decline less rapidly, allowing more time to recruit and maintain more preovulatory follicles before FSH levels become sub-threshold. The interval between CL regression and the ovulation inducing LH surge is also extended in these breeds (Baird and Campbell, 1998). The second strategy is by synchronising and increasing the number of small follicles in the ovaries, which are responsive to the threshold level of FSH. Recently, the importance of local factors in follicle development has been revealed in the prolific Booroola Merino, following the identification of a single gene mutation named the Fec^B gene in the BMP-1β receptor (Montgomery *et al.*, 2001; Monget *et al.*, 2002). Reduced BMP signalling by the BMP-1β receptor leads to physiological changes such as precocious follicular development, which results in increased ovulation rate (Souza *et al.*, 2001). In Booroola ewes (which carry the Fec^B gene) follicular cells are more sensitive to gonadotrophins *in vitro* (Webb, Campbell and Draincourt, 1995), as a result follicles develop and ovulate at a smaller size. Both oestradiol and inhibin A concentrations (Campbell *et al.*, 2003) prior to ovulation and blood progesterone concentrations from subsequent CLs, are similar to those reported in less prolific sheep. However, in Fec^B gene carriers, 2-4mm follicles

express significantly more mRNA for $P450_{arom}$ and inhibin α and β-A subunits (Webb *et al.*, 1999b). In addition BMP-2 has been shown to increase oestradiol production by ovine granulosa cells *in vitro* (Souza *et al.*, 2002). Similar studies in cattle demonstrated increased oestradiol, inhibin A, activin A and follistatin following individual incubation with BMP-4, -6 and -7, although no effects on FSH-induced hormone secretion were detected (Glister, Morgan and Knight, 2002). The observed precocious follicular development could therefore be due to both hormonal and/or local factors (Baird and Campbell, 1998). However, despite administration of identical concentrations of gonadotrophins to ewes rendered hypogonadotrophic, following GnRH antagonist treatment, Fec^B ewes were able to ovulate more follicles than controls. Thus, the Fec^B gene is thought to act at the ovarian level to enhance ovarian responsiveness to gonadotrophic stimulation (Campbell *et al.*, 2003).

Numerous studies (see sections 1.6.4-1.6.6) have demonstrated that, inhibin immunisation results in an increased ovulation rate and increased litter size in both sheep and cattle. These effects are probably elicited through increased super-threshold FSH levels and yet to be clarified intra-ovarian interactions (Campbell, Scaramuzzi and Webb, 1995; Bleach *et al.*, 1996). Intra-ovarian actions of inhibin and other factors, such as IGFs and IGFBPs, are complex and inhibins appear to have both positive and negative effects on folliculogenesis. For example, inhibin promotes LH and FSH stimulated steroid production from theca and granulosa cells, respectively (Armstrong and Webb, 1997), but there is recent evidence that free α subunit can function as an FSH-binding inhibitor and may also suppress oocyte maturation (Knight and Glister, 2001).

The current study aims to investigate the interaction between the GH/IGF and the gonadotrophin/inhibin axes on follicle growth and development.

5.2 MATERIALS AND METHODS

5.2.1 Experimental Animals

Sixteen ewe lambs, North Country mule (Scottish Blackface x Blueface Leicester) x Charolais tups (lambing rate 1.88) born on the University Farm between 6th and 26th March 1999, were kept on pasture and then group housed in September. All animals received a typical University Farm diet of ad-libitum hay and grass nuts. Throughout the experiment the live weight of the animals was monitored regularly, usually at the

time of blood sampling, and Mr. T Dingle, an experienced Stockman, condition scored all animals two days prior to slaughter.

Despite the small group sizes (n=4), this number of animals was justified by our primary intention, to carry out molecular studies on ovarian follicles, to investigate mRNA expression for P450 Aromatase ($P450_{AROM}$) and LH receptors. However, because the magnitude of the GnRH immunisation studies (Chapters 3 and 4), which began soon after the current trial ended; the degree of commitment to those studies and the time limitations of the Author's Studentship, the completion of ISH analysis has not been possible. Molecular investigations into the treatment effects on $P450_{AROM}$ and LH receptors, using Immunohistochemical analysis, are under way as an alternative to ISH. It is acknowledged that for follicular culture studies such low animal numbers are of marginal acceptability.

Following post mortem examination, the data from one ewe from group D (Ewe 14) was removed from the study because the animal presented with under developed ovaries. Antiserum from Ewe 14 was used in the subsequent Chapter 6 study and so the titration curves display Ewe 14 data. The treatment in this trial is not believed to be responsible for the ovarian abnormalities seen in this animal.

5.2.2 Experimental Protocol

The experimental period ran from August 1999 to April 2000 during seasonal anoestrus. This was because the antibody titre responses were lower than expected and the animals required three booster injections. Previous inhibin immunisation studies state that during seasonal anoestrous, the FSH rise is either absent (Kusina *et al.*, 1994) or reduced in size (Kusina *et al.*, 1995). While it is acknowledged that waiting another six months and conducting the experiment during the breeding season would have been ideal, as for example, this would be the time that prolificacy treatments would be administered in a commercial system, further booster vaccination of these animals was not permitted due to Home Office restrictions. The decision was made to conduct the experiment during anoestrous because our objective was to investigate the interaction between GH/IGF and inhibin. This objective was possible, and would add to current knowledge, regardless of the oestrous cycle status, because during seasonal anoestrous folliculogenesis continues, large antral follicles develop (Campbell, McNeilly and Baird, 1989; Noel, Bister and Paraquay, 1993; Kusina *et al.*, 1995) and ovulation rate mechanisms are maintained (Webb *et al.*, 1992b).

5.2.3 Inhibin vaccine preparation and immunisation

Inhibin vaccine was prepared just prior to administration. A detailed description of the method of vaccine preparation is given in Chapter 2.

Eight ewe lambs, 21.8 ± 1.2 (mean ± s.e.m.) weeks of age, were actively immunised against an inhibin α-C-subunit fraction, 1-26 tyr-gly conjugated to Imject® keyhole limpet haemocyanin (KLH) in Freund's non-ulcerative complete adjuvant (NUFCA; Guildhay, Guilford, Surrey). Booster vaccinations containing non-ulcerative Freund's adjuvant (NUFA; Guildhay, Guilford, Surrey) were given at 29.5, 36.1 and 54.7 ± 1.2 weeks of age (mean ± s.e.m.). Vaccines were administered via subcutaneous injection (0.4ml) into 4 sites (total 1.6ml) on the back of each animal. Control animals received the same KLH and Freund's adjuvant preparations minus the inhibin peptide.

5.2.4 Recombinant Bovine Growth Hormone (rbGH) Administration

Seven days after the 3rd inhibin booster, animals were sub-divided to give 4 groups, of ewes (n=4 per group) to give a 2x2 factorial design. Table 5.1 shows the four groups and summarises their respective treatments. 8 animals received a 4ml injection (1mg ml^{-1}) of rbGH in dorsal neck area (s.c.) at 09:00 for 6 days (days 1- 6). The control animals received vehicle only (see section 2.4).

Table 5.1 Summary of the four treatment groups

Group	Inhibin immunised	GH injections
A	No	No
B	Yes	No
C	No	Yes
D	Yes	Yes

5.2.5 Synchronisation of Follicular Waves

On day 4 of the rBGH treatment regime all animals were given a GnRH agonist (4μg; 1ml volume; Receptal®: Buserelin; i.m. injection in the near side hind leg), i.e., an amount considered adequate to induce a surge release of LH and hence synchronise the follicle waves (www.intervet.com; Intervet International).

5.2.6 Blood sampling

Blood samples were taken every 10-14 days by jugular venepuncture throughout the experiment and just prior to each immunisation to monitor inhibin antibody titres. To determine circulating FSH concentrations, the frequency of sampling was increased to twice-daily samples during the last 9 days of the trial.

5.2.7 Post-mortem

5.2.7.1 Inhibin antiserum and tissue collection

On day 7 ewes were transported to the abattoir, in lairage fresh water was made available. Animals were stunned and culled by exsanguination. In order to obtain inhibin antiserum, blood was collected from all animals into clean buckets and then decanted into 1litre plastic bottles, which were stored at 4°C. Subsequent to storage, for the following 5 days the clots retracted and the serum was then poured off into 50ml vials. To further reduce the amount of red blood cell contamination in the antiserum, the antiserum was transferred to 50ml vials and centrifuged for 30 minutes (4,000-x G), and the clear antiserum fraction was pipette into clean bottles and stored at -80°C.

The left ovaries were fixed in 4% Para formaldehyde, and left overnight. Then ovaries were cut open and placed in 70% alcohol and stored at RT for *in situ* hybridisation analysis. The right ovaries were placed in a 20ml universal flask containing warm (37°C) M199 culture media then immediately taken to the laboratory for follicle dissection and culture.

5.2.7.2 Follicle counts, culture and oestradiol production

Following dissection, follicles were then counted, measured and using 24 well culture plates (Nunclon Life Technologies) and an incubator (37°C; 95% O_2 and 5% CO_2) individually cultured in 2ml M199 culture media for 2h. After culture the follicles were stored in vials at -80°C. The culture media was stored (-80°C) until oestradiol was measured.

5.2.8 Radioimmunoassays

Details of RIA method for measuring reproductive hormones and antibody titres are given in Chapter 2 in sections 2.7 and 2.8, respectively.

5.2.9 Statistical Analyses

Statistical analyses of data, was carried out using Genstat version 6.1 Anti-inhibin titres were analysed using ANOVA. The follicle size data from each of the 4 groups was analysed using Poisson regression analysis (logarithmic link function), rather than ANOVA, as the data was not normally distributed and the number of follicles in the >3.5 group was small. Where data are transformed to a logarithmic scale to normalise the distribution of data, the textual description and statistical significance is based on transformed data. However, for ease of presentation, especially for presenting standard errors, the tables and figures display the unadjusted data unless otherwise stated. Where Poisson regression analysis is used, no standard errors are given. Due to small data sets for oestrogenic follicle numbers, the use of Friedman's non-parametric ANOVA was considered, but ruled out because this type of analysis is for one-way randomised block data. This data was two-way and completely randomised. Therefore, treatment interaction analysis was carried out on individual counts. A generalised linear model assuming Poisson errors was used to produce an analysis of deviance table for each variable. These values can be interpreted in the same way as an ANOVA. The raw averages and percentages are presented in section 5.34 to show which direction the significance effects and trends take.

5.2 RESULTS

5.3.1 Live weight and condition scores

The overall ewe live weights (mean ± s.e.m.) are presented in Figure 5.1. Ewe live weights were significantly increased with age ($P<0.001$). Neither inhibin immunisation ($P=0.49$) or rbGH treatment ($P=0.74$) had any influence on live weight.

Figure 5.1 Pooled ewe live weights.

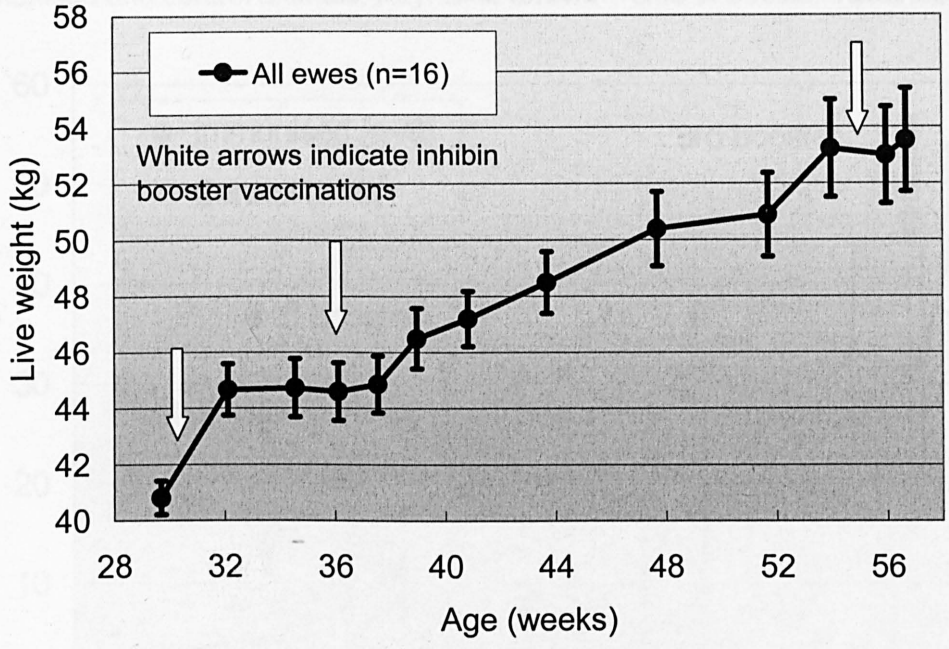


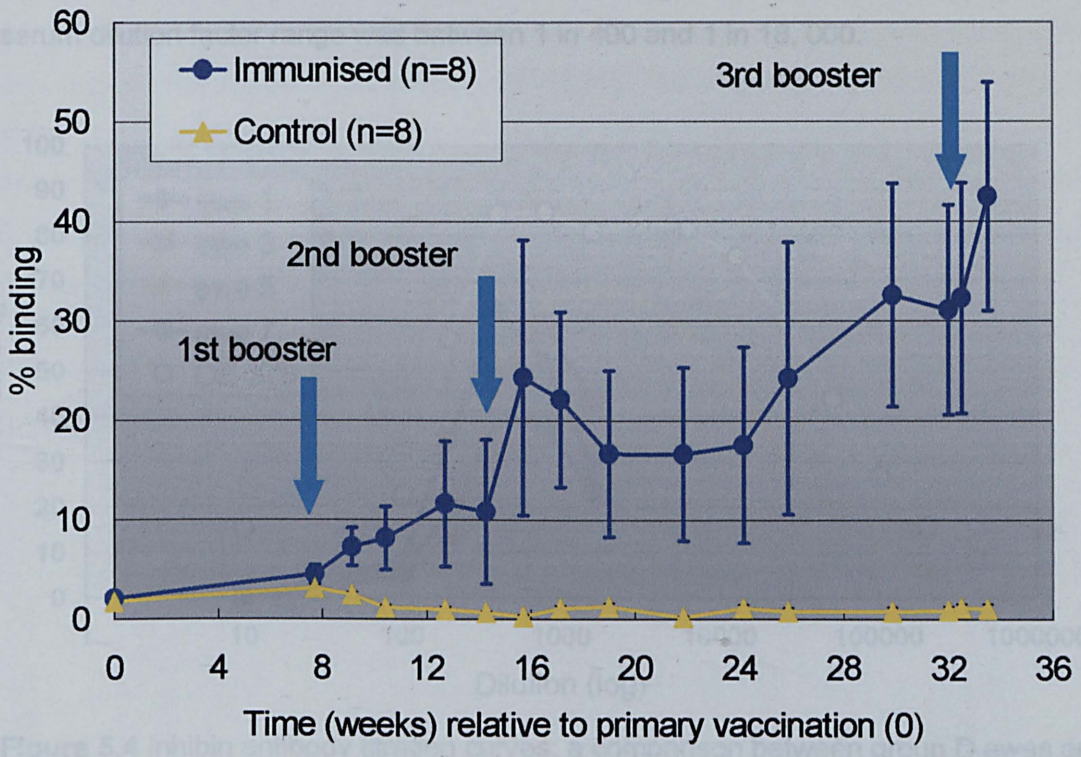
Table 5.2 Condition scores and live weights (mean ± s.e.m.) prior to culling. Letter after a condition score number indicate extremes within a classification grade e.g. L and H represent Low and High, respectively.

Group	Condition scores	Live weight (kg)
A	2, 3L, 3L, 3L	50.2 ± 3.3
B	2, 3L, 3H, 3H	51.3 ± 6.1
C	2, 2, 3L, 3L	51.6 ± 1.9
D	2, 3L, 3H, 3H	56.8 ± 3.1

5.3.2 Serum Inhibin antibody titres and pooled serum antibody titres

Inhibin antibody titres (mean ± s.e.m.) at 1 in 1,000 dilution over time are presented in Figure 5.2. Antibody titre responses to primary vaccination were not significantly ($P>0.05$) elevated, whereas responses to 1st, 2nd and 3rd booster vaccinations resulted in significant ($P<0.05$) antibody titres. Inhibin antibody titres remained elevated peaking on the day of slaughter (Figure 5.2). Individual inhibin antibody titre, defined as the dilution at which serum antibodies give 40% binding of ^{125}I-inhibin are presented in Figures 5.3 and 5.4. Mean inhibin antibody titres in serum collected at slaughter are shown in Chapter 6.

Figure 5.2 Comparison of inhibin antibody levels (1 in 1,000 dilution) between immunised and control animals. Key: Blue arrows = time of booster vaccination.



The 'Bell shaped curves' shown in Figures 5.3 and 5.4 have several phases: an increasing phase when antibodies are so highly concentrated that their ability to bind to the hapten (inhibin) is impaired. As the antiserum is diluted down the binding increases until a plateau phase is reached were binding efficiency peaks. The final phase is the 'decline phase' and this phase is used to indicate the antibody titre, as the rate of decline with increased dilutions indicates the affinity and number of antibodies, which can bind to the hapten.

Figure 5.3 Inhibin antibody titration curves in individual ewes: a comparison between group B ewes and OE 378 (positive control from a previous experiment; O'Shea and Webb, unpublished observations). At 40% binding (on the titre decline phase) the serum dilution factor range was between 1 in 400 and 1 in 18, 000.

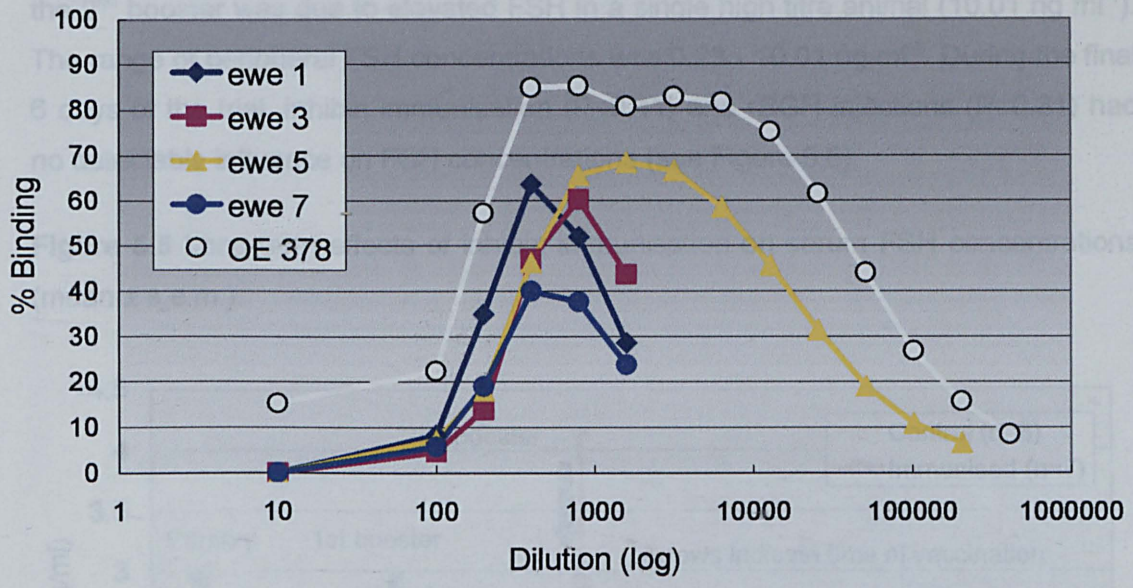
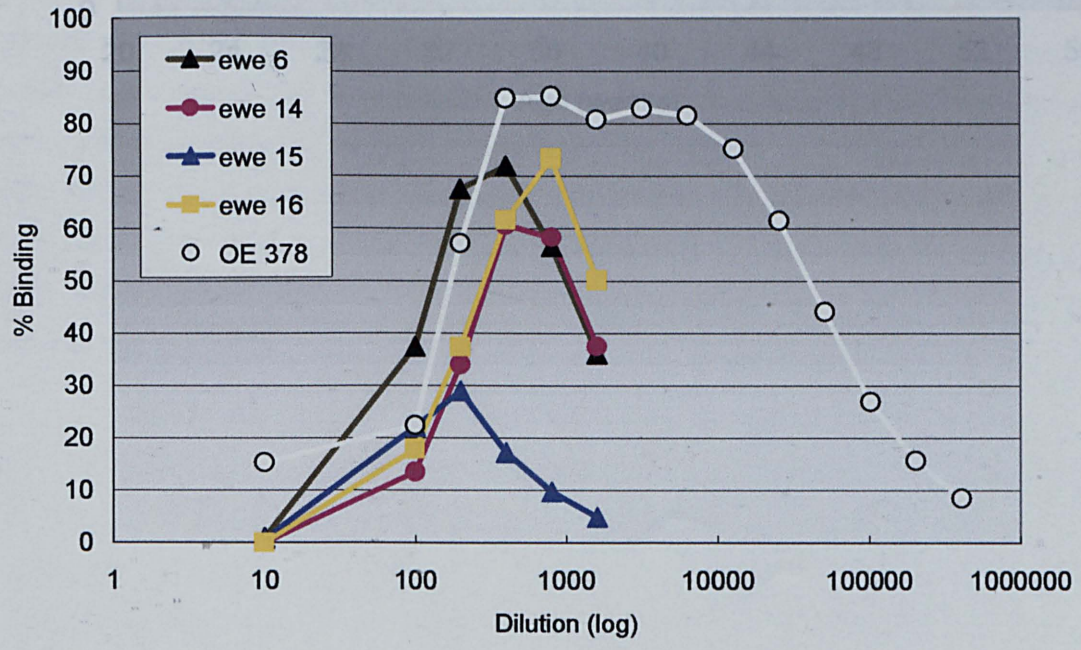


Figure 5.4 Inhibin antibody titration curves: a comparison between group D ewes and OE 378. At 40% binding the serum dilution factor range (excluding ewe 15) was between 1 in 1, 450 and 1 in 1, 900. Ewe 15 responded poorly to vaccination and antibody binding was less than 40%. The ewe 15 dilution factor at peak binding (~28%) was 1 in 200.

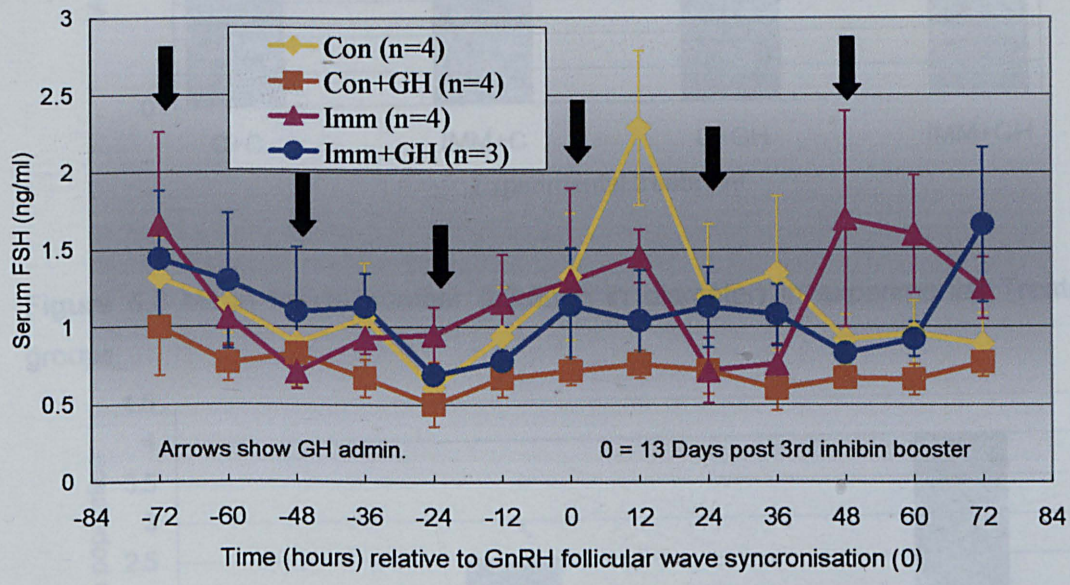


5.3.3 Follicle Stimulating Hormone

Long-term effects of inhibin immunisation on serum FSH concentrations are shown in Figure 5.5. Time (P=0.18) and inhibin immunisation (P=0.28) had no significant effect on FSH concentrations, despite the apparent increase in FSH concentrations. There was also no time/treatment interaction (P=0.21). The apparent increase in FSH after the 2nd booster was due to elevated FSH in a single high titre animal (10.01 ng ml^{-1}). The range of peripheral FSH concentrations was 0.23 - 10.01 ng ml^{-1}. During the final 6 days of the trial, inhibin immunisation (P=0.41) and rBGH injections (P=0.31) had no detectable influence on FSH concentrations (see Figure 5.6).

Figure 5.5 Long-term effects of inhibin immunisation on serum FSH concentrations (mean ± s.e.m.).

Figure 5.6 Serum FSH concentrations (mean ± s.e.m.), inhibin immunised and control ewes, with and without rbGH treatment. Inhibin immunisation (P=0.41) and rBGH injections (P=0.31) had no detectable influence on FSH concentrations. Key: black arrows = time of rbGH administration. Receptal® GnRH agonist injection was given to all ewes to synchronised follicular waves at time 0, i.e. 13 days after the 3rd inhibin booster vaccination.



5.3.3 Follicle counts

Administration of rbGH significantly (P<0.001) increased the mean number of follicles <3.5mm in diameter (Figure 5.7). The effects of rbGH were significantly (P<0.002) reduced when rBGH administration occurred in conjunction with inhibin immunisation. Inhibin immunisation had no significant effect on the small follicle population. Inhibin immunisation significantly (P<0.006) increased the number of follicles ≥3.5mm and rBGH had no effect on these large follicles (see Figure 5.8). Key= C + C, IMM + C, C + GH and IMM + GH represent; control (n=4 animals), inhibin immunised without rbGH (n=4), control with rbGH (n=4) and inhibin immunised with rbGH (n=3), respectively.

Figure 5.7 Mean numbers of small follicles (<3.5 mm) in the 4 treatment groups.

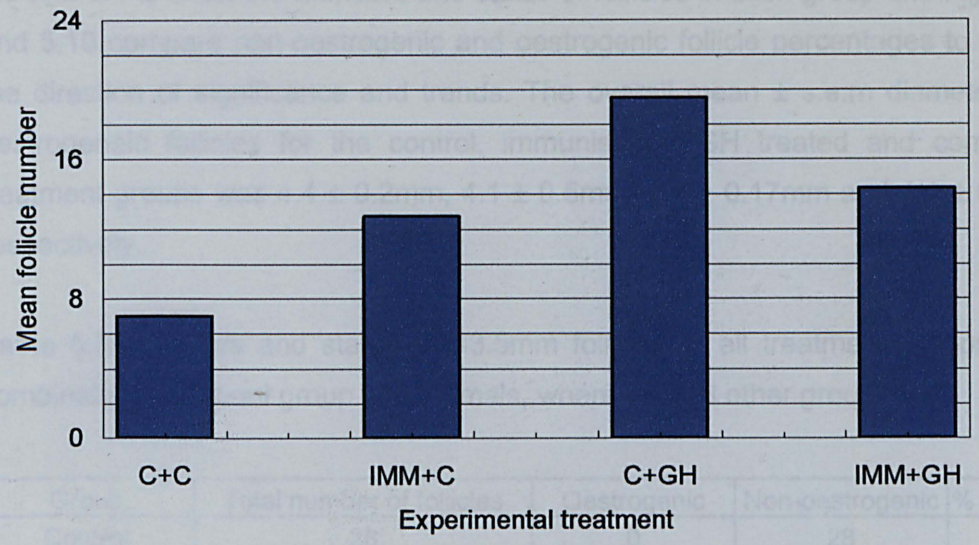
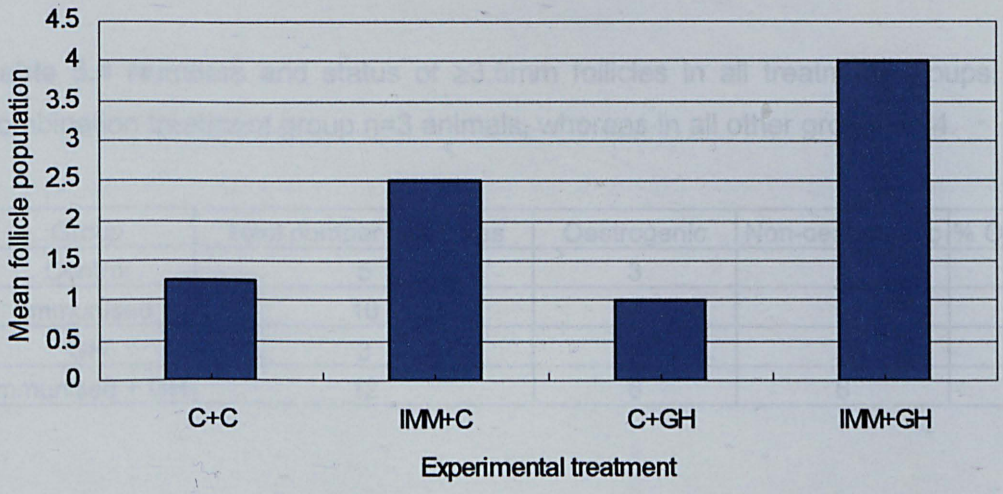


Figure 5.8 Mean follicle number (≥3.5mm in diameter) in experimental Treatment groups.



5.3.3 Oestrogenic follicles

Oestrogenic follicles were defined as those secreting >100pg/ml/h of oestradiol into culture media over a 2h period. This definition was arrived at based on a bimodal plot of oestrogen output from all follicles and based on earlier definitions by Webb and England, (1982).

Inhibin immunisation increased the numbers of both oestrogenic ($P<0.01$) and non-oestrogenic ≥3.5mm follicles ($P<0.01$). Whereas, Growth hormone increased the number of oestrogenic ($P<0.005$) and non-oestrogenic ($P<0.05$) <3.5mm follicles. The combination treatments of inhibin immunisation and bGH tended ($P=0.07$) to

increase the number of small (<3.5mm) follicles. The raw data is presented in Tables 5.3 and 5.4 to show the numbers and status of follicles in each group and Figures 5.9 and 5.10 compare non-oestrogenic and oestrogenic follicle percentages to illustrate the direction of significance and trends. The overall mean ± s.e.m diameter of the oestrogeneic follicles for the control, immunised, rbGH treated and combination treatment groups was 4.4 ± 0.2mm, 4.1 ± 0.5mm, 2.8 ± 0.17mm and 4.1 ± 0.49mm, respectively.

Table 5.3 Numbers and status of <3.5mm follicles in all treatment groups. In the combination treatment group n=3 animals, whereas in all other groups n=4.

Group	Total number of follicles	Oestrogenic	Non-oestrogenic	% Oestrogenic
Control	28	0	28	0
Immunised	51	1	50	1.9
GH	78	7	71	8.9
Immunised + GH	43	5	38	11.6

Table 5.4 Numbers and status of ≥3.5mm follicles in all treatment groups. In the combination treatment group n=3 animals, whereas in all other groups n=4.

Group	Total number of follicles	Oestrogenic	Non-oestrogenic	% Oestrogenic
Control	5	3	2	60
Immunised	10	4	6	40
GH	3	1	2	33.3
Immunised + GH	12	6	6	50

Figure 5.9 Percentage of non-oestrogenic and oestrogenic follicles in total <3.5mm follicle population. Total follicle number was obtained by pool of all <3.5mm follicles from 4 ewes per treatment group, except the combination group (Imm + C; n=3).

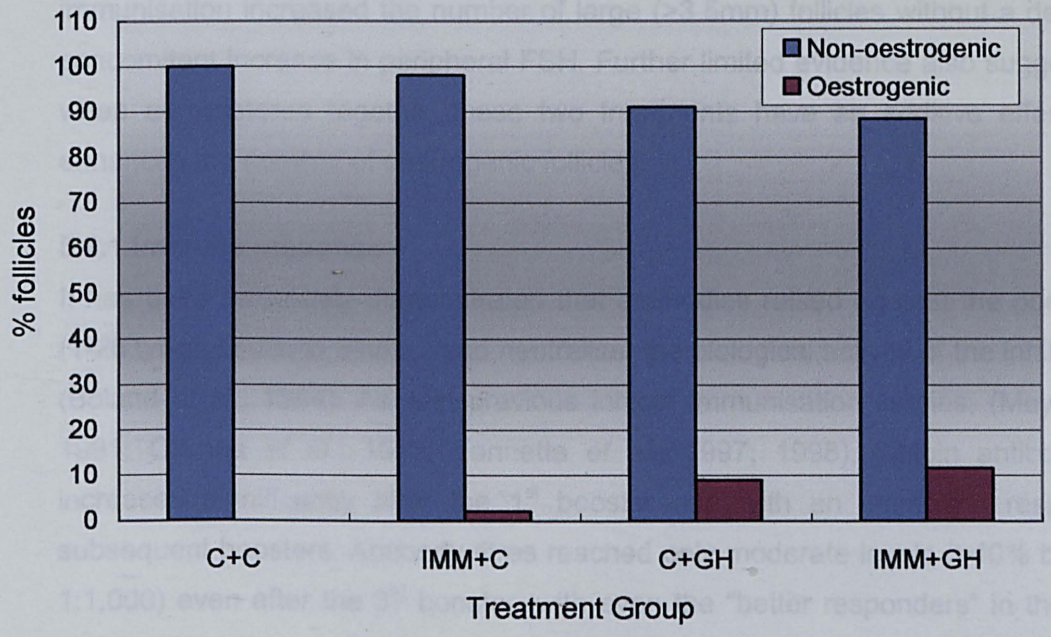
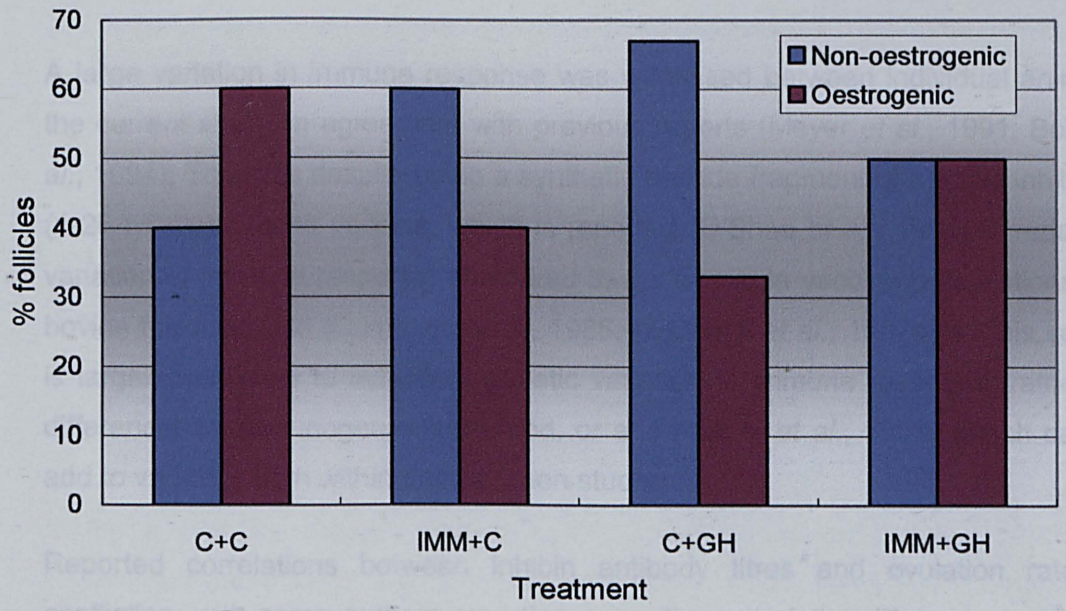


Figure 5.10 Percentage of non-oestrogenic and oestrogenic follicles in total ≥3.5mm follicle population. Total follicle number was obtained by pool of all ≥3.5mm follicles from 4 ewes per treatment group, except the combination group (Imm + C; n=3).



5.4 DISCUSSION

The present study has provided evidence to show that GH treatment in sheep increases the number of small (<3.5mm) follicles, while long-term inhibin immunisation increased the number of large (>3.5mm) follicles without a detectable, concomitant increase in peripheral FSH. Further limited evidence also suggests that, when administered together these two treatments have an additive effect, which enhances the number of oestrogenic follicles.

5.4.1 Immune response

It has been previously demonstrated that antibodies raised against the porcine α-C (1-26 tyr-gly) subunit bind to, and neutralise, the biological activity of the inhibin dimer (Boland *et al.*, 1994). As with previous inhibin immunisation studies, (Meyer *et al.*, 1991; O'Shea *et al.*, 1993; Tannetta *et al.*, 1997; 1998), inhibin antibody titres increased significantly after the 1st booster and with an increased response to subsequent boosters. Antibody titres reached only moderate levels (>40% binding at 1:1,000) even after the 3rd booster, with even the "better responders" in the current trial exhibiting much lower inhibin antibody titres relative to very high responding animals in previous studies, e.g. the positive control (OE 378) used in the current trial. This reduced response is probably due to the age of the ewes at immunisation, as in older ewes (age 4-5 years), immunised against bovine α-C (1-26 tyr-gly) subunit, similar titres were reported (Boland *et al.*, 1994) after only one booster was administered.

A large variation in immune response was witnessed between individual animals in the current study, in agreement with previous reports (Meyer *et al.*, 1991; Boland *et al.*, 1994). This was despite using a synthetic peptide fragment of porcine inhibin α-C (1-26 tyr-gly) subunit vaccine, which is reported (O'Shea *et al.*, 1994) to reduce the variation of immune response compared to earlier crude vaccine preparations using bovine follicular fluid (Cummins *et al.*, 1986; Al-Obaidi *et al.*, 1987a;b). This variation is largely attributed to individual genetic variation in immune response, rather than differences in immunogen dose, breed, or age (Meyer *et al.*, 1991), which can also add to variation both within and between studies.

Reported correlations between inhibin antibody titres and ovulation rates are conflicting, with some authors reporting a positive correlation (Boland *et al.*, 1994), while others find no correlation (O'Shea *et al.*, 1993). It appears that a positive correlation between inhibin antibody titres and ovulation rate can be detected when

the immune response and titres are variable (Boland *et al.*, 1994), whereas when most animals have a good response no significant correlation was observed (O'Shea *et al.*, 1993). This suggests that there is a threshold level at which inhibin is sufficiently neutralised to allow increased follicle recruitment and/or selection, leading to increased ovulation rate. Antibody titre levels beyond this threshold may not always result in further ovulations. However, the higher, and even superovulatory, responses of some animals may be due to individual animal variation in response.

5.4.2 FSH and follicle growth

The follicle recruitment window lasts ~24 hours (Draincourt, 2001) and has been shown to occur <8 hours after the start of the rise in FSH, when FSH concentrations reach a follicle specific threshold (Webb, Gould and Draincourt, 1989; Picton and McNeilly, 1991; Fry and Draincourt, 1996; Campbell, 1999). The FSH rise would not need to be large to stimulate increased recruitment and increased ovulation (Findlay *et al.*, 1989). Wrathall *et al.* (1990) suggested that the size of the FSH increment is of no consequence, whereas it is the prevention of the preovulatory decline in FSH that is important in increasing ovulation rate. A delay in the FSH decline would delay selection and allow more time for follicles to differentiate and be selected into the ovulatory pool. In contrast, several superovulation studies suggest that the size of the FSH rise is important in producing superovulatory responses (see Armstrong, 1993).

In the current trial a rise in peripheral FSH was seen in the control ewes approximately 12h after GnRH administration. Although FSH concentrations tended to increase with time in control animals (P=0.09), variation between animals was evident as previously reported (Al-Obaidi *et al.*, 1987a;b) and the expected FSH rise in the treated groups was not apparent. This indicates that there was a lack of synchronisation of follicle waves following GnRH administration, which would have induced ovulation or atresia in large antral follicles, and hence would provide the conditions for an increase in FSH and for the recruitment of the next wave of follicles. Passive inhibin immunisation consistently results in an abrupt elevation in circulating FSH concentrations (Mann *et al.*, 1989; 1993; Wrathall *et al.*, 1990; Campbell and Scaramuzzi, 1995; Campbell, Scaramuzzi and Webb, 1995). However, it was noted that, FSH levels soon declined (Mann *et al.*, 1989; 1993; Wrathall *et al.*, 1990; Campbell and Scaramuzzi, 1995) by day 6 (Campbell, Scaramuzzi and Webb, 1995), despite the presence of high antibody titres (Mann *et al.*, 1989; 1993; Campbell and Scaramuzzi, 1995; Campbell, Scaramuzzi and Webb, 1995). In contrast, previous publications on the effects of active immunisation against inhibin (as in the current study) on peripheral FSH levels are conflicting. For example, subsequent to active

inhibin immunisation, FSH concentrations were either increased (Al-Obadi *et al.*, 1987a;b; Findlay *et al.*, 1989; O'Shea *et al.*, 1989c; Mizumachi *et al.*, 1990; Wrathall *et al.*, 1990; 1992; Tannetta *et al.*, 1997) or remained unaltered (Henderson *et al.*, 1984; Schanbacher, 1988a; Meyer *et al.*, 1991; Schanbacher, Schemm and Rhind, 1991; Fray, Wrathall and Knight, 1994; Tannetta *et al.*, 1998). Findlay *et al.* (1989) suggested several explanations for these inconsistencies in FSH response; such as day of blood sampling relative to follicle recruitment and dominance, frequency of sampling, radioimmunoassays used and group sizes. Indeed, Wrathall *et al.* (1990) reported a 22% increase in FSH with random sampling, yet after frequent sampling (the sampling regime used in the current study), no change in peripheral FSH was observed in either the follicular or luteal phases. Furthermore, following long-term active inhibin immunisation (>6 months) FSH concentrations were no different to control ewes, despite increases in numbers of large oestrogenic follicles (Tannetta *et al.*, 1998) and/or ovulation rate (Wrathall *et al.*, 1990; Knight *et al.*, 1991; Fray, Wrathall and Knight, 1994). These findings are similar to those in our current trial, however, in the above trials during the initial active immunisation regime, the same ewes exhibited elevated FSH concentrations (Wrathall *et al.*, 1990; 1992), and the return of FSH to control levels may have resulted from homeostatic adjustment of the pituitary gland responsiveness to inhibin and steroid negative feedback with time.

Our failure to detect differences in FSH concentrations may be due to small group numbers, as was the case in the study by Henderson *et al.* (1984), although this is unlikely as comparisons between inhibin immunised and controls (n=8 per group) also showed no difference (P=0.33) in FSH concentrations. Alternatively, an inadequate sampling frequency may be responsible for our inability to detect differences in FSH. Bister and Paquay (1983) reported that although the mean FSH concentrations in cyclic and anoestrous ewes were similar, peripheral FSH was more variable in anoestrous ewes. A further explanation may be that the contribution of inhibin to negative feedback regulation of FSH may be reduced in anoestrous ewes relative to cycling animals (Kusina *et al.*, 1994; 1995), while oestradiol's feedback role may be increased (Legan and Karsch, 1980). For example, the magnitude of the FSH rise following inhibin passive immunisation was 50% less during seasonal anoestrous than during the breeding season (Kusina *et al.*, 1995). Moreover, active inhibin α-subunit immunisation increased peripheral FSH concentrations in cycling ewes, but not anoestrous ewes (Kusina *et al.*, 1994).

It is well established that FSH is inhibited by testicular and ovarian inhibin, and by oestradiol negative feedback (sections 1.1.3.4 and 1.1.4.4). The lack of elevated FSH in this and previous trials is likely the result of negative feedback from increased oestrogenic follicle numbers, as has been reported previously (Campbell *et al.*, 1995). Also difficulty in detecting FSH may be due to the fact that, increased FSH is transient, and only detected during the recruitment phase i.e., prior to negative feedback. Meyer *et al.* (1991) speculated that elevated oestradiol concentrations, arising from increased follicular mass following inhibin immunisation, may explain depressed LH concentrations, and absence of an increase in FSH concentrations in some studies. Indeed, higher oestradiol in inhibin immunised lambs, would also be consistent with their lower preovulatory LH surge values (Goodman and Karsch, 1980). The effects of this are increased ovulation rate and lambing rate despite no detectable increase in FSH. This may also have occurred in the present trial. More large oestrogenic or precocious (smaller oestrogenic) follicles in respective, inhibin immunised and rbGH treated animals may result in increased negative feedback on FSH and LH. This agrees with results from bovine follicular fluid treated ewes in which inhibin reduced the level of oestradiol negative feedback (Campbell and Scaramuzzi, 1996). However, rbGH treatment has previously been reported (Tannetta *et al.*, 1997) to reduce the inhibin antibody induced rise on FSH, probably due to more small follicles and hence more inhibin negative feedback on FSH.

5.4.5 Inhibin immunisation: mechanism of action

In the present study long-term inhibin immunisation alone resulted in increased numbers of large (>3.5mm) follicles 72h after follicle wave synchronisation (Figure 5.8). This is in agreement with previous work (O'Shea *et al.*, 1984; Al-Obaidi *et al.*, 1987a;b; Forage *et al.*, 1987a; Mizumachi *et al.*, 1990; Wrathall *et al.*, 1990; 1992; Campbell, Scaramuzzi and Webb, 1995; Tannetta *et al.*, 1997). It is unclear how inhibin immunisation in this and previous trials (Henderson *et al.*, 1984; Schanbacher, 1988; Meyer *et al.*, 1991; Schanbacher, Schemm and Rhind, 1991; Fray, Wrathall and Knight, 1994; Tannetta *et al.*, 1998) increased follicular development and ovulation rate, since alterations in FSH concentrations were not detected. These findings suggest that inhibin antibodies may act at the ovarian level. Alternatively, this may suggest that the increase in FSH due to immunisation were only transient and difficult to detect in animals with asynchronous follicular waves. Following immunisation, the hapten hormone concentrations have been known to be either reduced by antibody neutralisation or increase following a "rebound" effect, as described following steroid immunisation (Scaramuzzi *et al.*, 1980; Scaramuzzi,

Martinez and Van Look, 1980). Recently, Tannetta *et al*, (1998) reported that inhibin A and activin A were significantly increased following long-term active immunisation, without a concomitant significant increase in follistatin. Unfortunately, the effects of α-C-subunit inhibin antibodies in the current trial on inhibin, activin and follistatin concentrations, and more importantly, the bioavailability of these hormones is unknown.

Inhibin and the inhibin α-subunit are known to affect folliculogenesis at the level of the ovaries. For example, in addition to inhibiting pituitary FSH release (Baird *et al.*, 1989), inhibin may increase steroidogenesis, by enhancing LH induced androgen production by thecal cells, as demonstrated by *in vitro* studies in sheep (Campbell and Webb, 1995; see Webb *et al.*, 1999a) and several other species (rat; Hsueh *et al.*, 1987; human; Hillier, 1991; cow; Wrathall and Knight, 1993). Conversely, *in vivo* infusion of the ovine ovary via the ovarian artery with recombinant human inhibin reduced ovarian oestradiol and androstenedione secretion (Campbell, Scaramuzzi and Webb, 1995a), although FSH may have mediated these effects. Inhibin also competes with activin for, and may elicit its effects through, activin receptors (Knight and Glister, 2001), whereas the free α-subunit competes with FSH-R and with inhibin for the type II activin receptor (Robertson, Hertan and Farnworth, 2000; Knight and Glister, 2001; 2003). It may also reduce oocyte competence and the ability of the subsequent ovum to form blastocysts after fertilisation (Silva, Groome and Knight, 1999). Furthermore, given that activin A is increased following inhibin immunisation (Tanneta *et al.*, 1998), and activin A has been shown to up-regulate FSH-R and aromatase activity in rat granulosa cells, it may have a role in oocyte maturation (Knight and Glister, 2001). The increase in activin A, in the absence of a significant rise in its binding protein, follistatin, could explain how long-term inhibin immunisation enhances follicular development and ovulation rate, without necessarily promoting a sustained increase in peripheral FSH (Tannetta *et al.*, 1998). Recent findings support this suggestion as activin A and activin B may have roles in bovine oocyte maturation and fertilisation (Silva, Groome and Knight, 2003). Furthermore, it has recently been demonstrated in prolific sheep breeds that the oocyte plays a greater role in modulating folliculogenesis and ovulation rate than was previously anticipated (Montgomery *et al.*, 2001; Wilson *et al.*, 2001; Fabre *et al.*, 2003). Oocyte secreted BMPs have a role in modulating follicular development and ovulation rate (see section 1.1.5). Moreover, follistatin binds BMPs (Otsuka *et al.*, 2001a) and the evidence to date indicates that follistatin adversely affects prolificacy (Christensen, Redmond and Laarveld, 2000). By reducing the ratio of follistatin to activin (Tannetta

et al., 1998) the amount of follistatin available to bind BMPs may be reduced, thus promoting prolificacy. The exact mechanism through which α-C subunit immunisation induces increased inhibin A and activin A and the subsequent effects require further investigation. Current evidence suggests that inhibin and inhibin subunits appear to have both adverse and beneficial effects on follicular development, however further investigation is required. For example, the effects of immunisation against inhibin on TGF-β super family proteins and the relative importance of the intra-follicular actions of inhibins, activins and follistatin throughout antral follicular development requires clarification.

5.4.4 Effects of rbGH on follicles and interactions with inhibin

The increase in small follicle numbers in the current trial is in agreement with earlier findings following rbGH administration in ewes (Gong *et al.*, 1996b) and heifers (Gong, Bramley and Webb, 1991; Gong *et al.*, 1993; 1996; 1997). GH administration is thought to cause increased small follicle numbers by increasing liver and pancreatic production and release of IGF-I (Pell, 1990; Cohick, 1998; Armstrong *et al.*, 2000) and insulin (Gong *et al.*, 1996b; as described in 1.1.5.1). Indeed, rbGH administration has been observed to increase plasma IGF-I concentrations 2-3 fold (Davis, Smith and Gluckman, 1990; Gong, Bramley and Webb, 1991; Eckery *et al.*, 1994; Tannetta *et al.*, 1997), without affecting peripheral FSH, oestradiol or progesterone concentrations (Tannetta *et al.*, 1997) or ovulation rate in sheep (Davis, Smith and Gluckman, 1990; Eckery *et al.*, 1994; Gong *et al.*, 1996b) and cattle (Gong, Bramley and Webb, 1991; Gong *et al.*, 1993a). IGF-I increases proliferation and steroidogenesis (see Armstrong and Webb, 1997) and enhances the responsiveness of bovine and ovine granulosa cells to FSH (Monniaux and Pisselet, 1992; Spicer, Alpizar and Echternkamp, 1993; Campbell, Scaramuzzi and Webb, 1996). It does this by increasing gonadotrophin receptor expression and by stimulating the andenylate cyclase second messenger system (Webb *et al.*, 1999a; Lucy *et al.*, 1999). As a result, the number of gonadotrophic sensitive follicles responding to gonadotrophic drive, i.e., being recruited, differentiating and attaining oestrogenic status following GH and FSH treatment has recently been shown to increase in ewes (Gong *et al.*, 1993a). On the basis of rGH dose response study (Gong *et al.*, 1997) and the absence of GH receptors in bovine follicles (Lucy *et al.*, 1993), the direct actions of GH are considered to be less important than the ovarian actions of endocrine IGF-I (see Webb *et al.*, 1999a; Lucy *et al.*, 1999; Webb *et al.*, 2003a;b).

In conclusion, this work has confirmed and extended previous work showing that rbGH administration results in an increase in the number of small antral follicles in sheep and that active inhibin immunisation results in an increase in the number of large antral follicles. Preliminary evidence also suggests that the combination of these treatments results in earlier maturation of small antral follicles, based on literary evidence we can speculate that these effects are possibly due to an increase in FSH receptors, which are reported to be inhibited by inhibin α-subunit (Knight and Glister, 2003) and increased by IGF-I (Lucy *et al.*, 1999). However, it is unclear as to whether these treatments are additive or whether the effects are the result of a direct interaction of the two systems. The eagerly awaited immunohistochemical results may enable us to draw a stronger conclusion as to whether these two systems interact. Finally, the fact that these effects occurred independently of detectable changes in peripheral FSH concentrations suggests that they are mediated via intra-follicular control mechanisms.

CHAPTER SIX

THE EFFECT OF PASSIVE INHIBIN IMMUNISATION IN CATTLE ON OVARIAN FOLLICLE DEVELOPMENT

6.1 INTRODUCTION

One of the most effective ways of improving beef production efficiency is to increase twinning rate in suckler beef cattle (Echternkamp and Gregory, 1999a). Computer simulation and embryo transfer studies suggest that input costs per unit of beef output could be reduced and profit margins increased by 20-30% (Guerra-Martinez *et al.*, 1990), in herds with a 40% twinning rate (Diskin *et al.*, 1987). Twinning rate in cattle is a product of ovulation rate, conception rate and embryo (Van Vleck and Gregory, 1996) and foetal survival (Rutledge, 1975), and ovulation rate and twinning rate are positively correlated (r^2=0.75-9.0; Van Vleck, Gregory and Echternkamp, 1991; Gregory *et al.*, 1997). However, due to low incidence (0.5-4.5%; Pfau *et al.*, 1947) and heritability of twinning (0.04; Syrstad, 1984), the development of the Nebraskan Meat Animal Research Centre (MARC) ≥35% twin bearing suckler herd, through natural selection, has taken almost two decades (Echternkamp and Gregory, 1999b).

FSH is the primary hormone controlling follicle development (section 1.1.4.2) and immunoneutralisation of one of the major feedback hormones controlling FSH, namely inhibin, often leads to an increase in peripheral FSH concentrations (Glencross *et al.*, 1994; Campbell *et al.*, 1995; Terqui *et al.*, 1995; Akagi *et al.*, 1997; 2002; Takedomi *et al.*, 1997). As a consequence ovarian follicle growth is stimulated and the number of recruited follicles can be increased (Campbell, Scaramuzzi and Webb, 1995; Campbell and Scaramuzzi, 1995; Takedomi *et al.*, 1997). Furthermore, following a longer period of FSH elevation (Kaneko *et al.*, 1993b; 1995; Glencross *et al.*, 1994), cohort follicles have more time available to grow, differentiate and attain ovulatory status, thus increasing the chance of multiple ovulation. Further evidence (Campbell *et al.*, 1995; Bleach *et al.*, 1996) suggests that inhibin antibodies act at the ovarian level to enhance follicular development independently of peripheral FSH concentrations, but the exact mechanisms involved have not been fully elucidated.

Several alternatives to natural selection have been investigated with a view to developing faster, more practical approaches to increase twinning rates in ruminants. These approaches have included exogenous hormone treatment (Armstrong, 1993) and immunisation against reproductive hormones (Hillard *et al.*, 1995), however

extreme variation in animal response, both between animals and within a single animal across successive oestrous cycles (Webb and Morris, 1988), to exogenous gonadotrophins and active immunisation regimes, against both gonadal steroids (Price *et al.*, 1987a) and inhibin (Bindon *et al.*, 1988; Scanlon *et al.*, 1993), have resulted in a failure to consistently induce twin ovulations in cattle. In contrast, inhibin immunisation has proved relatively successful in sheep (O'Shea *et al.*, 1994). The possible reason for this difference between sheep and cattle are many. However, we hypothesised that the main cause was the overriding influence of the dominant follicle in cattle, i.e., the drive to produce a single egg. Thus, it was reasoned that the variation in response to vaccination was possibly due to immunising animals at an inappropriate stage of the follicular wave, which would lead either to a low response, if a dominant follicle was present (Straigmiller and England, 1982; Guilbault *et al.*, 1991; Huhtinen *et al.*, 1992), or an over stimulation if the immunisation boost coincides with follicular recruitment. This problem, allied with a more general difficulty in obtaining repeatable titres following immunisation in cattle, means that active inhibin immunisation is unlikely to succeed as a means of inducing twin ovulations in cattle.

Passive inhibin immunisation has received less research attention, due to the transient nature of the response to passive immunisation and the large quantities of antibody required in cattle (Hillard *et al.*, 1995). However, using a passive immunisation protocol in sheep, which involved an acute bolus injection of inhibin antiserum followed by supplementary smaller daily injections, FSH concentrations were increased, multiple follicles developed and multiple ovulations occurred (Campbell, Scaramuzzi and Webb, 1995; Campbell and Scaramuzzi, 1995). Thus, the acute and controllable nature of passive immunisation enables treatment of animals to occur at an optimum stage of the follicular wave, with long-term elevation in inhibin titre, as occurs with active immunisation. In a similar inhibin passive immunisation study, using polyclonal sheep antiserum, in Holstein-Angus cross heifers, a highly effective and repeatable induction of twin ovulations was obtained (Campbell, Gong and Webb, unpublished observations). Twin ovulations were observed in 4/6 heifers and one of the other two remaining animals had two dominant follicles, but only a single ovulation.

The objective of the current study was to investigate the affects of passive inhibin immunisation on circulating FSH, folliculogenesis, ovulation, corpora lutea (CLs) and progesterone concentrations in a larger group of heifers using antiserum raised in

ewes (Chapter 5). Also, as the antiserum in the current trial was of lower titre, we aim to compare the antibody titres between groups and the physiological responses exhibited.

6.2 MATERIALS AND METHODS

6.2.1 Experimental Animals

A total of 18 beef cross heifers aged 18 months and weighing 510.6 ± 17.2 kg were involved in the current experiment. The heifers had previously been used in the GnRH immunisation study (Chapter 4). There were several reasons, which justify the re-use of these animals. Firstly, the current study took place 15 weeks after the Chapter 4 study ended (>15 months since the last GnRH booster) and GnRH antibody titres were not detectable. Secondly, gonadotrophin and ovarian steroid concentrations, reproductive anatomy, follicle development, ovarian cycles and oestrus behaviour was similar (P>0.05) in both treatment groups. Thirdly, the heifers had been acclimatised to handling and ovarian scanning procedures to reduce stress. Finally, the UK was suffering from FMD outbreak and keeping the heifers for an additional trial was considered preferable to buying in new animals from outside sources. Animals were randomly selected for treatment by an independent adjudicator. The results of this random selection are shown in Figure 6.1. Animals were group housed on straw and fed twice daily with concentrate and ad libitum hay.

Figure 6.1 A schematic illustration of the results of random heifer selection. Treated (n=3) and control (n=6) heifers from the earlier study described in Chapter 4 were immunised against inhibin in the current study, while GnRH immunised animals (n=6) and Chapter 4 control animals (n=3) served as control heifers in the current study.

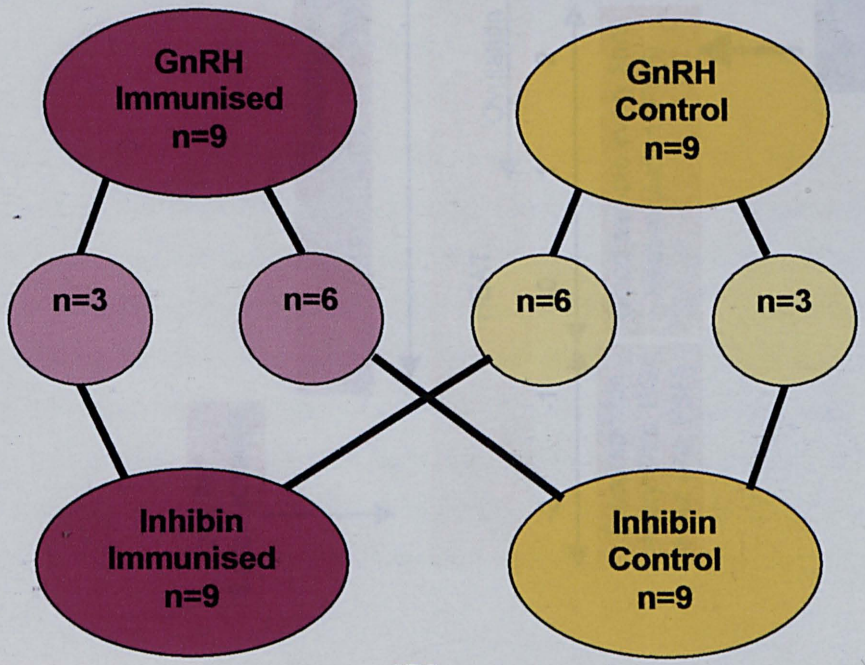
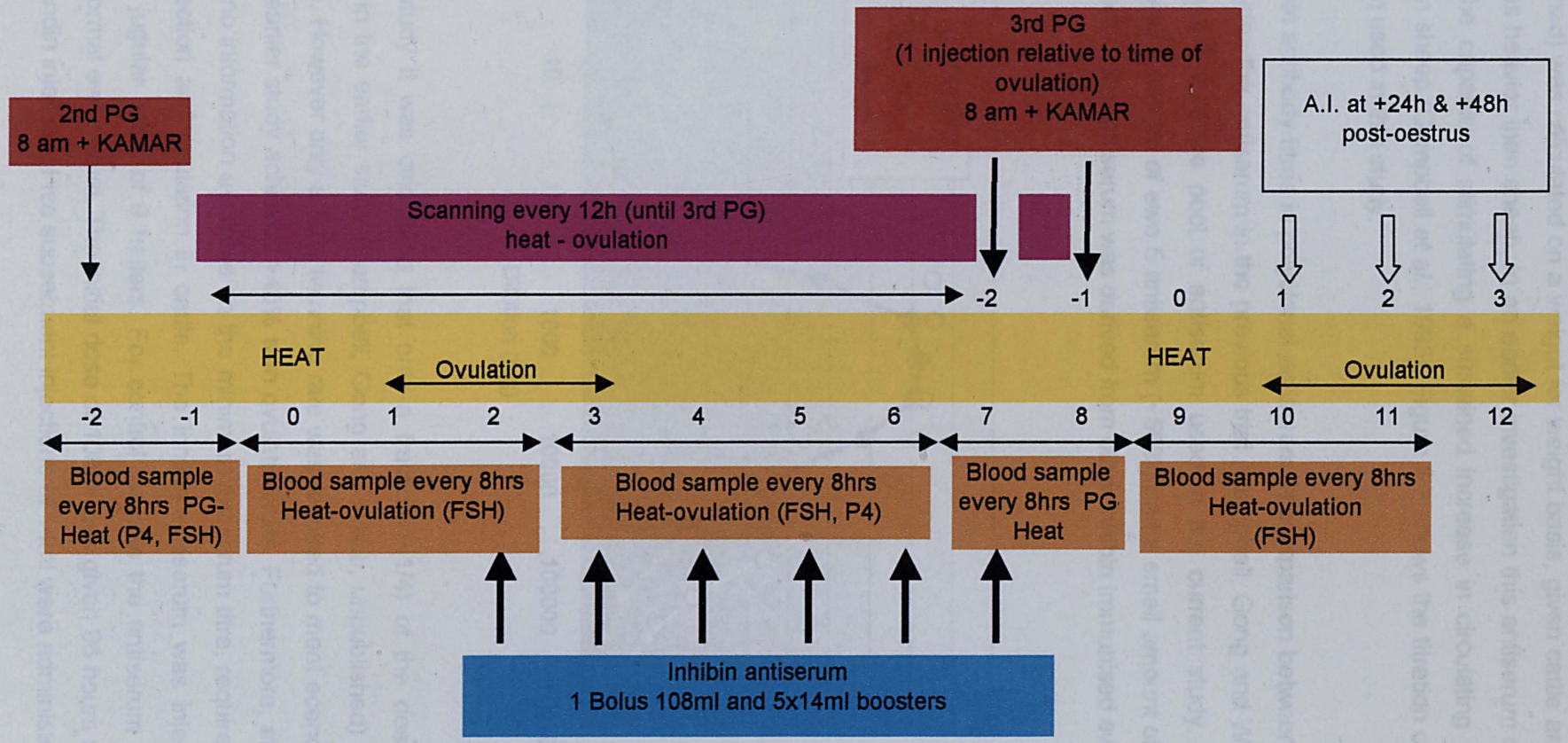


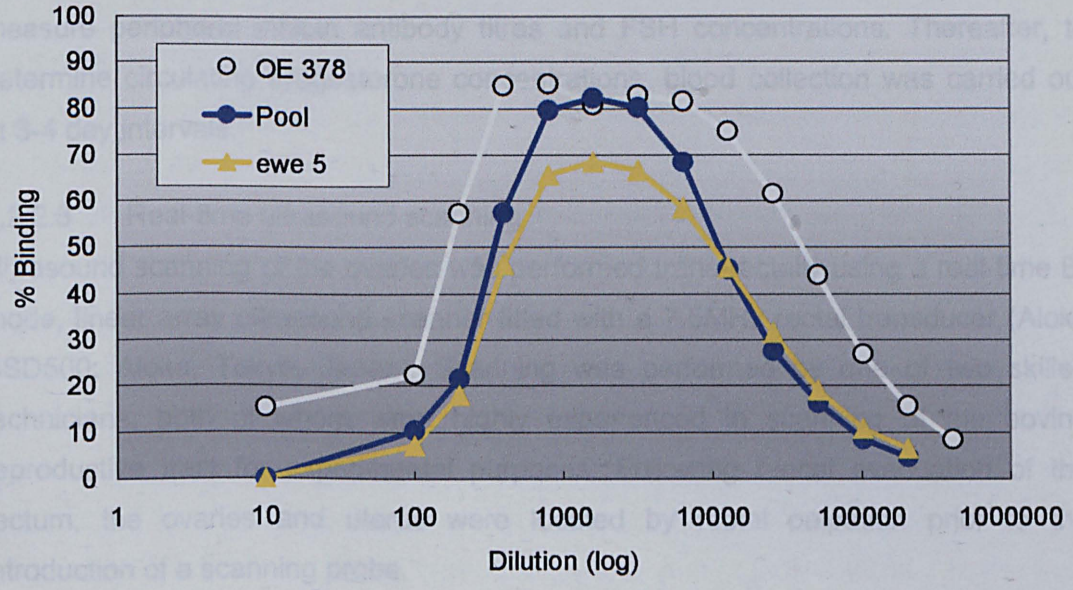
Figure 6.2 Experimental protocol



6.2.2 Passive immunisation

The dose of inhibin antiserum used in the previous cattle study (Campbell, Gong and Webb, unpublished) was calculated on a weight for weight basis, given cattle are on average 10 times heavier than sheep. In an earlier investigation this antiserum dose was shown to be capable of stimulating a sustained increase in circulating FSH concentrations in sheep (Campbell *et al.*, 1995). Figure 6.3 shows the titration curve for the antiserum used in this study.

Figure 6.3 Inhibin antibody titres in polyclonal antiserum: a comparison between OE 378, which provided the antiserum in the previous trial (Campbell, Gong and Webb, unpublished), ewe 5 and the pool of antiserum used in the current study. The antiserum pool was made up of ewe 5 antiserum (~56%) and a small amount of OE 378, the remainder of the antiserum was derived from other inhibin immunised ewes.



In the current study it was calculated that only a fraction (~1/4) of the dose of antiserum used in the earlier study (Campbell, Gong and Webb, unpublished) was available for use. However only a 40% twinning rate was required to meet economic targets and the earlier study achieved >40% twin ovulation rate. Furthermore, at the time, there was no information available on the minimum antiserum titre, required to induce twin selection and ovulation in cattle. The inhibin antiserum was injected directly into the jugular veins of 9 heifers. For control animals the antiserum was substituted for normal ewe serum. The initial dose of 108ml was given 96 hours after the 2nd prostaglandin injection. Five subsequent injections of 14ml were administered

at 24 hourly intervals. For details on vaccine preparation and production of antiserum see Chapters 2 and 5, respectively.

6.2.2.1 Oestrous synchronisation and artificial insemination

The animals were synchronised by giving 2 injections of $PGF_{2\alpha}$ 12 days apart. Following ovulation, a 3rd $PGF_{2\alpha}$ injection was given to induce luteolysis and allow the dominant follicle(s) of the first follicular wave after oestrus to ovulate. All heifers were artificially inseminated (A.I.) at fixed times, 72 and 96 hours, relative to the 3rd $PGF_{2\alpha}$ injection. If the corpus luteum (CL), which formed after 2nd $PGF_{2\alpha}$ was not able to respond to the 3rd $PGF_{2\alpha}$ injection on Day 7 (Figure 6.2), a further $PGF_{2\alpha}$ injection was given on Day 8 to induce CL regression, and timing of A.I. was adjusted accordingly.

6.2.2.2 Blood sampling

Blood samples were collected by jugular venepuncture (section 2.5.1) every 8 hours from 24 hours after the 2nd $PGF_{2\alpha}$ injection until the 2nd artificial insemination, to measure peripheral inhibin antibody titres and FSH concentrations. Thereafter, to determine circulating progesterone concentrations, blood collection was carried out at 3-4 day intervals.

6.2.2.3 Real-time ultrasound scanning

Ultrasound scanning of the ovaries was performed trans-rectally using a real-time B-mode, linear array ultrasound scanner fitted with a 7.5MHz rectal transducer (Aloka SSD500; Aloka, Tokyo, Japan). Scanning was performed by one of two skilled technicians, both of whom were highly experienced in scanning of the bovine reproductive tract for experimental purposes. Following faecal evacuation of the rectum, the ovaries and uterus were located by rectal palpation prior to the introduction of a scanning probe.

The presence of all antral follicles on each ovary was recorded. The diameters of small (<4mm) and medium-sized (4-7.9mm) follicles were estimated against the centimetre scale on the monitor. Larger follicles (≥8mm) and CLs were measured using the electronic calliper feature on the machine along the longest and shortest axis of the follicle/CL, the mean of these two measurements being the recorded size.

Scanning was initiated 24 hours after the 2nd $PGF_{2\alpha}$ injection and was performed every 12 hours until 48 hours prior to the 1st A.I. To ascertain the number and size of the CL(s), which formed subsequent to 3rd $PGF_{2\alpha}$ injection, additional scans were

performed on Days, 5, 10, 16 and 31, relative to oestrus (Day 0) after 3rd $PGF_{2\alpha}$ injection.

6.2.2.4 Oestrus

Oestrus was determined using KAMAR™ heat mount detectors and observations of behaviour as described previously (sections 1.3.4.1 and 2.9.1).

6.2.2.5 Post-mortem

Heifers were slaughtered 14-16 weeks after A.I.

6.2.2.5.1 Foetus and reproductive tract assessment

Following slaughter the foetus, ovaries, uterus and ovaries of each animal were weighed and measured, prior to examination. The results of carcass assessments, which were also conducted, are presented with results of the previous GnRH immunisation trial in Chapter 4.

6.2.3 Statistical Analyses

Prior to any statistical analysis a general ANOVA was performed on the data and the distribution of the data was checked on residual plots. Where necessary the data was log transformed (ln). FSH data was log transformed and analysed using Genstat 6.1 (Lawes Agricultural Trust, 2001, IACR Rothamsted), using split-plot analysis with repeated measures and degrees of freedom adjusted for the GGE effect. Ovarian follicle counts of medium-sized (4-7.9mm) and large (≥8mm) were analysed in Genstat 6.1, using generalised linear regression analysis, with Poisson distribution and logarithmic (ln) link function. This analysis was selected due to the small mean numbers of large (>8mm) follicles. Poisson analysis gives no standard errors of difference (s.e.d) or standard errors of means (s.e.m.). Anti-inhibin titres were analysed using Genstat 6.1, ANOVA. Where data are transformed to a logarithmic scale to normalise the distribution of data, the textual description and statistical significance is based on transformed data. However, for ease of presentation, especially for presenting standard errors, the tables and figures display the unadjusted data unless otherwise stated.

6.3 RESULTS

With permission, some unpublished results from a previous trial (B.K. Campbell, J. G. Gong and R. Webb) are presented in a comparative way with the results of the

present trial. Antibody titres from the current study and the earlier study are comparable as samples from both studies were assayed together.

6.3.1 Antibody titres

Mean antibody titres, 8 hours after inhibin antiserum injection, are compared with those of the previous (Campbell, Gong and Webb, unpublished) and shown in Figure 6.4. Heifers in the Campbell, Gong and Webb trial had significantly ($P<0.001$) higher anti-inhibin titres than those of the present trial, (Figure 6.4). Mean control heifer titre was <2.0% binding at each dilution. Mean inhibin antibody titres (1 in 20 dilution) over time are illustrated in Figure 6.5. Inhibin antibody titres remained constant for 6.5 days after the antiserum bolus injection.

Figure 6.4 A comparison of mean (± s.e.m.) inhibin antibody titres, in peripheral serum 8 hours after a bolus injection of antiserum, between the current experiment (referred to as Williams) and the previous experiment (Campbell, Gong and Webb, unpublished observations) referred to as Campbell. Heifers in the Campbell trail had significantly ($P<0.001$) higher inhibin antibody titres than heifers in the current study.

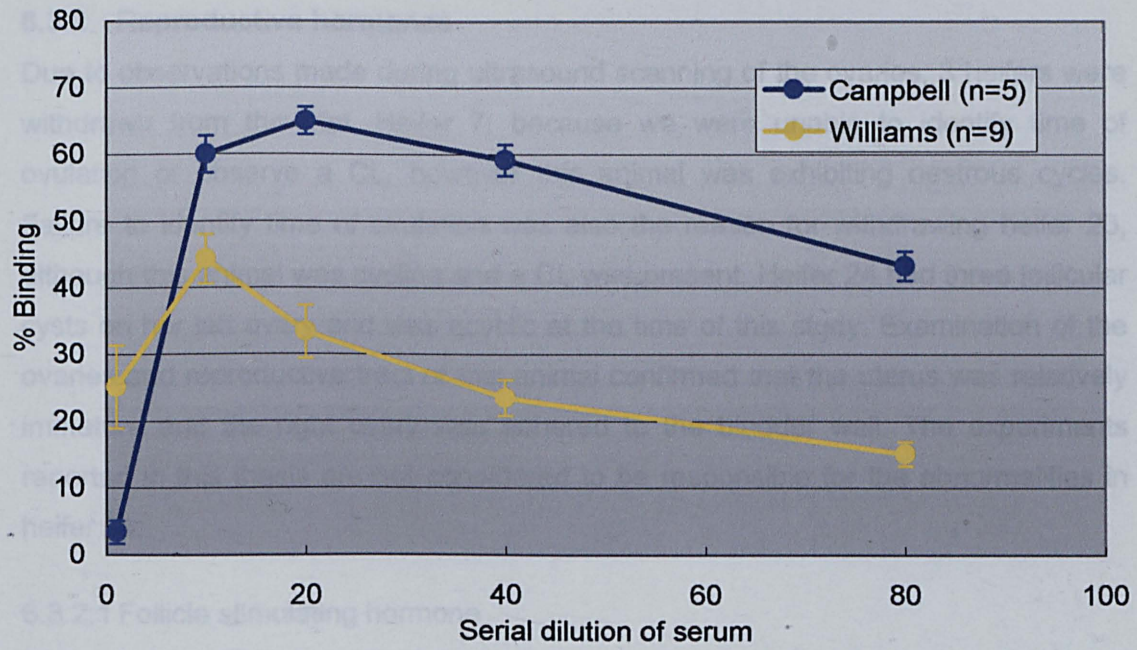
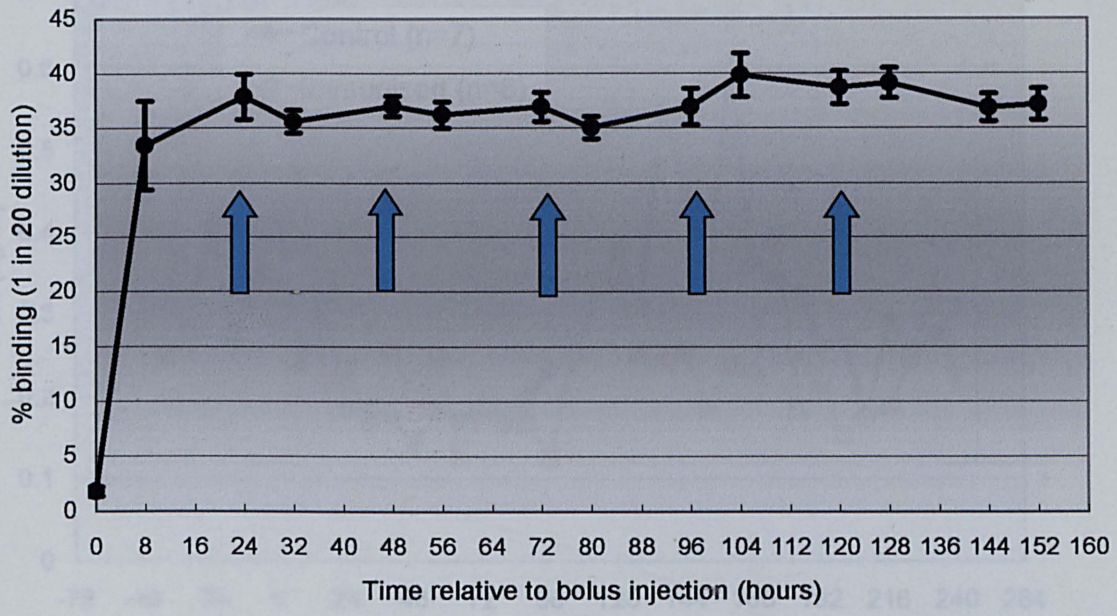


Figure 6.5 Mean (± s.e.m.) inhibin antibody titres in the peripheral serum of 9 heifers (1:20 dilution) over time. Key: blue arrows represent booster antiserum injections, relative to bolus administration (time 0). 24h after the bolus antiserum was administered the mean anti-inhibin titres stabilised between 35-40% binding.



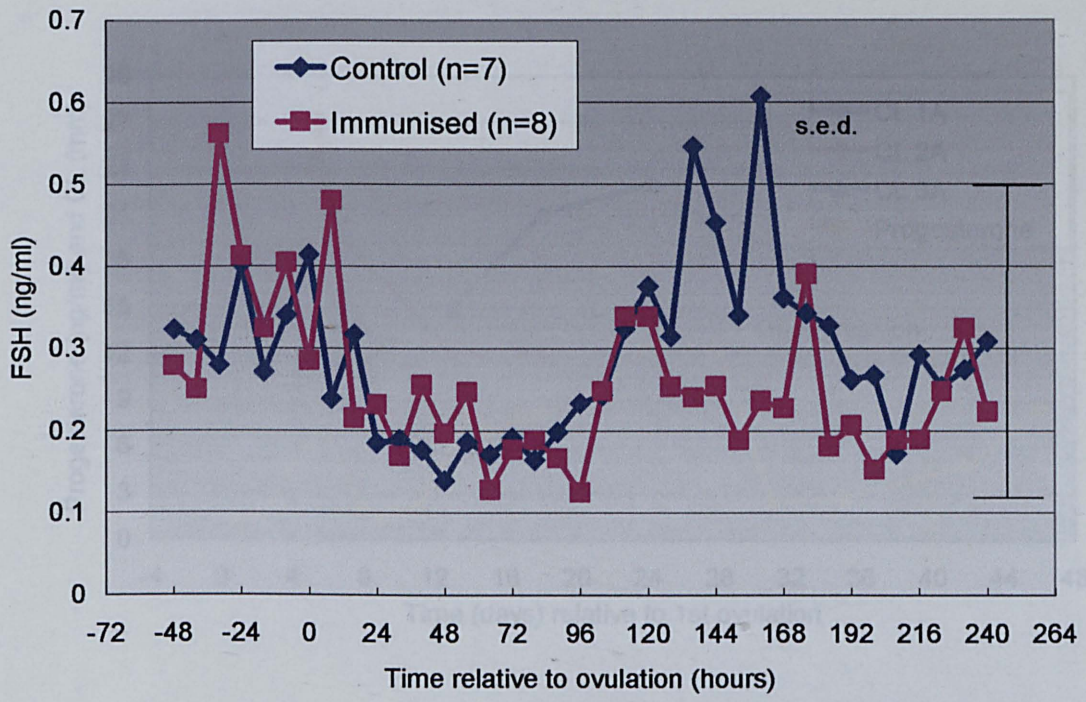
6.3.2 Reproductive hormones

Due to observations made during ultrasound scanning of the ovaries, 3 heifers were withdrawn from the trial. Heifer 7: because we were unable to identify time of ovulation or observe a CL, however this animal was exhibiting oestrous cycles. Failure to identify time of ovulation was also the reason for withdrawing heifer 23, although this animal was cycling and a CL was present. Heifer 24 had three follicular cysts on her left ovary and was acyclic at the time of this study. Examination of the ovaries and reproductive tract of this animal confirmed that the uterus was relatively immature and the right ovary was adhered to the bladder wall. The experiments reported in this thesis are not considered to be responsible for the abnormalities in heifer 24.

6.3.2.1 Follicle stimulating hormone

Mean serum FSH in both treatment groups is presented in Figure 6.6. FSH concentrations did not change significantly throughout the sampling period (P=0.12). Furthermore, treatment had no significant effect on serum FSH concentrations (P=0.27), and there was no time/ treatment interaction (P=0.36).

Figure 6.6 Mean FSH concentrations in inhibin immunised and control heifers with time (P=0.12). Standard error of the differences of means were; 0.26, 0.12, 0.41 and 0.39 for time, treatment and time / treatment interactions, respectively.



6.3.2.2 Progesterone

Examples of typical pregnant and non-pregnant progesterone profiles and growth of associated CL are presented in Figures 6.7 and 6.8, respectively. Figure 6.9 shows the one and only immunised heifer, which exhibited a twin ovulation and associate CL. In all three Figures, A.I. occurred 24 and 48h after Oestrus (Heat).

Figure 6.7 Progesterone concentration in plasma and associated growth of CL diameter relative to oestrous cycle synchronisation treatments and ovulation in a non-pregnant heifer (2475). Key: PG3 = 3rd $PGF_{2\alpha}$ injection. H = approximate time of heat. A.I. occurred at Heat +24 and +48h.

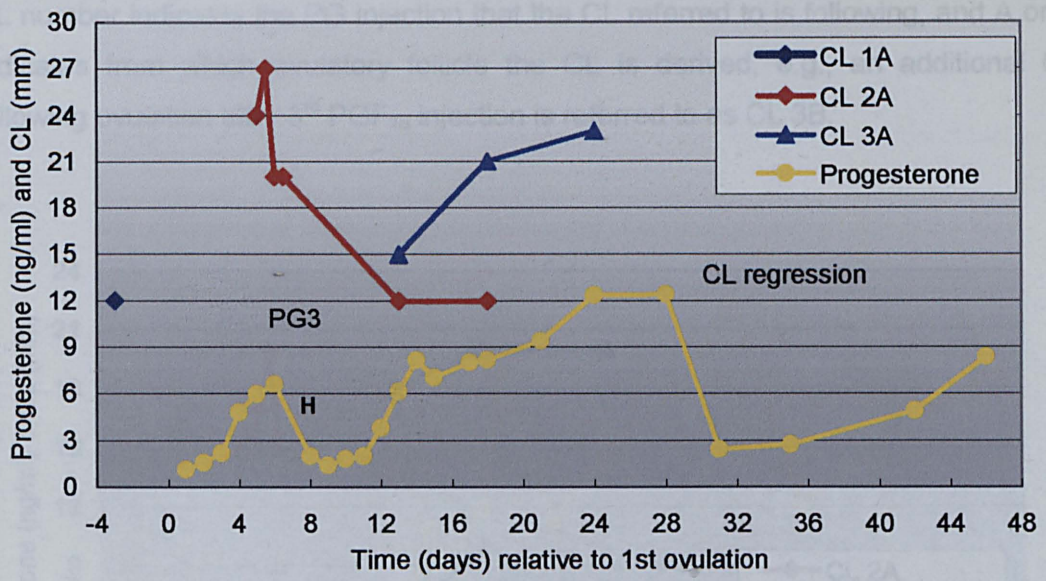


Figure 6.8 Progesterone concentration in plasma and associated growth of CL diameter relative to oestrous cycle synchronisation treatments and ovulation in a pregnant heifer (39). Key: PG3 = 3rd $PGF_{2\alpha}$ injection. H = approximate time of heat. A.I. occurred at Heat +24 and +48h.

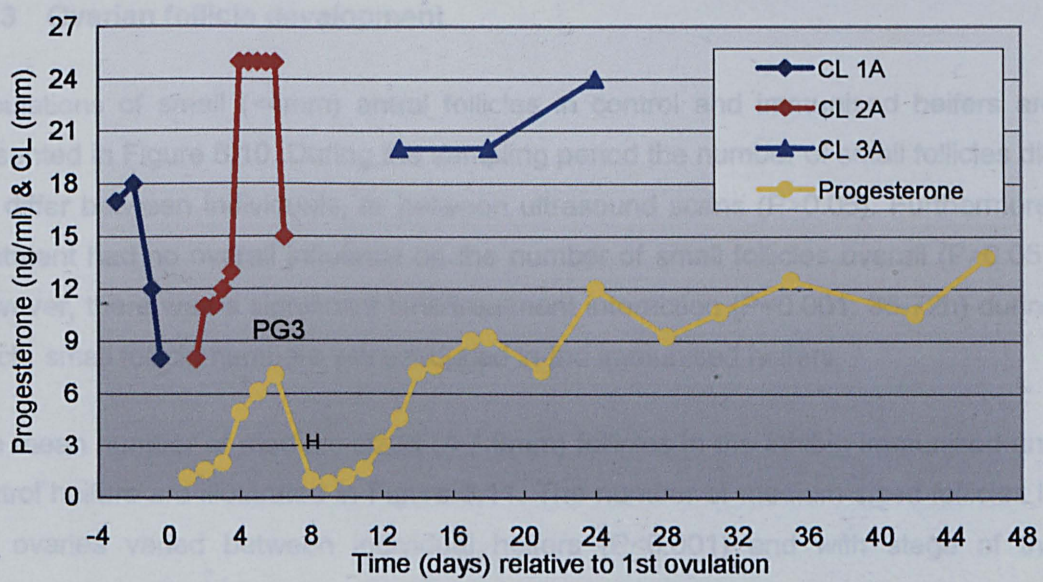
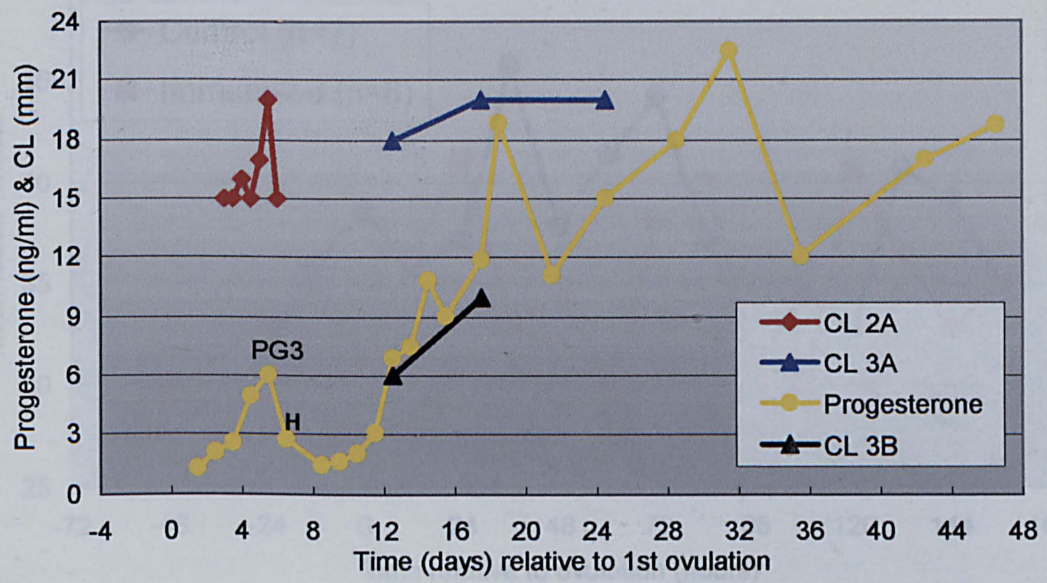


Figure 6.9 Progesterone concentration in plasma and associated growth of CL relative to oestrous cycle synchronisation treatments and ovulation in a pregnant heifer (73). Note the presence of an additional CL (CL 3B) indicative of a double ovulation. The life span of the additional CL was short (5 days). Key: PG3 = 3rd $PGF_{2\alpha}$ injection. H = approximate time of heat. A.I. occurred at Heat +24 and +48h. CL number indicates the PG injection that the CL referred to is following, and A or B indicates from which ovulatory follicle the CL is derived, e.g., an additional CL following ovulation after 3rd $PGF_{2\alpha}$ injection is referred to as CL 3B.



6.3.3 Ovarian follicle development

Populations of small (<4mm) antral follicles in control and immunised heifers are presented in Figure 6.10. During the sampling period the number of small follicles did not differ between individuals, or between ultrasound scans (P>0.05). Furthermore, treatment had no overall influence on the number of small follicles overall (P>0.05). However, there was a significant time/treatment interaction (P<0.001; 36-72h) during which, small follicle numbers were reduced in the immunised heifers.

The mean number of medium-sized (4-7.9mm) follicles in the inhibin immunised and control heifers are illustrated in Figure 6.11. The number of medium-sized follicles in the ovaries varied between individual heifers (P<0.001) and with stage of the oestrous cycle (P<0.001). Passive inhibin immunisation caused a significant increase

in the number of medium-sized follicles (P<0.001) overall and there was a significant time/treatment interaction (P<0.001; 0-48h).

Numbers of large follicles (≥8mm) in treated and control groups are presented in Figure 6.13 the follicle numbers varied between individuals (P<0.01) and with day relative to ovulation (P<0.001). Treatment had no affect on numbers of large follicles (P=0.68) and there was no time/treatment interaction (P=0.94).

Figure 6.10 Mean numbers of small antral follicles in control and immunised heifers.

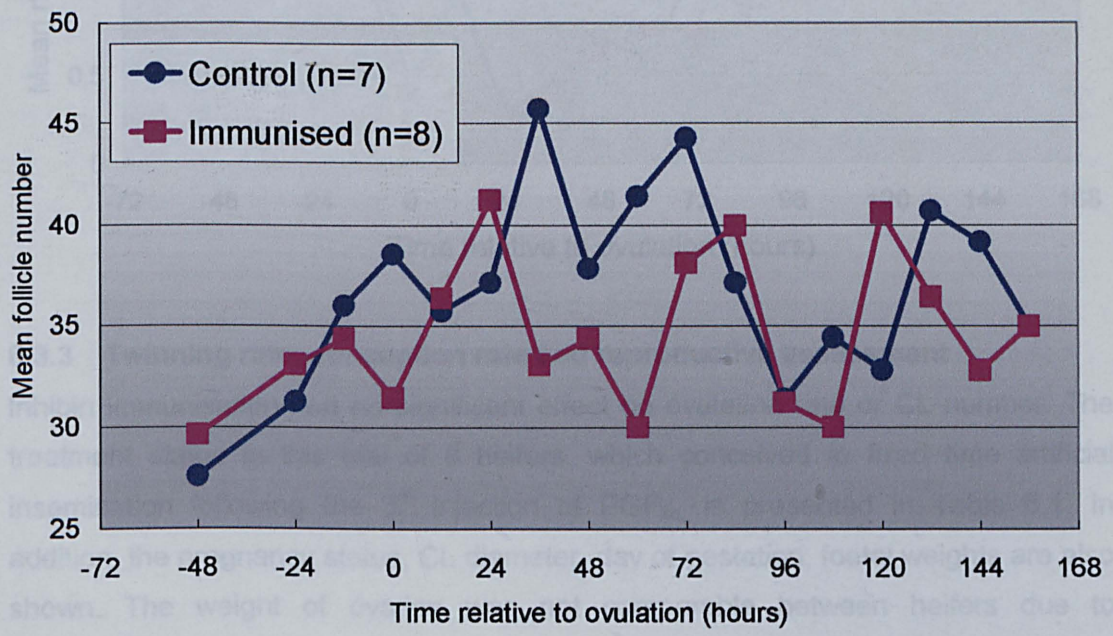


Figure 6.11 Mean number of medium-sized follicles in control and inhibin immunised heifers.

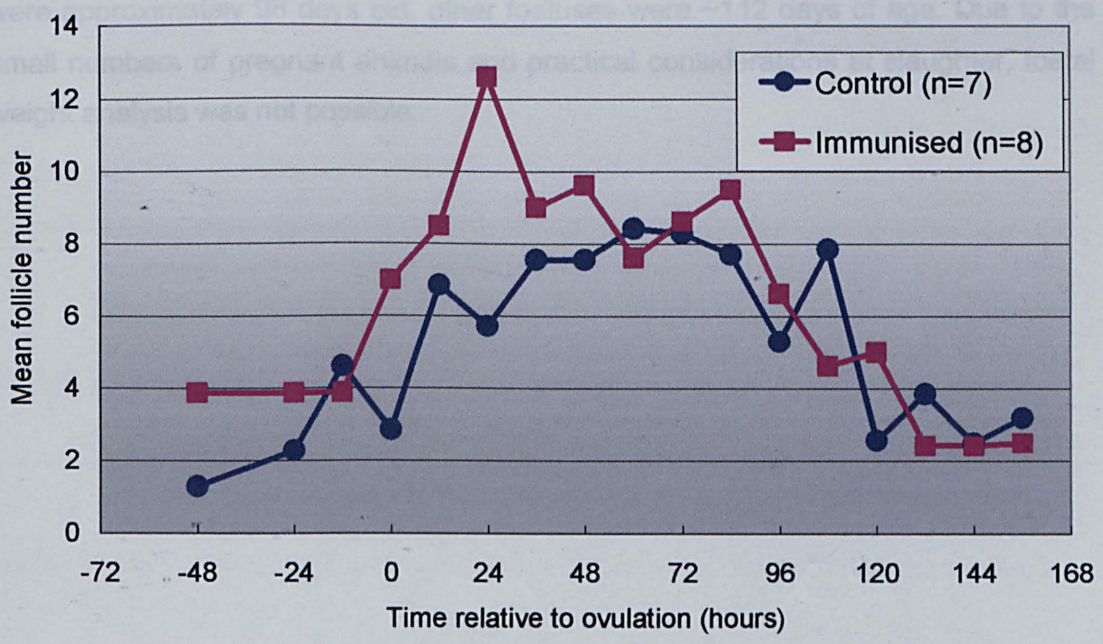
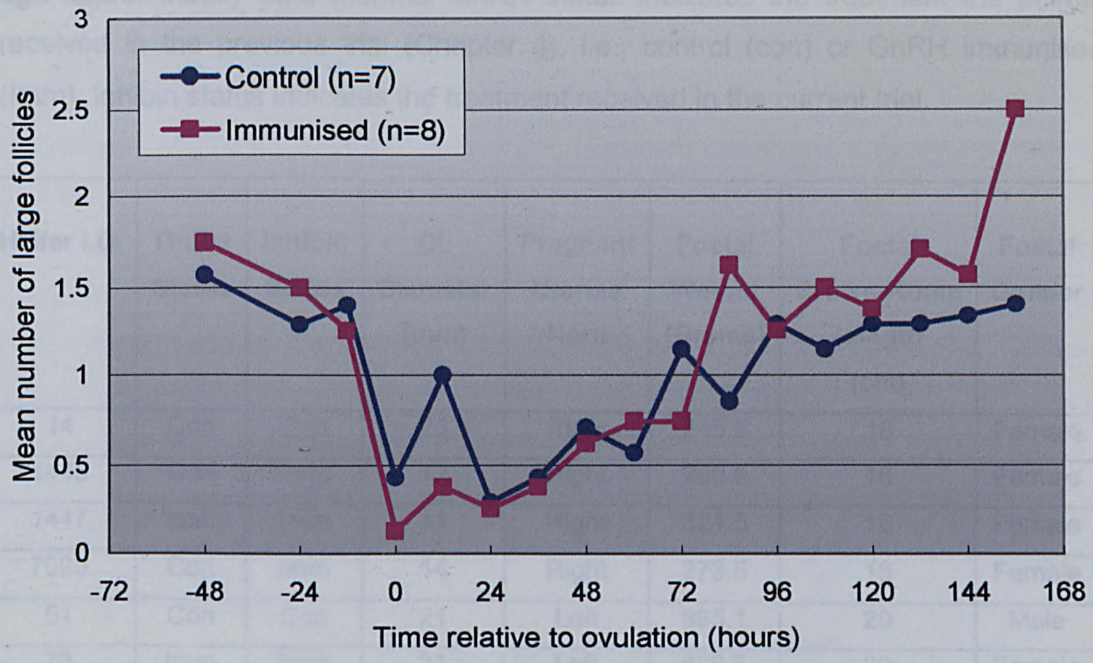


Figure 6.12 Mean number of large follicles in control and inhibin immunised heifers.



6.3.3 Twinning rate, conception rate and reproductive assessment

Inhibin immunisation had no significant effect on ovulation rate or CL number. The treatment status in this trial of 8 heifers, which conceived to fixed time artificial insemination following the 3rd injection of $PGF_{2\alpha}$, is presented in Table 6.1. In addition, the pregnancy status, CL diameter, day of gestation, foetal weights are also shown. The weight of ovaries was not comparable between heifers due to asynchrony of folliculogenesis at the time of slaughter, i.e., at time of slaughter heifers were at different stages of follicular waves, and the number and size of antral follicles and CLs affects ovarian weight. Foetuses with a crown-rump length of 16 cm were approximately 98 days old, other foetuses were ~112 days of age. Due to the small numbers of pregnant animals and practical considerations at slaughter, foetal weight analysis was not possible.

Table 6.1 Post-mortem results and immunisation status from eight pregnant heifers age approximately 22.5 months. GnRH status indicates the treatment the animal received in the previous trial (Chapter 4), i.e., control (con) or GnRH immunised (Imm). Inhibin status indicates the treatment received in the current trial.

Heifer I.D.	GnRH Status	Inhibin Status	CL Diameter (mm)	Pregnant Uterine Horn	Foetal Weight (Grams)	Foetal Crown-Rump Length (cm)	Foetal Gender
14	Con	Con	25	Right	245.8	16	Female
1445	Con	Imm	12	Right	260.8	16	Female
1447	Imm	Imm	11	Right	324.5	16	Female
7020	Con	Imm	14	Right	273.5	16	Female
51	Con	Con	21	Left	695.1	20	Male
73	Imm	Imm	21	Left	686.7	20	Female
39	Imm	Con	12	Left	700.8	20.5	Male
338	Imm	Con	23	Right	777.2	21	Male

6.4 DISCUSSION

The current study on the effects of passive immunisation against inhibin in heifers has provided some evidence that inhibin antibodies act at the ovarian level to enhance the recruitment of follicles, without a detectable increase in pituitary FSH release. Furthermore, the evidence suggests that while lower levels of inhibin antibodies are adequate to enhance recruitment and/or reduce the rate of atresia in medium-sized follicles. Higher inhibin antibody titres may be necessary to over-ride the strong dominant follicle selection mechanism in heifers. In addition, the results of this and a previous trial (Campbell, Gong and Webb, unpublished), demonstrate that a threshold inhibin antibody titre range is required to obtain a reproducible increase in ovulation rate on which future passive inhibin immunisation studies may be based. Finally, this approach of a bolus followed by subsequent small antiserum injections has successfully been shown to give a steady and consistent concentration of circulating inhibin antibody titres.

6.4.1 Follicle stimulating hormone response

In the current study there was no detectable increase in peripheral FSH concentrations following immunisation. Earlier studies report variations in the FSH

response to active inhibin immunisation. For example, some authors observed an elevation in peripheral FSH concentrations (Glencross, 1992; Glencross *et al.*, 1994; Scanlon *et al.*, 1993; Akagi *et al.*, 2002), while others reported no significant change in FSH (Schanbacher, 1989; Glencross *et al.*, 1992; Morris and Grealy, 1993; Scanlon *et al.*, 1993), or intermittent FSH increases (Morris, Grealy and Screenan, 1995a;b). Previous experiments involving passive inhibin immunisation of cattle have also reported an increase in peripheral FSH concentrations (Kaneko *et al.*, 1993; 1995; Takedomi *et al.*, 1997; Akagi *et al.*, 1997). Considerable variation in plasma FSH concentrations, both within and between animals, coupled with inadequate blood sampling regimes, has previously been blamed for the lack of detectable changes in FSH concentrations (Glencross *et al.*, 1994), however, we believe our sampling regime to have been adequate. Therefore variation is likely to be linked to follicle waves and the transient nature of the FSH rise in relation to the stage of folliculogenesis.

The peak FSH concentrations prior to ovulation (Akbar *et al.*, 1974; Schams and Schallenberger, 1976), the secondary FSH peak, which typically occurs 24 hours after an LH surge (Dobson, 1978; Webb *et al.*, 1980; see Peters, 1985), preceding follicle wave emergence (Adams *et al.*, 1992; Sunderland *et al.*, 1994), and the decrease in FSH around the time of selection (Sunderland et al., 1994) were undetectable because of the large variation in FSH concentrations between individuals. This large variation in FSH is probably due to asynchrony of follicle waves between animals. After ovulation follicle recruitment follows, however the time of ovulation relative to the 2nd $PGF_{2\alpha}$ injection, and hence recruitment varied between individuals by 2.5 days. We acknowledge that presenting FSH waves relative to an LH peak may have enabled us to detect any FSH differences that might have occurred, however, we did not measure LH in this study.

6.4.2 Follicle Response

Asynchrony of follicular waves in the current study led to a large variation in the time of antiserum administration relative to ovulation (range: -24 to +36 hours) and the emergence of the subsequent follicular wave. However, treatment effects included; a reduction in small follicles (36-72h), increased medium-sized follicles (0-48h), but no effect on large follicles or ovulation rates. These data may indicate that initially inhibin antibodies reduced the rate of atresia in medium-sized follicles and subsequently increased the number of recruited follicles. Treatment appears to have increased the numbers of small follicles, which made the transition into the medium-size category.

Interestingly, although the number of small follicles was similar in individual heifers, there were significant individual differences in the numbers of both recruited and dominant follicles (Figures 6.11 and 6.12). As inhibin antibody titres were similar in all animals, unlike previous active inhibin immunisation trials (Sunderland *et al.*, 1991; Scanlon *et al.*, 1993; Morris and Grealy, 1993; Mantovani *et al.*, 1997), this suggests that, individual animals (and/or follicles) respond differently to passive immunisation with anti-inhibin serum. However, the asynchrony between heifers, with respect to time of ovulation relative to the $PGF_{2\alpha}$ injection may be responsible for some of this variation. In contrast to earlier active inhibin immunisation studies (Price *et al.*, 1987b; Morris, McDermot and Screenan, 1991; Morris *et al.*, 1993; Morris, Grealy and Screenan, 1995; Sunderland *et al.*, 1991; Glencross, 1992; Glencross *et al.*, 1992; 1994; Scanlon *et al.*, 1993; Akagi *et al.*, 2002), no increase in ovulation rate was observed in the current study. However, one heifer exhibited a small and short-lived accessory CL (Figure 6.9).

6.4.3 Ovulation rate

Previous passive inhibin immunisation experiments (Takedomi *et al.*, 1997; Akagi *et al.*, 1997) report extreme variability and superovulatory responses when excessively high doses (75ml; Takedomi *et al.*, 1997; 37.5 and 50ml; Akagi *et al.*, 1997) of high antibody titre antiserum are administered (50% binding at 1: 96,000; Kaneko *et al.*, 1993) and large increases in peripheral FSH concentrations observed (Kaneko *et al.*, 1993; 1995; Takedomi *et al.*, 1997; Akagi *et al.*, 1997). The lack of an increased ovulation rate in the current study and the high incidence of twin ovulations in the previous (Campbell, Gong and Webb, unpublished) study, confirm that antiserum dose is critical to the physiological response.

Despite no affect of treatment on ovulation rate, evidence in the current and previous studies (Glencross *et al.*, 1992; Kaneko *et al.*, 1993; Bleach *et al.*, 1996) suggests that the stimulatory effects of inhibin antibodies on small and medium-sized follicles act at the ovarian level. Inhibin antibodies may enhance the responsiveness of small and medium-sized follicles to gonadotrophic drive (as discussed in Chapter 5) thus promoting development and reducing atresia, without a detectable increase in FSH. However, to over-ride the selection mechanism and reduce atresia in selected follicles, higher inhibin antibody titre (Akagi *et al.*, 1997) may be necessary and a small increase in FSH required. Indeed, earlier work (Kaneko *et al.*, 1993) supports the current findings that small and medium-sized follicles are more responsive to inhibin antibodies than large or dominant follicles. This may be because medium-

sized follicles are gonadotrophin dependent, but due to the lack of LH receptors they are totally FSH dependent. For example, after passive inhibin immunisation, when titres had returned to pre-treatment levels dominant follicle numbers, which had been increased (4.7 ± 0.9), returned to control levels within 24 hours. In contrast, small and medium-sized follicles continued to be increased relative to control heifers for several days (Kaneko *et al.*, 1993).

6.4.4 Inhibin Antiserum Dose and Inhibin Antibody Titres

The current passive immunisation protocol demonstrates that, inhibin antibody titres can be maintained at elevated levels, as reported in ewes (Campbell and Scaramuzzi, 1995). The small variations in antibody titres may be further reduced by the use of a herd of more uniform weight, or by varying the dose of antiserum according to weight of the animal, although this is unlikely to be practical. A problem with earlier studies (Kaneko *et al.*, 1993; 1995; Akagi *et al.*, 1997; Takedomi *et al.*, 1997) is that animal numbers are small (3-5 animals per treatment group), which makes comparisons between trial and groups within trials more difficult. However, when comparing the findings between studies (Akagi *et al.*, 1997, Campbell, Gong and Webb, unpublished and the current trial), it is clear that inhibin antibody titres in the current trial may have been too low. For example, at 1:30 plasma dilution, on Day 1-2 of the oestrous cycle, anti-inhibin titres were in the previous (Campbell, Gong and Webb, unpublished) and current studies about 62% and 29% binding, respectively (Figure 6.3). Antibody titres in the earlier trials (Kaneko *et al.*, 1993; 1995; Takedomi *et al.*, 1997; Akagi *et al.*, 1997) are not directly comparable to our study because of different assays used. Differences in persistence of titres around 3 days after oestrus, the breed of cattle used, and the protocol used might also be partly responsible for differences in ovulation rates and FSH responses to vaccination between trials. In the previous (Campbell, Gong and Webb, unpublished) and current studies, the bolus injection was given on the day of ovulation and daily boosters thereafter for 5 days, until a PG injection, to which all animals responded. This longer exposure to a steady level of inhibin α-subunit antibodies resulted in increased follicular growth. Furthermore changes, for example, in the activin: follistatin ratio (Tannetta *et al.*, 1998) or the expression of α and β-subunits, or inhibin dimer concentrations relative to α-subunits may have occurred during the more prolonged period of inhibin antibody in the current trial, relative to the earlier study (Akagi *et al.*, 1997). The more abrupt introduction of inhibin antibodies in the Akagi *et al.* (1997) trial may not have allowed the time for the aforementioned suggested changes to occur. The most obvious difference between the previous studies i.e., Akagi *et al.*,

(1997) and Campbell, Gong and Webb (unpublished) is the immunogen used (32kDa inhibin vs. α-C inhibin subunit). The choice of immunogen has been shown to affect the physiological response dramatically (Knight *et al.*, 1989; Scanlon *et al.*, 1993; see O'Shea *et al.*, 1994; Terqui *et al.*, 1995). Indeed, decreased variability and high proportion of twin ovulators in the UK study relative to the Japanese studies (Akagi *et al.*, 1997, Takedomi *et al.*, 1997) was observed. Furthermore, the current study provides further evidence to support the evidence (Scanlon *et al.*, 1993; Bleach *et al.*, 1996) that the effects of α-C subunit antibodies act at the ovarian level, although the exact mode(s) of action have yet to be elucidated.

The data indicates that inhibin α-subunit antibodies might act at the ovarian level to enhance follicle recruitment and/or reduce atresia. However, an inhibin immunisation effect mediated through FSH cannot be ruled out. An increase in twin ovulation rate may be achieved following this passive inhibin immunisation protocol if antibody titres in heifers are >30 and <65% binding at 1 in 30 dilution, and this treatment can be applied accurately at the correct stage of follicular recruitment.

6.4.5 Future work

To attain consistent double ovulations, an improvement in follicular wave synchronisation is required. This may be achieved by using a controlled intra-vaginal progesterone releasing device (CIDR) treatment regime or PG injections coupled with GnRH treatment. In addition, larger animal numbers are required and higher titre antiserum. A suggested alternative protocol may be a two-tier anti-inhibin titre protocol. A lower antiserum volume as in the current trial to increase recruited follicle number, without over stimulating the ovaries, followed by a higher titre antiserum (Campbell, Gong and Webb, unpublished), to overcome the mono-ovulatory drive and prevent atresia by increasing the number of follicles which are able to 'switch' their dependency from FSH to LH when FSH concentrations decline.

Future investigations into inhibin antibody modes of action at the ovarian level and the subsequent effects on prolificacy should adopt the use of *in vivo* physiological models. For example, using long-term GnRH agonists to inhibit pituitary gland gonadotrophin release, followed by exogenous FSH and LH injections to mimic the typical patterns of gonadotrophic release, and passive inhibin immunisation, the intra-ovarian role of inhibin antibodies may be elucidated. However, care must be taken to avoid over-stimulation of the ovaries with exogenous FSH. For a dose response study, see Murphy, Boland and Roche, (1998).

CHAPTER SEVEN

GENERAL SUMMARY AND DISCUSSION

The main finding of this thesis is that, neonatal active GnRH immunisation of calves did not permanently impair reproductive function, and with the exception of limited improvements to carcass classification, all inhibited characteristics were similar to control animals by 17 months (bulls; Chapter 3) and 22.5 months (heifers; Chapter 4). As discussed, these findings are in contrast to earlier GnRH immunisation trials on reproductive function in sheep (Brown and Mattner, 1992; Brown *et al.*, 1994; 1995; Clarke *et al.*, 1998) and pre- and post-pubertal zebu bulls (D'Occhio, Aspden and Trigg, 2001) were neonatal and early active immunisation protocols resulted in a permanent suppression of reproductive function in a gender specific proportion of animals. The timing, in relation to hypothalamic maturation, and magnitude of the elevation in numbers of unbound high affinity GnRH antibodies in the circulation (GnRH antibody titre) may be responsible for the lack of long-term physiological responses in the studies herein, compared with earlier trials. For example, antibody titres were greater in studies by Brown *et al.* (1994; ewes; 50% binding at 1:1,500-1:18,300) and Brown *et al.* (1995; rams; 50% binding at 1:2000-1:50,000) than those reported in Chapters 3 and 4. Future neonatal immunisation studies should adopt new approaches to ensure satisfactory immune responses and elevation in titres.

In chapter 4, we report on an "autoimmune response/titre rebound" to the early immunisation regime. The incorporation of a significant and predictable "self-booster" response into an immunisation protocol might be extremely useful when designing an immunisation protocol, which for practical reasons, must keep the number of booster vaccinations to a minimum. The cause of the observed elevation in titres, in the absence of a booster vaccination is unclear, and although we have discussed and speculated on the possible causes in Chapter 4, there is no direct evidence to indicate that there was an autoimmune response.

In addition, these two chapters (3 and 4) describe one of the most comprehensive studies on the acquisition of sexual maturity in bull and heifer calves and make a significant contribution to knowledge in this field.

The Chapter 5 study has confirmed and extended previous work showing that rbGH administration results in an increase in the number of <3.5mm antral follicles in sheep and that active inhibin immunisation results in an increase in the number of ≥3.5mm antral follicles. Preliminary evidence also suggests that the combination of these treatments results in earlier maturation of small antral follicles, it is unclear as to whether these treatments are independent and simply additive, or whether the effects are the result of a direct interaction of the two systems. On going immunohistochemical results may enable us to draw a stronger conclusion as to whether these two systems interact. Finally, the fact that these effects occurred independently of detectable changes in peripheral FSH concentrations suggests that they might be mediated, to some extent at least, via intra-follicular control mechanisms.

As with Chapter 5, passive inhibin immunisation in heifers (Chapter 6) did not result in a detectable increase in circulating FSH concentrations. Thus, this study has also provided some evidence that inhibin antibodies act at the ovarian level to enhance follicle recruitment. However, the key finding of this study comes from the comparison between it, and a previous trial (Campbell, Gong and Webb, unpublished). Evidence suggests that while lower levels of inhibin antibodies are adequate to enhance recruitment and/or reduce the rate of atresia in medium-sized follicles. Higher inhibin antibody titres may be necessary to over-ride the strong dominant follicle selection mechanism in heifers. Moreover, the results of this and a previous trial (Campbell, Gong and Webb, unpublished), demonstrate that a threshold inhibin antibody titre range is required to obtain a reproducible increase in ovulation rate, on which future passive inhibin immunisation studies may be based. Finally, this approach of a larger bolus antiserum injection followed by subsequent small antiserum injections has successfully been shown to give a steady and consistent concentration of circulating inhibin antibody titres.

BIBLIOGRAPHY

ABDEL-RAOUF, M. (1960). The postnatal development of the reproductive organs in bulls with special reference to puberty. *Acta Endocrinologic, (Suppl.)* **49**, 1-109.

ADA, G. (2003). The immunology of vaccination. In: *Vaccines*. Eds: S.A. Plotkin, W.A. Orenstein and P.A. Offit. Pg: 31-63.

ADAMS, T. E. and Adams, B.M (1990). Reproductive function and feedlot performance of beef heifers actively immunised against GnRH. *Journal of Animal Science*, **68**: 2793-2802.

ADAMS, T. E., Dunbar, J. R., Berry, S. L., Garrett, W. N., Famula, T. R. and Lee, Y. B. (1990). Feedlot performance of beef heifers implanted with Synovex-H: effect of melengestrol acetate, ovariectomy or active immunization against GnRH. *Journal of Animal Science*, **68**: 3079-3085.

ADAMS, T. E. and Adams, B.M (1992). Feedlot performance of steers and bulls actively immunized against gonadotropin-releasing hormone. *Journal of Animal Science*, 70: 1691-1698.

ADAMS, T. E., Daley, C. A., Adams, B. M. and Sakurai, H. (1993a). Testis function and feedlot performance of bulls actively immunized against gonadotrophin-releasing hormone: effects of implants containing progesterone and estradiol benzoate. *Journal of Animal Science*, **71**: 811-817.

ADAMS, T. E., Daley, C. A., Adams, B. M. and Sakurai, H. (1996). Testis function and feedlot performance of bulls actively immunized against gonadotrophin-releasing hormone: effect of age at immunization. *Journal of Animal Science*, **74**: 950-954.

ADAMS, G. P., Matteri, R. L., Kastelic, J. P., Ko, J. C. H. and Ginther, O. J. (1992). Association between surges of follicle-stimulating hormone and the emergence of follicular waves in heifers. *Journal of Reproduction and Fertility*, **94**, 177-188.

ADAMS, G. P., Kot, K., Smith, C. A. and Ginther, O. J. (1993b). Selection of a dominant follicle and suppression of follicular growth in heifers. *Animal Reproduction Science*, **30**; 259-271.

ADAMS, G. P., Kot, K., Smith, C. A. and Ginther, O. J. (1993c). Effect of the dominant follicle on regression of its subordinates in heifers. *Canadian Journal of Animal Science*, **73**, 267-275.

ADAMS, G. P., Evans, A. C. O. and Rawlings N. C. (1994). Follicular waves and circulating gonadotrophins in 8 month-old prepubertal heifers. *Journal of Reproduction and Fertility*, **100**; 27-33.

ADELMAN, J. P., Mason, A. J., Hayflick, J. S and Seeburg, P. H. (1986). Isolation of the gene and hypothalamic cDNA for the common precursor of gonadotrophin-releasing hormone and prolactin-releasing factor in the human and the rat. *Proceedings of the National Academy of Sciences of the United States of America*, **83**, 179-183.

ADKINS, B., Ghanei, K. and Hamilton, K. (1993). Developmental regulation of IL-4, IL-2 and IFN-gamma production by murine peripheral T lymphocytes. *Journal of Immunology*, **151**: 6617-6626.

AKAGI, S., Kaneko, H., Nakanishi, Y., Takedomi, T., Watanabe, G. and Taya, K. (1997). Ovarian response and FSH profile in cows following injection of various doses of inhibin antiserum. *Journal of Veterinary Medicine*, **55**: (12): 1129-1135.

AKAGI, S., Shi, F., Kaneko, H., Watanabe, G., Tsonis, C. G., Kazuyoshi, T. (2002). Ovarian Response and Hormonal Profiles in Heifers After Immunization and Re-immunization Against Inhibin α-Subunit. *Journal of Reproduction and Development*, **48** : (6): 600 - 605

AKBAR , A. M., Reichert Jr, L.E., Dunn, T.G, Kaltenbach, C. C. and Niswender, G. D. (1974). Serum levels of follicle-stimulating hormone during the bovine estrous cycle. *Journal of Animal Science*, **39**, 360-365. -

ALEXANDER, J. and Brewer, J. M. (1995). Adjuvants and their modes of action. *Livestock Production Science*, **42**, 153-162.

ALMQUIST, J. O. and Amann, R. P. (1961). Reproductive capacity of dairy bulls II. Gonadal and extra-gonadal sperm reserves, as determined by direct counts and depletion trials; dimensions and weight of genitalia. *Journal of Dairy Science*, **44**: 1668.

ALMQUIST, J. O. (1982). Effects of long term ejaculation at high frequency on output of sperm, sexual behaviour, and fertility of Holstein bulls; relation of reproductive capacity to high nutrient allowance. *Journal of Dairy Science*, **65**: 814.

AL-OBAIDI, S. A. R., Bindon, B. M., Findlay, J. K., Hillard, M. A. and O'Shea, T. (1987a). Plasma follicle stimulating hormone in Merino ewes immunised with an inhibin-enriched fraction from bovine follicular fluid. *Animal Reproduction Science*, **14**, 39-51.

AL-OBAIDI, S. A. R., Bindon, B. M., Hillard, M. A. and O'Shea, T. (1987b). Reproductive characteristics of lambs actively immunised early in life with inhibin-enriched preparations from follicular fluid of cows. *Journal of Reproduction and Fertility*, **81**, 403-414.

AMANN, R. P. (1983). Endocrine changes associated with onset of spermatogenesis in Holstein bulls. *Journal of Dairy Science*, **66**: 2606-2622.

AMANN, R. P. and Schanbacher, B. D. (1983). *Journal of Animal Science, (Supplement 2)*, **57**, 380.

AMANN, R. P. and Walker, O. A. (1983). Changes in the pituitary-gonadal axis associated with puberty in Holstein bulls. *Journal of Animal Science*, 433-442.

AMANN, R. P., Wise, W. E., Glass, J. D. and Nett, T. M. (1986). Prepubertal changes in the hypothalamic pituitary in Holstein bulls. *Biology of Reproduction*, **34**: 71-80.

ANDERSON, S.T., Bindon, B.M., Hillard, M.A. and O'Shea, T. (1996). Advancement of puberty in ewe lambs by active immunisation against inhibin early in life. *Animal Reproduction Science*, **44**: 111-125.

ANDREWS, W.W., Advis, J.P. and Ojeda, S. R. (1981). The maturation of oestradiol-negative feedback in female rats: evidence that the resetting of the hypothalamic "gonadostat" does not precede the first preovulatory surge of gonadotrophins. *Endocrinology*, **109**: 2022-2031.

ARAVINDAKSHAN, J.P., Honaromooz, A., Bartlewski, P.M., Beard, A.P., Pierson, R.C. and Rawlings, N.C. (2000). Gonadotrophin secretion in prepubertal bull calves born in spring and autumn. *Journal of Reproduction and Fertility*, **120**, 159-167.

ARMSTRONG, D. T. (1993). Recent advances in superovulation of cattle. *Theriogenology*, **39**, 7-24.

ARMSTRONG, D. G. and Webb, R. (1997). Ovarian follicular dominance: the role of intra-ovarian growth factors and novel proteins. *Reviews of Reproduction*, **2** :139-146.

ARMSTRONG, D.G., Baxter, G., Gutierrez C. G., Hogg, C. O., Glazyrin, A.L., Campbell, B. K., Bramley, T. A. and Webb, R. (1998). Insulin-like growth factor binding protein -2 and -4 mRNA expression in bovine ovarian follicles: effect of gonadotrophins and developmental status. *Endocrinology*, **139**, 2146-2154.

ARMSTRONG, D. G., Gutierrez, C. G., Baxter, G., Glazyrin, A. L., Mann, G. E., Woad, K. J., Hogg, C. O. and Webb, R. (2000). Expression of mRNA encoding IGF-I, IGF-II and type 1 IGF receptor in bovine ovarian follicles. *Journal of Endocrinology*, **165**: 101-113.

ARMSTRONG, D.G., Gong, J. G. and Webb, R. (2003). Interactions between nutrition and ovarian activity in cattle: physiological, cellular and molecular mechanisms. *Reproduction Supplement*, **61**: 403-414.

ASDELL, S. A. (1964) *Patterns of Mammalian Reproduction*. Cornell University Press, Ithaca, New York. Pgs 670.

ASHDOWN, R. R. (1960). Development of penis and sheath in the bull calf. *Journal of Agricultural Science*, **54**, 348.

ASHDOWN, R. R. (1962). Adherence of penis and sheath in beef calves at the time of castration. *Journal of Agricultural Science*, **58**, 71.

ASPDEN, W. J., Rodgers, R. J., Stocco, D. M., Scott, P. T., Wreford, N. G., Trigg, T. E., Walsh, J. and D'Occhio, M. J. (1998). Changes in testicular steroidogenic acute regulatory protein (STAR), steroidogenic enzymes and testicular morphology associated with increased testosterone secretion in bulls receiving the luteinizing hormone releasing hormone agonist deslorelin. *Domestic Animal Endocrinology*, **15**: (4): 227-238.

AUSTIN, E. J., Mihm, M., Evans, A. C. O., Knight, P. G., Ireland, J. J. and Roche, J. F. (2001). Alterations in intrafollicular regulatory factors and apoptosis during selection of follicles in the first follicular wave of the bovine estrous cycle. *Biology of Reproduction*, **64**: 839-848.

AWONIYI, C.A., Chandrashekar, V., Arthur, R.D., Schanbacher, B.D., Amador, A.G. and Favio, R. E. (1988a). Pituitary and leydig cell function in boars actively immunized against gonadotrophin-releasing hormone. *Journal of Reproduction and Fertility*, **84**: 295:302.

AWONIYI, C.A., Chandrashekar, V., Arthur, R.D., Schanbacher, B.D. and Favio, R.E. (1988b). Changes in testicular morphology in boars actively immunised against gonadotrophin hormone-releasing hormone. *Journal of Andrology*, **9**: 160-171.

BAIRD, D.T. (1983). Factors regulating the growth of the preovulatory follicle in the sheep and human. *Journal of Reproduction and Fertility*, **69**: 343:352.

BAIRD, D.T., Campbell, B. K., Mann, G. E. and McNeilly, A. S. (1991). Gonadotrophin control of follicular growth in the ewe. *Journal of Reproduction and Fertility*, (suppl.) **43**: 125-138.

BAIRD, D.T. and Smith, K.B. (1993). Inhibin and related peptides in the regulation of reproduction. *Oxford Reviews of Reproductive Biology*, **15**: 191-232.

BAIRD, D. T. and Campbell, B. K. (1998). Follicle selection in sheep with breed differences in ovulation rate. *Molecular and Cellular Endocrinology*, **145**: 89-95.

BAKER, J., Hardy, M. P., Zhou, J., Bondy, C., Lupu, F., Bellve, A. R. and Efstratiadis, A. (1996). Effects of an IGF-I gene null mutation on mouse reproduction. *Molecular Endocrinology*, **10**: 903-918.

BAME, J. H., Dalton, J. C., Degelos, S. D., Good, T. E. M., Ireland, J. L. H., Jimenez-Krassel, F., Sweeney, T., Saacke, R.G. and Ireland, J.J. (1999). Effects of long-term immunisation against inhibin on sperm output in bulls. *Biology of Reproduction*, **60**: 1360-1366.

BAO, B., Garverick, H. A., Smith, G.W., Smith M. F., Salfen, B. E. and Youngquist, R. S. (1997a). Expression of messenger RNA encoding 3β-hydroxysteroid dehydrogenase Δ^5-Δ^4 isomerase during recruitment and selection of bovine ovarian follicles: identification of dominant follicles by expression of 3β-HSD mRNA within the granulosa cell layer. *Biology of Reproduction*, **56**: 1466-1473.

BAO, B., Garverick, H. A., Smith, G.W., Smith M. F., Salfen, B. E. and Youngquist, R. S. (1997b). Changes in messenger RNA encoding LH receptor, cytochrome P450 side chain cleavage, and aromatase are associated with recruitment and selection of bovine ovarian follicles. *Biology of Reproduction*, **56**: 1158-1168.

BAO, B. and Gaverick, H. A. (1998). Expression of steroidogenic enzyme and gonadotrophin receptor genes in bovine follicles during ovarian follicular waves: A review. *Journal of Animal Science*, **76**: 1903-1921.

BARNES, M. A., Bierley, S. T., Halman, R. D. and Henricks, D. M. (1980). Follicle stimulating hormone, luteinising hormone and oestradiol-17β response in GnRH treated prepubertal Holstein heifers. *Biology of Reproduction*, **22**, 459-465.

BARRIOS, C., Brawand, P., Berney, M., Brandt, C., Lambert, P. H. and Siegrist, C. A. (1996). Neonatal and early life immune responses to various forms of vaccine antigens qualitatively differ from adult responses: predominance of a Th2-biased pattern which persists after adult boosting. *European Journal of Immunology*, **26**: 1489-96.

BARTH, A. D. and Oko, R. (1989). Abnormal morphology of bovine spermatozoa. Iowa State University Press, Ames, IA, pp. 130-266.

BASS, J. J., Peterson, A.J., Payne, E. and Jarnet, M.P. (1977). The effect of neonatal oestrogen treatment on plasma levels and behaviour in pre- and post-pubertal bulls. *Theriogenology*, **8**: 59-71.

BASS, J. J., McNeilly, A. S. and Moreton, H. E. (1979). Plasma concentrations of FSH and LH in entire and castrated prepubertal bull calves treated with GnRH. *Journal of Reproduction and Fertility*, **57**: 219

BAXTER, G., O'Shea, T., Campbell, B. K. and Webb, R. (1995). Effects of bovine follicular fluid (bFF) fractions, which delay oestrus in sheep and cattle, and proliferation and steroid production in cultured granulosa cells. *Journal of Reproduction and Fertility, Abstract Series*, **15**: *Abstract* 189.

BEACH, F. A. (1970). Coital behaviour in dogs. VI. Long-term effects of castration upon mating in the male. *Journal of Comparative Physiological Psychology*, **70**: 1-32.

BEARD, A. J., Savva, D., Glencross, R. G., McLeod, B. J. and Knight, P. G. (1989). Treatment of ovariectomized heifers with bovine follicular fluid specifically suppresses pituitary levels of FSH-β mRNA. *Journal of Molecular Endocrinology* **3**: 85-91

BELL, M., Daley, C. A., Berry, S. L. and Adams, T. E. (1997). Pregnancy status and feedlot performance of beef heifers actively immunised against gonadotrophin-releasing hormone. *Journal of Animal Science*, **75**,-1185-1189.

BERARDINELLI, J. G., Ford, J. J. and Christenson, R. K. and Anderson, L. L. (1984). Luteinising hormone secretion in ovariectomised gilts: effects of age, reproductive state and oestrogen replacement. *Journal of Animal Science*, **58**, 165-173.

BERCU, B.B., Jackson, I.M.D., Sawin, C.T., Saffaii, H. and Reichlin, S. (1977). Permanent impairment of testicular development after transient blockade of endogenous luteinising hormone releasing hormone in the neonatal rat. *Endocrinology*, **101**: (6): 1871-1879.

BERCU, B.B. and Jackson, I.M.D. (1980). Response of adult male rats to LH-RH after neonatal immunisation with antiserum to LH-RH. *Journal of Reproduction and Fertility*, **59**: 501-507.

BERGFELD, E. G. M., Kojima, F. N., Cupp, A. S., Wehrman, M. E., Peters, K. E., Garcia-Winder, M. and Kinder, J. E. (1994). Ovarian follicular development in prepubertal heifers is influenced by level of dietary intake. *Biology of Reproduction*, **51**, 1051-1057.

BERNARD, C., Vallet, J. P., Beland, R. and Lambert, R. D. (1983). Prediction of bovine ovulation by rapid radioimmunoassay for plasma LH. *Journal of Reproduction and Fertility*, **68**, 425-430.

BERNDTSON, W.E., Igboeli, G. and Parker, W. G. (1987). The number of Sertoli cells in mature Holstein bulls and their relationship to quantitative aspects of spermatogenesis. *Biology of Reproduction*, **37**, 60-67.

BICSAK, T. A., Tucker, E. M., Cappel, S., Vaughan, J., Rivier, J., Vale, W. and HSUEH, A. J. W. (1986). *Endocrinology*, **119**, 2711.

BILEZIKJIAN, L. M., Corrigan, A.Z., Blount, A.L. and Vale, W.W. (1996). Pituitary follistatin and inhibin subunit messenger ribonucleic acid levels are differentially regulated by local and hormonal factors. Endocrinology, 137: 4277-4284.

BILLINGHAM, R. E., Brent, L. and Medawar, P. B. (1953). Actively acquired tolerance of foreign cells. *Nature*, **172**: 603-606.

BINDON, B. M., O'Shea, T., Miyamoto, K. Hillard, M., Piper, L. R., Nethary, R. D. and Uphill, G. (1988). Superovulation in pubertal heifers immunised against ovine inhibin purified by

monoclonal antibody affinity chromatography. *Proceedings of Australian Society of Reproductive Biology*, **20**, *Abstract*, 28.

BINDON, B. M., Anderson, S. T., Cummins, L. J., Findlay, J. K., Hillard, M. A., O'Shea, T., Paull, D., Tsonis, C. G. and Wilkins, J. F. (1994). Manipulation of reproduction of sheep and cattle by vaccination against inhibin-related peptides. *Vaccines in Agriculture: Immunological Applications to Animal Health and Production*, pp 85-96 (Ed. P.R. Woods, P. Willadsen, J.E. Vercoe, R.M. Hoskinson and D. Demeyer) CSIRO Publications, Melbourne.

BISHOP, M. W. H. (1970). *Journal of Reproduction and Fertility, Supplement*, **12**, 65.

BISTER, J. L. and Paquay, R. (1983). Fluctuations in the plasma levels of the follicle-stimulating hormone during estrous cycle, anoestrus, gestation and lactation in the ewe: evidence for an endogenous rhythm of FSH release. *Theriogenology*, **19**: 565-82.

BISTER, J. L., Noel, B., Perrad, B. Mandiki, S. N. M., Mbayahaga, J. and Paquay, R. (1999). Control of ovarian follicle activity in the ewe. *Domestic Animal Endocrinology*, **17**: 315-328.

BLEACH, E.C L., Muttukrishna, S. Cunningham, F.J., Knight, P.G. and Glencross, R.G. (1996) Effect of inhibin immunisation using different synthetic peptide fragments of the bovine α_c –subunit on plasma anti – inhibin titres, plasma FSH concentrations and the incidence of multiple ovulations in heifers. *Animal Reproduction Science*, **41**: 1-12.

BLOCKLEY, M., Ade, B. and Galloway, D. B. (1978). Hormonal control of serving capacity in bulls. *Theriogenology*, **9**, 143-151.

BLOSS, R.E., Northam, J.I., Smith, L.W. and Zimbleman, R.G. (1966). Effects of oral melengestrol acetate on the performance of feedlot cattle. *Journal of Animal Science*, **25**, 1048.

BLOWEY, R. W. (1993). A veterinary book for dairy farmers (second edition). Farming Press, Ltd, United Kingdom, pp, 413-416.

BO, G.A., Adams, G.P., Pierson, R.A. and Mapletoft, R.J. (1995). Exogenous control of follicular wave emergence in cattle. *Theriogenology*, **43**: 31-40.

BOLAND, M.P., Goulding, D. and Roche, J.F. (1991). Alternative gonadotrophins for superovulation in cattle. *Theriogenology*, **35**, (1): 5-17.

BOLAND, M.P., Sunderland, S.J., Williams, D.H., Kane, M., Headon, D.R. and Roche, J.F. (1994). Effect of immunisation of ewes against α 1-26 inhibin fragment on antibody titres, ovulation and lambing rate. *Animal Reproduction Science*, **34**: 241-251.

BOLT, D. J. (1971). Changes in the concentration of luteinising hormone in plasma of rams following administration of oestradiol, progesterone or testosterone. *Journal of Reproduction and Fertility*, **24**: 435.

BONNEAU, M. and Enright, W. J. (1995). Immunocastration in cattle and pigs. *Livestock Production Science*, **42**: 193-200.

BOT, A., Antohi, S., Bot, S., Garcia-Sastre, A. and Bona, C. (1997). Induction of humoral and cellular immunity against influenza virus by immunization of newborn mice with plasmid bearing a hemagglutinin gene. *International Immunology*, **9**: 1641-1650.

BOT, A. and Bona, C. (2002). Genetic immunisation of neonates. *Microbes and Infection*, **4**:511-520.

BOULTON, M.I., Haley, C.S., Springbett, A.J. and Webb, R. (1995). The effect of the Booroola (Fec^B) gene on peripheral FSH concentrations and ovulation rates during oestrus, seasonal anoestrus and on FSH concentrations following ovariectomy in Scottish Blackface ewes. *Journal of Reproduction and Fertility*, **103**: 199-207.

BRAR, J. S., Johnson, D. W., Muscoplat, C. C., Shope, R. E. and Meiske, J. C. (1978). Maternal immunity to infectious bovine rhinotracheitis and bovine viral diarrhea viruses. *American Journal of Veterinary Research*, **60**: 1098-1101.

BRATTON, R.W., Musgrave, S. D., Dunn, H.O. and Foote, R.H. (1959). Causes and preventions of reproductive failures in dairy cattle. II. Influence of underfeeding and overfeeding from birth to 80 weeks of age on growth, sexual development, and semen production of Holstein bulls. *Cornell University Agricultural Experimental Station Bulletin* **940**.

BREMNER, W. J., Findlay, J. K., Lee, V. W. K., de Kretser, D. M. and Cummins, I. A. (1980). Feedback effects of the testis on pituitary responsiveness to luteinising-hormone releasing hormone infusions in the ram. *Endocrinology*, **106**: 329.

BRIMIJOIN, S. and Lennon, V. A. (1990). Autoimmune preganglionic sympathectomy induced by acetylcholine esterase antibodies. *Proceedings of National Academic Science, USA*, 87, 9630-9634.

BROOKS, J. and McNeilly, A. S. (1994). Regulation of GnRH receptor mRNA expression in the sheep. *Journal of Endocrinology*, **143**, 175-182.

BROOKS, A. N., McNeilly, A. S. and Thomas, G. B. (1995). Role of GnRH in the ontogeny and regulation of the fetal hypothalamo-pituitary-gonadal axis in sheep. *Journal of Reproduction and Fertility, Supplement*, **49**, 163-175.

BROOKS, A. N., Hagan, D. M., Sheng, C., McNeilly, A. S. and Sweeney, T. (1996). Prenatal gonadotrophins in the sheep. *Animal Reproduction Science*, **42**, 471-481.

BROWN, B. W. and Mattner, P. E. (1992). Anti GnRH immunisation in males early life. Proceedings 12th International Congress of Animal Reproduction. Vol. 3, 1198-1200.

BROWN, B. W., Mattner, P. E., Carroll, P. A., Holland, E. J. Paull, D. R., Hoskinson, R. M. and Rigby, R. D. G. (1994). Immunization of sheep against GnRH early in life: effects on reproductive function and hormones in rams. *Journal of Reproduction and Fertility*, **103**: 131-135.

BROWN, B. W., Mattner, P. E., Carroll, P. A., Hoskinson, R. M. and Rigby, R. D. G. (1995). Immunization of sheep against GnRH early in life: effects on reproductive function and hormones in ewes. *Journal of Reproduction and Fertility*, **103**: 131-135.

BURGER, G. H. (1988) Inhibin: definition and nomenclature. *Journal of Endocrinology* **117**: 159.

BURGUS, R. and Guilleman, R. (1970). Hypothalamic releasing factors. Annual Reviews of Biochemistry, **39**, 499-526.

CALDANI, M., Antoine, M., Batailler, M. and Duittoz, A. (1995). Ontogeny of GnRH systems. *Journal of Reproduction and Fertility (Suppl.)*, **49**:147-162.

CAMERON, V., Nishimura, E., Mathews, L., Lewis, K., Sawchenco, P. and Vale, W. (1994). Hybridization histochemical localization of activin receptor subtypes in rat brain, pituitary, ovary and testis. *Endocrinology*, **134**: 799-808.

CAMPBELL, B. K., Mann, G. E., McNeilly, A. S. and Baird, D. T. (1990) The pattern of ovarian inhibin estradiol and androstenedione secretion during the estrous cycle in the ewe. *Endocrinology*, **127**: 227-235.

CAMPBELL, B. K., Scaramuzzi, R. J., Evans, G. and Downing, J. A. (1991a). Increased ovulation rate in androstenedione-immune ewes is not due to elevated plasma concentrations of FSH. *Journal of Reproduction and Fertility*, **91**, 655-666.

CAMPBELL, B. K., Picton, H. M., Mann, G. E., McNeilly, A. S. and Baird, D. T. (1991b). Effect of steroid- and inhibin- free ovine follicular fluid on ovarian follicles and ovarian hormone secretion. *Journal of Reproduction and Fertility*, **93**, 83-96.

CAMPBELL, B. K., Gordon, B. M., Tsonis, C. G. and Scaramuzzi, R. J. (1995). The effect of acute immuno-neutralisation of inhibin in ewes during the early luteal phase of the oestrous cycle on ovarian hormone secretion and follicular development. *Journal of Endocrinology*, **145**: (3): 479-490.

CAMPBELL, B. K., Scaramuzzi, R. J. and Webb, R. (1995). Control of antral follicle development and selection in sheep and cattle. *Journal of Reproduction and Fertility, (suppl.)*, **49**; 335-350.

CAMPBELL, B. K. and Scaramuzzi, R. J. (1995). Effect of acute immunoneutralization of inhibin in ewes during late luteal phase of the oestrous cycle on ovarian hormone secretion and follicular development during the subsequent follicular phase. *Journal of Reproduction and Fertility,* **104**: 337-345.

CAMPBELL, B.K. and Webb, R.W. (1995). Evidence that inhibin has paracrine and autocrine actions in controlling ovarian function in sheep. *Journal of Reproduction and Fertility, Abstract*, **15**: 140.

CAMPBELL, B. K. and Scaramuzzi, R. J. (1996). The effect of arterial infusion of human recombinant inhibin and bovine follicular fluid on ovarian follicles and ovarian hormone secretion. *Journal of Endocrinology*, **148**; 531-540.

CAMPBELL, B. K., Scaramuzzi, R. J. and Webb, R. (1996). Induction and maintenance of oestradiol and immunoreactive inhibin production with FSH by ovine granulosa cells cultured in serum- free media. *Journal of Reproduction and Fertility,* **106**; 7-16.

CAMPBELL, B. K. (1997). Differential effects of insulin- like growth factor I (IGF-I) and II (IGF-II) on proliferation and differentiation of cultured sheep granulosa cells. *Journal of Endocrinology (suppl. Abstract)*, **152**; 236.

CAMPBELL, B.K., Engelhardt, H., McNeilly, A.S., Harkness, L.M., Fukuoka, M. and Baird, D.T. (1999a). Direct effect of ovine follicular fluid on ovarian steroid secretion and expression

of markers of cellular differentiation in sheep. *Journal of Reproduction and Fertility*, **117**: 259-269.

CAMPBELL, B. K., Dobson, H., Baird, D. T. and Scaramuzzi, R. J. (1999b). Examination of the relative role of FSH and LH in the mechanism of ovulatory follicle secretion in sheep. *Journal of Reproduction and Fertility*, **117**: 355-367.

CAMPBELL, B. K. (1999). The modulation of gonadotrophic hormone action on the ovary by paracrine and autocrine factors. *Reproduction in Domestic Animals*, **34** (3-4): 147-153.

CAMPBELL, B. K. and Baird, D. T. (2001). Inhibin A is a follicle stimulating hormone-responsive marker of granulosa cell differentiation, which has both autocrine and paracrine actions in sheep. *Journal of Endocrinology*, **169**: 333-345.

CAMPBELL, B.K., Baird, D. T., Souza, C.J.H. and Webb, R. (2003). The FecB (Booroola) gene acts at the ovary: in vivo evidence. *Reproduction*, **126**, 101-111.

CARRAGHER, J. F., Knight, T. W., Death, A. F., Fisher, A. D. and Matthews, L. R. (1997). Measures of stress and growth suppression in surgically castrated bulls. *Proceedings of the New Zealand Society of Animal Production*, **57**: 100-104.

CARSON, R. S., Findlay, J. K., Burger, H. G. and Trounson, A. O. (1979). Gonadotrophin receptors of the ovine ovarian follicle during follicular growth and atresia. *Biology of Reproduction*, **21**: 75-87.

CARSON, R. S., Findlay, J. K., Clarke, I. J. and Burger, H. G. (1981). Estradiol, testosterone and androstenendione in ovine follicular fluid during growth and atresia of ovarian follicles. *Biology of Reproduction*, **24**; 105-113.

CARSON, A. F., McCaughey, W. J. and Steen, R. W. J. (1994). Active immunisation of young male calves and post-pubertal bulls against LHRH with various conjugate doses. *Proceedings of the British Society of Animal Production*, (*winter meeting*), **65**: 1-2.

CATT, K. J., Loumaye, E., Wynn, P. B., Iwashua, M., Hirota, K., Morgan, R. O. and Chang, J. P. (1985). GnRH receptors and actions in the control of reproductive functions. *Journal of Steroid Biochemistry*, **23**: 677-689.

CHANDOLIA, R. K., Bartlewski, P. M., Omeke, B. C., Beard, A. P., Rawlings, N. C. and Pierson, R. A. (1997a). Ultrasonography of the developing reproductive tract in ram lambs: effects of a GnRH agonist. *Theriogenology*, **48**: 99-117.

CHANDOLIA, R. K., Honaramooz, A., Omeke, B. C., Beard, A. P. and Rawlings, N. C. (1997b). Assessment of development of the testes and accessory glands by ultrasonography in bull calves and associated endocrine changes. *Theriogenology*, **48**: 119-132.

CHANDOLIA, R. K., Evans, A. C. O. and Rawlings, N. C. (1997). Effect of inhibition of increased gonadotrophin secretion before 20 weeks of age in bull calves on testicular development. *Journal of Reproduction and Fertility*, **109**: 65-71.

CHANG, C.F., McFarland, S.Y. and Reeves, J.J. (1987). Active immunisation of heifers against prostaglandin F-2 α: Potential as a sterilisation vaccine. *Journal of Animal Science*, **64**: 1090-8.

CHAPPUIS, G. (1998). Neonatal immunity and immunisation in early age: lessons from veterinary medicine. *Vaccine*, **16**:14/15: 1468-1472.

CHICOTEAU, P., Thiombiano, D., Boly, H. and Cloe, C. (1990). Contribution to the study of puberty in Baoule cattle. *Revue d'Elevage et de Medecine Veterinaire des Pays Tropicaux*, **43**, 535-539.

CHRISTENSEN, C.R., Redmond, M.J. and Laarveld, B. (2000). Vaccination against follistatin affects reproductive potential in cycling gilts. Canadian *Journal of Animal Science*, **80**: 337-342.

CLARKE, I. J., Fraser, H. M. and McNeilly, A.S. (1978). Active immunisation of ewes against luteinising hormone releasing hormone, and its effects on ovulation and gonadotrophin, prolactin and ovarian steroid secretion. *Journal of Endocrinology*, **78**, 39-47.

CLARKE, I. J. and Cummins, J. T. (1982). The temporal relationship between gonadotrophin releasing hormone (GnRH) and luteinising hormone (LH) secretion in ovariectomised ewes. *Endocrinology*, **111**: 1737-1739.

CLARKE, I. J., Cummins, J. T. and de Kretser, D. M. (1983). Pituitary gland function after disconnection from direct hypothalamic influences in the sheep. *Neuroendocrinology*, **36**, 376-384.

CLARKE, I. J. and Cummins, J. T. (1984). Direct pituitary effects of estrogen and progesterone on gonadotrophin secretion in the ewe. *Neuroendocrinology*, **39**: 267-274.

CLARKE, I. J. and Cummins, J. T. (1985). GnRH pulse frequency determines LH pulse amplitude by altering the amount of releasable LH in the pituitary gland. *Journal of Reproduction and Fertility*, **73**: 425-431.

CLARKE, I. J., Findlay, J. K., Cummins, T. J. and Ewens, W. J. (1986). Effects of ovine follicular fluid on plasma FSH secretions in ovariectomized ewes to indicate the site of action of inhibin. *Journal of Reproduction and Fertility*, **77**: 575-585.

CLARKE, I. J., Brown, B. W., Tran, V. V., Scott, C. J., Fry, R., Millar, R. P. and Rao, A. (1998). Neonatal immunization against gonadotropin-releasing hormone (GnRH) results in diminished GnRH secretion in adulthood. *Endocrinology*, **139** (4): 2007-2014.

CLARKE, I. J., (2002). Two decades of measuring GnRH secretion. *Reproduction Supplement*, **59**, 1-13.

CLAUS, R. and Weiler, U. (1994). Endocrine regulation of growth and metabolism in the pig: a review. *Livestock Production Science*, **37**, 245-260.

CLAUS, R. (2003). Effect of active immunisation against gonadotrophin releasing hormone on anabolic hormones in boars. *Reproduction in Domestic Animals*, **38**, (4). *Abstract*, W4.2, pp; 321.

CLAYPOOL, L. E. and Foster, D. L. (1990) Sexual differentiation of the mechanism controlling pulsatile secretion of luteinising hormone contributes to sexual differences in the timing of puberty in sheep. *Endocrinology*, **126**, 1206-1215.

CLAYTON, R. N. and Catt, K. J. (1981). Gonadotrophin-releasing hormone receptors; characterisation, physiological regulation and relationship to reproductive function. *Endocrine Reviews*, **2**, 186-209.

CLAYTON, R. N. (1982). Gonadotrophin-releasing hormone modulation of its own pituitary receptors: evidence of biphasic regulation. *Endocrinology*, **111**, 152-161.

COHICK, W.S. (1998). Role of insulin-like growth factors and their binding proteins in lactation. *Journal of Dairy Science*, **81**: 1769-1777.

COLLINS, W. E., Inskeep, E. K., Dreher, W. H., Tyler, W. J. and Casida, L. E. (1962). Effect of age on fertility of bulls in artificial insemination. *Journal of Dairy Science*, **45**, 1015.

COOK, M. E and Pariza, M. (1998). The role of conjugated linoleic acid in health. *International Dairy Journal*, **8**: 459-462.

COOK, B. R., Popp, J. D., Kastelic, J. P., Robbins, S. and Harland, R. (2000). The effects of active immunisation against GnRH on testicular development, feedlot performance and carcass characteristics of beef bulls. *Journal of Animal Science*, **78**: 2778-2783.

CORRIE, J. E. T., Hunter, W. M. and Macpherson, J. S. (1981). A strategy for raioimmunoassay of plasma progesterone with use of a homologous-site 125I-labeled radioligand. *Clinical Chemistry*, **27**, (4): 594-599.

CRIM, L. W. and Geschwind, I. I. (1972). Patterns of FSH and LH secretion in the developing ram: the influence of castration and replacement therapy with testosterone proprionate. *Biology of Reproduction*, **7**, 47.

CROKER, K. P., Cox, R. I., Johns, M. A., Johnson, T. J., Roberts, D., Salerian, M. and Sunderman, F. (1988). Potential for Fecundin to influence the reproductive performance of Merino ewes in Western Australia. *Australian Journal of Biological Science*, **41**, 47-55.

CROWE, M. A., Enright, W. J. and Roche, J. F. (1995). Immunisation of heifers against prostaglandin F2α. *Livestock Production Science*, **42**:213-221.

CROWE, M. A., Enright, W.J., Boland, M. P. and Roche, J. F. (2001a). Folicular growth and serum follicle stimulating hormone (FSH) responses to recombinant bovine FSH in GnRH-immunised anoestrous heifers. Animal Science, 73, 115-122.

CROWE, M. A., Kelly, P., Draincourt, M. A., Boland, M. P. and Roche J. F. (2001b). Effects of follicle stimulating hormone on serum hormone concentrations, follicle growth and intrafollicular estradiol and aromatase activity in Gonadotrophin-releasing hormone immunized heifers. *Biology of Reproduction*, **64**: 368-374.

CUMMINS, L. J., O'Shea, T, Bindon, B. M., Lea, V. W. K. and Findlay, J. K. (1983). Ovarian inhibin content and sensitivity to inhibin in Booroola and control strain Merino ewes. *Journal of Reproduction and Fertility,* **67**: 1-7.

CUMMINS, L. J., O'Shea, T, Al-Obaidi, S. A. R., Bindon, B. M. and Findlay, J. K. (1986). Increase in ovulation rate after immunisation of Merino ewes with a fraction of bovine follicular fluid containing inhibin activity. *Journal of Reproduction and Fertility*, **77**, 365-372.

CUMMINS, L. J., O'Shea, T, and Bindon, B. M. (1986). Increased ovulation rates in cattle vaccinated with a partially purified fraction of ovine follicular fluid. *Proceedings of Australian Society of Reproductive Biology*, **18**, *Abstract*, 39.

CURRIE, B.L., Sievertsson, H., Bogentoft, C., Chang, J-K, Folkers, K., Bowers, C.Y., Doolittle, R.F. (1971). Data on structure of bovine LHRH by inactivation. *Biochemical and Biophysical Research Communications*, **42**, 1180-1184.

CURTIS, S. K. and Amann, R. P. (1981). Testicular development and establishment of spermatogenesis in Holstein bulls. *Journal of Animal Science*, **53**, 1645-1657.

CUSHMAN, R. A., Hedgpeth, V. S., Echternkamp, S. E. and Britt, J. H. (2000). Evaluation of numbers of microscopic and macroscopic follicles in cattle selected for twinning. *Journal of Animal Science*, **78**, 1564-1567.

DANZER, S.C., McMullen, N.T. and Rance, N.E. (2001). Testosterone modulates the dendritic architecture of arcuate neuroendocrine neurons in adult male rats. *Brain Research*, **890**: 78-85.

DAVIS, S. R., Smith, J. F. and Gluckman, P. D. (1990). Effects of growth hormone injections on ovulation rate in ewes. *Reproduction, Fertility and Development*, **2**: 173-178.

DAY, M. L., Imakawa, K., Garcia-Winder, M., Zalesky, D. D., Schanbacher, B. D., Kittok, R. J. and Kinder, J. E. (1984). Endocrine mechanisms of puberty in heifers: oestradiol negative feedback regulation of luteinizing hormone secretion. *Biology of Reproduction*. **31**, 332-341.

DAY, M. L., Imakawa, K., Wolf, P. L., Kittok, R. J. and Kinder, J. E. (1987). Endocrine mechanisms of puberty in heifers: role of hypothalamo-pituitary oestradiol receptors in the negative feedback of oestradiol on luteinising hormone secretions. *Biology of Reproduction*, **37**, 1054-1065.

DEJONG, F. H. and Sharpe, K. M. (1976) Evidence for inhibin like activity in bovine folicular fluid. *Nature* **263**: 71-72.

DE SILVA, M., Tjoelker, L., Chang, C. F. and Reeves, J. J. (1986). Ovarian response to active immunisation against luteinising hormone in the cow. *Theriogenology*, **26**, 89.

DESJARDINS, C. and Hafs, H. D. (1969). Maturation of bovine female genetalia from birth through puberty. Journal of Animal Science, 28, 502-507.

DIELEMAN, S. J., Bevers, M. M., Vos, P. L. A. M. and de Loos, F. A. M. (1993). PMSG/anti-PMSG in cattle: a simple and efficient superovulatory treatment? *Theriogenology*, **39**, 25-41.

DISKIN, M. G., McEvoy, T. G., Hickey, B. C. and Sreenan, J. M. (1987). More twins in the beef herd: impact on beef output and financial returns. *Farm and Food Research*, **10**, 4-6.

DIXIT, V.D., Yang, H., Udhayakumar, V. and Sridaran, R. (2003). GnRH alters the T-helper cytokine balance in the pregnant rat. *Biology of Reproduction*, **102**: in press.

DOBSON, H. (1978). Plasma gonadotrophins and oestradiol during oestrus in the cow. *Journal of Reproduction and Fertility*, **52**, 5-53.

DODSON, S. E., McLeod, B. J., Haresign, W., Peters, A. R. and Lamming, G. E. (1988). Endocrine changes from birth to puberty in the heifer. *Journal of Reproduction and Fertility*, **82**, 527-538.

D'OCCHIO, M. J., Schanbacher, B. D. and Kinder, J. E. (1982). Relationship between serum testosterone concentration and patterns of LH secretion in male sheep. *Endocrinology*, **110**, (5): 1547-1554.

D'OCCHIO, M. J., Gifford, D. R., Hoskinson, R. M., Weatherby, T., Flavel, P. F., Mattner, P. E. and Setchell, B. P. (1987). Reproductive hormone secreation and testicular growth in bull calves actively immunized against testosterone and oestradiol-17β. *Journal of Reproduction and Fertility*, **79**: 315-324.

D'OCCHIO, M. J., Fordyce, G., O'Leary, B. M. Trigg, T. E. and Lindsey, M. J. (1992) Suppression of fertility in female cattle under range conditions with an anti-GnRH vaccine. In 'Proceedings of the 12th International Congress on Animal Reproduction', 23-27 August 1992, The Hague, The Netherlands, Vol. 3, 1207-1209.

D'OCCHIO, M. J. (1993). Immunological suppression of reproductive function in male and female mammals. *Animal Reproductive Science*, 33: 345-372.

D'OCCHIO, M. J. (1994). Endocrine, gamete and conceptus antigens as targets for immunocontraceptive vaccines. *Vaccines in Agriculture- Reproduction*, Chapter 9.

D'OCCHIO, M. J., Aspden, W. J. and Whyte, T. R. (1996). Controlled, reversible suppression of estrous cycles in beef heifers and cows using agonists of gonadotrophin-releasing hormone. *Journal of Animal Science*, **74**: 218-225.

D'OCCHIO, M. J. and Aspden, W. J. (1999). Endocrine and reproductive responses of male and female cattle to agonists of gonadotrophin releasing hormone. *Journal of Reproduction and Fertility, suppl.* **54**: 101-114.

D'OCCHIO, M. J., Aspden, W. J. and Trigg, T. E. (2001). Sustained testicular atrophy in bulls actively immunised against GnRH: potential to control carcass characteristics. *Animal Reproduction Science*, **66**: 47-58.

DONG, J., Albertini, D.F., Nishimori, K., Kumar, T.R., Lu, N. and Matzuk, M.M. (1996). Growth differentiation factor 9 is required during early folliculogenesis. *Nature*, **383**: 531-535.

DOW, J. S. Jr., Moore, J. D., Bailey, C. M. and Foote, W. D. (1982). Onset of puberty in heifers of diverse breeds and crosses. *Journal of Animal Science*, **55**, 1041-1047.

DOWNING, J. A. and Scaramuzzi, R. J. (1991). Nutrient effects on ovulation rate, ovarian function and the secretion of gonadotrophin and metabolic hormones in sheep. *Journal of Reproduction and Fertility, Supplement*, **43**, 209-227.

DRAINCOURT, M. A., Fry, R. C., Clarke, L. J. and Cahill, L. P (1987). Follicular growth and regression during the 8 days after hypophysectomy in sheep. *Journal of Reproduction and Fertility*, **79**: 635-641.

DRAINCOURT, M. A., Hermier, D. and Hanrahan, J. P. (1996). Alterations in follicular function associated with selection on ovulation rate in Finn ewes. *Journal of Animal Science*, **74**: 199-210.

DRAINCOURT, M. A., Reynaud, K., Cortrindt, R. and Smitz, J. (2000). Roles of KIT and KIT LIGAND in ovarian function. *Reviews of Reproduction*, **5**: 143-152.

DRAINCOURT, M. A. (2001). Regulation of ovarian follicular dynamics in farm animals. Implications for manipulation of reproduction. *Theriogenology*, **55**: 1211-1239.

DRAINCOURT, M. A., Reynaud, K. and Smitz, J. (2001). Differences in follicular function of 3 month old calves and mature cows. *Reproduction*, **121**: 463 –474.

DUFOUR, J., Cahill, L. P. and Mauleon, P. (1979). Short- and long-term effects of hypophysectomy and unilateral ovariectomy on ovarian follicular populations in sheep. *Journal of Reproduction and Fertility*, **57**: 301-309.

DUNSHEA, F. R., Colantoni, C., Howard, K., McCauley, I., Jackson, P., Long, K. A., Lopaticki, S., Nugent, E. A., Simons, J. A., Walker, J. and Hennessy, D. P. (2001). Vaccination of boars with a GnRH vaccine (Improvac) eliminates boar taint and increase growth performance. *Journal of Animal Science*, **79**: 2524-2535.

DURANDY, A., De Saint Basilē, G., Lisowska-Grospierre, G., Gauchat, G. F., Forveille, M., Kroczek, R. A., Bonnefoy, A. anf Fisher, A. (1995). Undetectable CD40 ligand expression on T cells and low B cell response to CD40 binding agonists in human newborns. *Journal of Immunology*, **154**: 1560-1568.

DYKEMAN, D. A., Katz, L. S. and Foote, R. H. (1982). Behavioural characteristics of beef steers administered oestradiol, oestrone, testosterone, and dihydrotestosterone. *Journal of Animal Science*, **55**, 1303-1309.

ECHTERNKAMP, S. E., Gregory, K. E., Dickerson, G. E., Cundiff, L. V., Koch, R. M. and Van Vleck, L.D. (1990a) Twinning in cattle:II. Genetic and environmental effects on ovulation rate in pubertal heifers and postpartum cows and the effects of ovulation rate on embryonic survival. *Journal of Animal Science* **68**: 1877-1888.

ECHTERNKAMP, S. E., Spicer, L. J., Gregory, K. E., Canning, S. F. and Hammond, J. M. (1990b). Concentrations of insulin-like growth factor-I in blood and ovarian follicular fluid of cattle selected for twins. *Biology of Reproduction*, **43**: 8-14.

ECHTERNKAMP, S. E. (1992). Fetal development in cattle with multiple ovulations. *Journal of Animal Science* **70**: 2309-2321.

ECHTERNKAMP, S. E. (1993) Relationships between placental development and calf birth weight in beef cattle. *Animal Reproduction Science* **32**: 1-13.

ECHTERNKAMP, S. E., Howard, R. J., Roberts, A. J., Grizzle, J. and Wise, T. (1994). Relationships among concentrations of steroids, insulin-like growth factor I and insulin-like growth factor binding proteins in ovarian follicular fluid of beef cattle. *Biology of Reproduction*, **51**: 971-981.

ECHTERNKAMP, S. E. and Gregory, K. E. (1998). Increased ovarian follicular development in cattle selected for twin births. *Journal of Animal Science*, **76**, (1), 222.

ECHTERNKAMP, S. E. and Gregory, K. E. (1999a) Effects of Twinning on Gestation Length, Retained Placenta, and Dystocia. *Journal of Animal Science*, **77**: 39-47.

ECHTERNKAMP, S. E. and Gregory, K. E. (1999b) Effects of Twinning on Postpartum Reproductive Performance in Cattle Selected for Twin Births. *Journal of Animal Science*, **77**: 48-60..

ECKERY, D. C., Moeller, C. L., Nett, T. M. and Sawyer, H. R. (1994). Recombinant bovine somatotropin does not improve superovulatory response in sheep. *Journal of Animal Science*, **72**: 2425-2430

ELLENBERGER and Baum (1943). Handbuch der vergleichenden. *Anatomie der Haustiere*, 18th Edition, Zietzschmann, Eds: Ackerknecht and Grau. Springer-Verlag, New York.

ENGLE, T. E., Spears, J. W., Brown, T. T. and Lloyd, K. E. (1999). Effect of breed (Angus vs. Simmental) on immune function and response to a disease challenge in stressed steers and preweaned calves. *Journal of Animal Science*, **77**, 516-521.

EPPIG, J. J. (2001). Oocyte control of ovarian follicular development and function in mammals. *Reproduction*, **122**: 829-838.

ESKOLA, J. and Kayhty, H. (1998). Early immunisation with conjugate vaccines. *Vaccine*, **16**:14/15:1433-1438.

EVANS, A. C. O., Currie, W. D. and Rawlings, N. C. (1992). Effects of naloxone on circulating gonadotrophin concentrations in prepubertal heifers. *Journal of Reproduction and Fertility*, **96**, 847-855.

EVANS, A. C. O., Currie, W. D. and Rawlings, N. C. (1993). Opioidergic regulation of gonadotrophin secretion in the early prepubertal bull calf. *Journal of Reproduction and Fertility*, **99**: 45-51.

EVANS, A. C. O. and Rawlings, N. C. (1994). Effects of a long-acting gonadotrophin-releasing hormone agonist (leuprolide) on ovarian follicular development in prepubertal heifer calves. *Canadian Journal of Animal Science*, **74**: 649-656

EVANS, A. C. O., Adams, G. P. and Rawlings, N. C. (1994a). Endocrine and ovarian follicular changes leading up to the first ovulation in prepubertal heifers. Journal of *Reproduction and Fertility*, **100**, 187-194.

EVANS, A. C. O., Adams, G. P. and Rawlings, N. C. (1994b). Follicular and hormonal development in prepubertal heifers from 2 – 36 weeks of age. Journal of *Reproduction and Fertility*, **102**, 463-470.

EVANS, A. C. O., Davies, F. J., Nasser, L. F., Bowman, P. and Rawlings, N. C. (1995). Differences in early patterns of gonadotrophin secretion between early and late maturing bulls and changes in semen characteristics at puberty. *Theriogenology*, **43**:569-578.

EVANS, A. C. O., Pierson, R.A., Garcia, A., McDougall, L.M., Hrudka, F. and Rawlings, N.C. (1996). - Changes in circulating hormone concentrations, testes histology and testes ultrasonography during sexual maturation in beef bulls. *Theriogenology*, **46**: 345-357.

EVANS, A. C. O., Duffy, P., Hynes, N. and Boland, M. P. (1999). Waves of follicle development during the estrous cycle in sheep. *Theriogenology*, **53**: 699-715.

EVANS, A. C. O. and O'Doherty, J. V. (2001). Endocrine changes and management factors affecting puberty in gilts. *Livestock Production Science*, **68**: 1-12.

EVANS, N. P., Land, R. B., McNeilly, J. R. and Webb, R. (1991). Role of gonadal negative feedback on the gonadotrophin responses to gonadotrophin-releasing hormone (GnRH) in

ram lambs from two lines of sheep selected for their luteinizing hormone response to GnRH. *Journal of Reproduction and Fertility*, **93**: 549-558.

EVANS, N.P., McNeilly, J.R. and Webb, R. (1994). Alterations in endogenous gonadotrophin secretion and pituitary responsiveness to gonadotrophin-releasing hormone in adult ewes, following indirect selection in prepubertal male lambs. *Biology of Reproduction*, **51**, 913-919.

EVANS, N. P., Dahl, G. E., Padmanabhan, V., Thrun, L. A and Karsch, F. J. (1997). Estradiol requirements for induction and maintenance of the GnRH surge: implications for neuroendocrine processingof the oestradiol signal. *Endocrinology*, **138**: 5408-5414.

EVANS, N. P., Richter, T. A., Skinner, D. C. and Robinson, J. E. (2002). Neuroendocrine mechanisms underlying the effects of progesterone on the oestradiol-induced GnRH/LH surge. *Reproduction Supplement*, **59**, 57-66.

FABRE, S., Pierre, A., Pisselet, C., Mulsant, P., Lecerf, F., Pohl, J., Monget, P. and Monniaux. (2003). The Booroola mutation in sheep is associated with an alteration of the bone morphogenetic protein receptor-IB functionality. *Journal of Endocrinology*, **177**, 435-444.

FARNWORTH, P.G. (1995). Gonadotrophin secretion revisited. How many ways can FSH leave a gonadotroph? *Journal of Endocrinology*, **145**, 387-395.

FAVLO, R. E., Chandrashakar, V., Arthur, R. D., Kuenstler, A. R., Hasson, T., Woniyi, C. and Schanbacher, B. D. (1986). Effects of active immunisation against LHRH or LH in boars: reproductive consequences and performance traits. *Journal of Animal Science*, **63**, 986-994.

FERRO, V. A., Mohammad, A. H. Khan, V. S., Brown, D., Urbanski, H. F. and Stimson, W. H. (2001). Immunoneutralisation of GnRH-I, without cross-reactivity to GnRH-II, in the development of a highly specific anti-fertility vaccine for clinical and veterinary use. *Journal of Reproductive Immunology*, **51**, 109-129.

FERRO, V. A., Mohammad, A. H. Khan, E.R., Earl, M., Harvey, M. J. A., Colston, A. and Stimson, W. H. (2002a). Influence of carrier protein conjugation site and terminal modification of a GnRH-I peptide sequence in the development of a highly specific anti-fertility vaccine. Part I. *American Journal of Reproductive Immunology*, **48**: 361-371.

FERRO, V. A., Harvey, M. J. A., Colston, A. and Stimson, W. H. (2002b). Part II: Influence of dimerization of a modified GnRH-I peptide sequence on a male antifertility vaccine. *American Journal of Reproductive Immunology*, **48**: 372-380.

FIELD, R. A. (1971). Effect of castration on meat quality. Journal of Animal Science, 32, 849-858.

FINDLAY, J. K. and Cumming, L. A. (1976) FSH in the ewe: effects of season, liveweight and plane of nutrition on plasma FSH and ovulation rate. *Biology of Reproduction*, **15**: 335-342

FINDLAY, J. K. and Clarke, I. J. (1987) Regulation of the secretion of FSH in domestic ruminants. *Journal of Reproduction and Fertility (suppl.)* **34**: 27-37

FINDLAY, J. K., Doughton, B., Robertson, D. M. and Forage, R. G. (1989). Effects of immunisation against recombinant bovine α-subunit on circulating concentrations of gonadotrophins in ewes. *Journal of Endocrinology*, **120**: 59-65.

FINDLAY, J. K., Doughton, B. W., Tsonis, C. G., Brown, R. W., Hungerford, J. W., Greenwood, P. E. and Forage, R. G. (1993). Inhibin as a fecundity vaccine. *Animal Reproduction Science*, **33**: 325-343.

FINDLAY, J. K., Drummond, A. E., Dyson, M.L., Baille, D. M., Robertson, J. F. Ethier., (2003). Recruitment and development of the follicle: the roles of the transforming growthfactor-β superfamily. *Molecullar and Cellular Endocrinology*, **191**: 35-43.

FINNERTY, M., Enright, W. J., McArthur, A. and Roche, J. F. (1991). Luteinizing secretion in bulls with different antibody titres against gonadotrophin releasing hormone (GnRH), induced by active immunisation. *Journal of Reproduction and Fertility, (Abstr.)* **7**: 37.

FINNERTY, M., Enright, W. J., Morrison, C. A. and Roche, J. F. (1994). Immunisation of bull calves with a GnRH analogue-human serum albumin conjugate: effect of conjugate dose, type of adjuvant and booster interval on immune, endocrine and testicular growth responses. *Journal of Reproduction and Fertility,* **101**: 333-343.

FINNERTY, M., Enright, W. J., Prendville, D. J., Spicer, L. J., Crowe, M. A. and Roche, J. F. (1995). Immunisation of post-pubertal bulls against gonadotrophin releasing hormone (GnRH): effect on hormones, behaviour and performance. *Abstracts: Agricultural Research Forum; Proceedings of the Irish Grassland and Animal Production, 21st Meeting*, 101-102.

FINNERTY, M., Enright, W. J., Prendville, D. J., Spicer, L. J. and Roche, J. F. (1996). The effect of different levels of gonadotrophin-releasing hormone antibody titres on plasma hormone concentrations, sexual and aggressive behaviour, testis size and performance of bulls. *Animal Science*, **63**: 51-63.

FINNERTY, M., Enright, W. J. and Roche, J. F. (1997). Immune response, testes function, body growth and carcass characteristics of bulls immunised against GnRH at 3 to 11 months of age. *Journal of Animal Science*, **75**: (1): 307 –308.

FINNERTY, M., Enright, W.J. and Roche, J.F. (1998). Testosterone, LH and FSH episodic secretory patterns in GnRH-immunized bulls. *Journal of Reproduction and Fertility*, **114**: 85-94.

FISHER, A.D., Crowe, M.A., Alonso de la Varga, M.E. and Enright, W.J. (1996). Effect of castration method and the provision of local anesthesia on plasma cortisol, scrotal circumference. Growth, and feed intake of bull calves. *Journal of Animal Science*, **74**: 2336-2343.

FOOTE, R. H. (1975). Oestrus detection and oestrus detection aids. *Journal of Dairy Science*, **58**: 248-256.

FOOTE, R. H. Munkenbeck, N and Green, W. A. (1976) Testosterone and libido in Holstein bulls of various ages. *Journal of Dairy Science*, **59**, 2011-2013.

FOOTE, R. H. (1978). *Journal of Animal Science*, **47**, (2): 1.

FORAGE, R. G., Brown, R. W., Oliver, K. J., Atrache, B. T., Devine, P. L., Hudson, G. C., Goss, N. H., Bertram, K. C., Tolstoshev, P., Robertson, D. M., de Kretser, D. M., Doughton, B., Burger, H. G. and Findlay, J. K. (1987a). Immunisation against an inhibin subunit

produced by recombinant DNA techniques results in increased ovulation rate in sheep. *Journal of Endocrinology*, 114, R1-R4.

FORAGE, R. G., Brown, R. W., Ring, J. M., Stewart, A. G., Milborrow, H. M., Oliver, K. J., Atrache, B. T., Devine, P. L., Hudson, G. C., Goss, N. H., Tolstoshev, P., Robertson, D. M., Doughton, B., de Kretser, D. M., Burger, H. G. and Findlay, J. K. (1987b). The clonoing and expression of inhibin genes: subunit use as a fecundity vaccine. In: Inhibin-Non-steroidal Regulation of Follicle Stimulating Hormone Secretion, pp. 89-103 (Eds. H. G. Burger, J. K. Findlay, D. M. de Kretser and M. Igarashi) Ares-Serono Symposium Series No. 42, Raven Press, New York.

FORAGE, R. G., Tsonis, C. G., Brown, R. W., Hungerford, J. W., Greenwood, P. E. and Findlay, J. K. (1993). Inhibin: a novel fecundity vaccine. In: *Animal health and production for the 21st century.* Ed; K. J. Beh. © CSIRO Australia. Chapter 14: pg 122-130.

FORD, J. J. and D'Occhio, M. J. (1989). Differentiation of sexual behaviour in cattle, sheep and swine. *Journal of Animal Science*, **67**, 1816-1823.

FORDYCE, G., Hodge, P. B., Beaman, N. J., Laing, A. R., Campero, C. and Shepherd, R. K. (1989). An evaluation of calf castration by intra-testicular injection of a lactic acid solution. *Australian Veterinary Journal*, **66**, 272-276.

FORDYCE, G., Jubb, T. F., Fitzpatrick, L. A., Whyte, T. R., Cooper, N. J., Bolam, M. J., Haddon, D. J., Hill, F. & D'Occhio, M. J. (2001). Contraceptive efficiency of an intra-uterine device in Brahman cattle. *Animal Reproduction Science*, **65**; 193-204.

FORTUNE, J. E. (2003). The early stages of follicular development: activation of primordial follicles and growth of pre-antral follicles. *Animal Reproduction Science*, **78**: 135-163.

FOSTER, D.L., Cruz, T.A.C., Jackson, G.L., Cook, B. and Nalbandov, A.V. (1972). Regulation of LH in the foetal and neonatal lamb. III. Release of LH by the pituitary in Vivo in response to crude ovine hypothalamic extraction or purified porcine gonadotrophin releasing factor. *Endocrinology*, **90**:3: 673-683.

FOSTER, D. L., Lemons, J. A., Jaffe, R. B. and Niswender, G. D. (1975). Sequential patterns of circulating luteinising hormone and follicle stimulating hormone in the female sheep from early postnatal life through the first oestrous cycle. *Endocrinology*, **97**, 985-994.

FOSTER, J. P., (1978). Plasma LH concentrations after single or double injections of LH-RH in dairy cows. *Journal of Reproduction and Fertility*, **54**, 119-121.

FOSTER, D. L. and Ryan, K. D. (1979). Endocrine mechanisms governing transition into adulthood: a marked decrease in inhibitory feedback action of oestradiol on tonic secretion of luteinising hormone in the lamb during puberty. *Endocrinology*, **105**, 896-904.

FOSTER, D. L. and Ryan, K. D. (1981) Endocrine mechanisms governing transition into adulthood in the female sheep. *Journal of Reproduction and Fertility, (Suppl.)* **30**: 75-90.

FOSTER, D.L., Ryan, K. D. and Papkoff, H. (1984). Hourly administration of luteinising hormone induces ovulation in prepubertal female sheep. *Endocrinology*, **115**, 1179-1185.

FOSTER, D. L., Karsch, F. J., Olster, D. H., Ryan, K. D. and Yellon, S. M. (1986). Determinants of puberty in a seasonal breeder. Recent Progress in Hormone Research, 42, 331-384.

FRANCHIMONT, P., Chari, S., Hangelstein, M. T. and Duraiswami, S. (1975). Existence of a follicle stimulating hormone inhibiting factor "inhibin" in bull seminal plasma. *Nature*, **257**, 402-404.

FRASER, H. M., Gunn, A., Jeffcoate, S. L. and Holland, D. T. (1974). Effect of active immunisation to luteinizing releasing hormone on serum and pituitary gonadotrophins, testes and accessory sex organs in the male rat. *Journal of Endocrinology*, **63**, 399.

FRASER, H. M. (1980). Inhibition of reproductive function by antibodies to luteinizing hormone releasing hormone. In: Immunological aspects of reproduction and fertility control. Ed; J. P. Hearn. MTP Press Ltd. Chapter **6**, pp; 143-171.

FRASER, H. M., Popkin, R. M., McNeilly, A. S. and Sharpe, R. M. (1982). Changes in pituitary LHRH receptor levels in situations of increased or decreased gonadotrophin secretion in the male rat. *Molecular and Cellular Endocrinology*, **28**, 321-333.

FRASER, H. M. and McNeilly, A. S. (1983). Differential effects of LH-RH immunoneutralization on LH and FSH secretion in the ewe. *Journal of Reproduction and Fertility*, **69**: 569-577.

FRAY, M. D., Wrathall, J. H. M. and Knight, P. G. (1994). Active immunisation against inhibin promotes a recurrent increase in litter size in sheep. *Veterinary Record*, **134**, 19-20.

FRY, R. C., Cahill, L. P., Cummins, J. T., Bindon, B. M., Piper, L. R. and Clarke, I. J. (1987). The half-life of follicle-stimulating hormone in ovary intact and ovariectomized Booroola and control Merino ewes. *Journal of Reproduction and Fertility*, **81**: 611-615.

FRY, R.C. and Draincourt, M. A. (1996). Relationship between Follicle Stimulating Hormone, follicle growth and ovulation rate in sheep. *Reproduction Fertility and Development*, **8**: 279-286.

FUKUDA, M., Miyamoto, K., Hasegawa, Y., Nomura, M., Igarashi, M., Kangawa, K. and Matsuo, H. (1986). Isolation of bovine follicular fluid inhibin of about 32kDa. *Molecular and Cellular Endocrinology*, **44**, 55-60.

FULTON, R.W., Briggs, R.E., Payton, M.E., Confer, A.W., Saliki, J.T., Ridpath, J.F., Burge, L.J. and Duff, G.C. (2004). Maternally derived humoral immunity to bovine viral diarrhea virus (BVDV) 1a, BVDV1b, BVDV2, bovine herpesvirus-1, parainfluenza-3 virus bovine respiratory syncytial virus, Mannheimia haemolytica and Pasteurella multocida in beef calves, antibody decline by half-life studies and effect on response to vaccination. *Vaccine*, **22**:643-649.

GALLOWAY, S. M., Gregan, S. M., Wilson, T., McNatty, K. P., Juengel, J. L., Ritvos, O. and Davis, G. H. (2002). BMP 15 mutations and ovarian function. *Molecular and Cellular Endocrinology*, **00**, 1-4.

GALYEAN, M. L., Perino, L. J. and Duff, G. C. (1999). Interaction between cattle health/immunity and nutrition. *Journal of Animal Science*, **77**: 1120-1134.

GANONG, W. F. (1996). The gonads: development and function of the reproductive system. In: *Review of Medical Physiology*, 18th edition, pp. 408. (Eds W, F. Ganong). Appleton & Lange, Stamford, Connecticut.

GANGWAR, P. D., Branton, C. and Evans, D. L. (1965). Reproductive and physiological responces of Holstein Heifers to controlled and natural climate conditions. *Journal of Dairy Science*, **48**: 222-227.

GARNIER, D. H., Terqui, M. and Pelletier, J. (1977). Plasma concentrations of LH and testosterone in castrated rams treated with testosterone or testosterone proprionate. *Journal of Reproduction and Fertility*, **49**, 359.

GAVERICK, H. A., Baxter, G., Gong, J., Armstrong, D. G., Campbell, B. K., Gutierrez, C. G. and Webb, R. (2002). Regulation of expression of ovarian mRNA encoding steroidogenic enzymes and gonadotrophin receptors by FSH and GH in hypogonadotrophic cattle. *Reproduction*, **123**(5): 651-661.

GESCHWIND, I. I. and Dewey, F. (1968). Dynamics of luteinizing hormone (LH) secretion in the cycling ewe: a radioimmunoassay study. *Proceedings of the Society of Experimental Biology and Medicine*, **129**: 451-455.

GETTYS, T. W., D'Occhio, M. J., Hendricks, D. M. and Schanbacher, B. D. (1984). Suppression of LH secretion by oestradiol, dihydrotestosterone, and trenbolone acetate in the acutely castrated bull. *Journal of Endocrinology*, **100**, 107-112.

GIBBONS, J. R., Kot, K., Thomas, D. L., Wiltbank, M. C. and Ginther, O. J. (1999). Follicular and FSH dynamics in ewes with a history of high and low ovulation rates. *Theriogenology*, **52**: 1005-1020.

GINTHER, O.J., Kot, K and Wiltbank, M.C. (1995). Association between emergence of follicular waves and fluctuations in FSH concentrationsduring the oestrous cycles in ewes. *Theriogenology*, **43**: 689-703.

GINTHER, O.J., Wiltbank, M. C. Fricke, P.M., Gibbons, J.R. and Kot, K. (1996). Selection of the dominant follicle in cattle. *Biology of Reproduction*, **55**, 1187-1194.

GINTHER, O. J., Bergfelt, D. R., Kulick, L. J. and Kot, K. (2000). Selection of the dominant follicle in cattle: role of oestradiol. *Biology of Reproduction*, **63**, 383-389.

GINTHER, O. J., Beg, M. A., Bergfelt, D. R. and Kot, K. (2002). Activin A, estradiol and free insulin-like growth factor I in follicular fluid proceding the experimental assumptions of follicle dominance in cattle. *Biology of Reproduction*, **67**: 14-19.

GLENCROSS, R. G. (1992). Effect on reproductive function in sheep and cattle of inhibin immunoneutralisation. *Annales de Zootechnie*, **41**: 287-290.

GLENCROSS, R. G., Bleach, E. C. L., McLeod, B. J., Beard, A. J. and Knight, P. G. (1992). Effect of active immunisation of heifers against inhibin on plasma FSH concentrations, ovarian follicular development and ovulation rate. *Journal of Endocrinology*, **134**, 11-18.

GLENCROSS, R. G., Bleach, E. C. L., Wood, S. C. and Knight, P. G. (1994). Active immunisation of heifers against inhibin: effects on plasma concentrations of gonadotrophins,

steroids and ovarian follicular dynamics during prostaglandin-synchronised cycles. *Journal of Reproduction and Fertility*, **100**, 599-605.

GLEZEN, W.P. (2003). Effect of maternal antibodies on infant immune response. *Vaccine*, 21:3389-3392.

GLISTER, C., Tannetta, D. S., Groome, N. P. and Knight, P. (2001). Interactions between follicle stimulating hormone and growth factors in modulating secretion of steroids and inhibin-related peptides by nonluteinized bovine granulosa cells. *Biology of Reproduction*, **65**: 1020-1028.

GLISTER, C., Morgan, A. L. and Knight, P. (2002). Bone morphogenetic protein (BMP)-mediated modification of steroid and inhibin-related peptide secretion by non-luteinized bovine granulosa cells. *Reproduction Abstracts Series* **28**:70.

GODFREY, R. W., Lunstra, D. D. and Schanbacher, B. D. (1992). Effect of implanting bull calves with testosterone proprionate, dihydrotesterone proprionate or oestradiol-17β prepubertally on the pituitary-testicular axis and on postpubertal social and sexual behaviour. *Journal of Reproduction and Fertility*, **94**, 57-69.

GODINE, J. E., Chin, W. W. and Habener, J. F. (1980). Luteinizing and follicle stimulating hormones. Cell-free translations of messenger RNAs coding for subunit precursors. *Journal of Biological Chemistry*, **225**: 8780-8783.

GOLDMAN, B. D., Quadagno, D. M., Shryne, J. and Gorski, R. A. (1972). Modification of phallus development and sexual behaviour in rats treated with gonadotrophin antiserum neonatally. *Endocrinology*, **90**, 1025.

GONG, J. G., Bramley, T. A. and Webb, R. (1991). The effect of recombinant bovine somatotropin on ovarian function in heifers: Follicular populations and peripheral hormones. *Biology of Reproduction*, **45**: 941-949.

GONG, J. G., Bramley, T. A. and Webb, R. (1993). The effect of recombinant bovine somatotropin on ovarian follicular growth and development in heifers. *Journal of Reproduction and Fertility*, **97**: 247-254.

GONG, J. G., Bramley, T. A., Wilmut, I. and Webb, R. (1993). The effect of recombinant bovine somatotropin on superovulatory response to pregnant mare serum gonadotropin in heifers. *Biology of Reproduction,* **48**: 1141-1149.

GONG, J. G., Bramley, T. A., Gutierrez, C. G., Peters, A. R. and Webb, R. (1995). Effects of chronic treatment with a gonadotrophin-releasing hormone agonist on peripheral concentrations of FSH and LH, and ovulation function in heifers. *Journal of Reproduction and Fertility*, **105**: 263-270.

GONG, J. G., Campbell, B. K., Bramley, T. A., Gutierrez, C. G., Peters, A. R. and Webb, R. (1996a). Suppression in the secretion of follicle- stimulating hormone and luteinising hormone, and ovarian follicle development in heifers continuously infused with a gonadotropin- releasing hormone agonist. *Biology of Reproduction,* **55**: 68-74.

GONG, J. G., Campbell, B. K., Bramley, T. A. and Webb, R. (1996b). Treatment with recombinant bovine somatotrophin enhances ovarian follicular development and increases

the secretion of insulin- like growth factor- I by ovarian follicle in ewes. *Animal Reproduction Science*, **41**: 13-26.

GONG, J. G., Wilmut, I., Bramley, T. A. and Webb, R. (1996c). Pre-treatment with recombinant bovine somatotropin enhances the superovulatory response to FSH in heifers. *Theriogenology*, 45: 611-622.

GONG, J. G., Baxter, G., Bramley, T. A. and Webb, R. (1997). Enhancement of ovarian follicular development in heifers by treatment with recombinant bovine somatotrophin: a dose-response study. *Journal of Reproduction and Fertility*, **110**: 91-97.

GONZALEZ-PADILLA, E., Ruiz, R., Lefever, D., Denham, A. and Wiltbank, J. N. (1975). Puberty in beef heifers. III Induction of fertile oestrus. *Journal of Animal Science*, **40**, 1110-1118.

GOODMAN, R.L. and Karsch, F. J. (1980). Pulsatile secretion of luteinizing hormone: differential suppression by ovarian steroids. *Endocrinology*, **107**: 1286-1290.

GOSH, S. and Jackson, D.C. (1999). Antigenic and immunogenic properties of totally synthetic peptide-based anti-fertility vaccines. *International Immunology*, **11**:1103-1110.

GOUBAU, S., Silversides, D. W., Gonzalez, A., Laarveld, B., Mapletoft, R. J. and Murphy, B. D. (1989). Immunization of cattle against modified peptides of gonadotropin releasing hormone conjugated to carriers. Effectiveness of Freund's and alternative adjuvants. *Theriogenology*, **32**: 4: 557-567.

GOULDING, D., Williams, D. H., Roche, J. F. and Boland, M. P. (1996). Factors affecting superovulation in heifers treated with PMSG. *Theriogenology*, **45**, 765-773.

GREENE, W. A., Mogil, L. and Foote, R. H. (1978). Behavioral characteristics of freemartins administered estradiol, estrone, testosterone, and dihydrotestosterone. *Hormones and Behaviour*, **10**: 71-84

GREGORY, K.E. and Ford, J. J. (1983). Effects of late castration, zeranol and breed group on growth, feed efficiency and carcass characteristics of late maturing bovine males. *Journal of Animal Science*, **56**, 771-780.

GREGORY, K. E., Bennett, G. L.., Van Vleck, L. D., Echternkamp, S. E. and Cundiff, L. V. (1997). Genetic and environmental perameters for ovulation rate, twinning rate, and weight traits in a cattle population selected for twinning. *Journal of Animal Science*, **75**: 1213-1222.

GRIEGER, D. M. and Reeves, J.J. (1988). Sterilisation of beef heifers immunised against luteinising hormone. *Proceedings of Western Section American Society of Animal Science*, **39**, 305-8.

GRIEGER, D. M. and Reeves, J.J. (1990). Active immunisation of beef heifers against lutenising hormone:II Evaluation of conjugation technique on antigenicity of LH. *Journal of Animal Science*, **68**, 3747-54.

GRIEGER, D. M., Scarborough, R., deAvila, D. M., Johnson, H. E. and Reeves J. J. (1990). Active immunisation of beef heifers against luteinizing hormone: III. Evaluation of dose and longevity. *Journal of Animal Science*, **68**: 3755-3764.

GRIER, H. T. and Marion, G. B. (1970). Development of the mammalian testis. In: The Testis (volume I, Chapter 1). (Eds): A. D. Johnson, W. R. Gomes and N. L. Vandemark. Academic Press, New York and London. Pp: 39.

GROOME, N., Illingworth, P., O'Brien, M., Pai, R., Rodger, F.E., Mather, J. and McNeilly, A. (1996). Measurement of dimeric inhibin B throughout the human menstrual cycle. *Journal of Clinical Endocrinology and Metabolism*, **81**: 1400-1405.

GUERRA-MARTINEZ, P., Dickerson, G. E., Anderson, G. B. and Green, R. D. (1990) Embryo transfer twinning and performance efficiency in beef production. *Journal of Animal Science* **68**: 4039-4050.

GUO, Q., Kumar, T. R. Woodruff, T., Hadsell, L. A., DeMayo, F.J. and Matzuk, M.M. (1998). Overexpression of mouse follistatin causes reproductive defects in transgenic mice. *Molecular Endocrinology*, **12**: 96-106.

GUILBAULT, L. A., Grasso, F., Lussier, J. G., Rouiller, P. and Matton, P. (1991). Decreased superovulatory responses in heifers superovulated in the presence of a dominant follicle. *Journal of Reproduction and Fertility*, **91**: 81-89.

GUTIERREZ, C. G., Oldham, J., Bramley, T. A., Gong, J. G., Campbell, B. K. and Webb, R. (1997a). The recruitment of ovarian follicles is enhanced by increased dietary intake in heifers. *Journal of Animal Science*, 1876-1884.

GUTIERREZ, C. G., Campbell., B. K. and Webb., R. (1997b). Development of a long-term bovine granulosa cell culture system: induction and maintenance of estradiol production, response to FSH and morphalogical characteristics. *Biology of Reproduction*, **56**: 608-616.

HAFEZ, E. S. E. and Sugie, T. (1963). Behavioural oestrus and ovulatory cycles in beef cattle with a note on the clay model technique. *Acta Zoologica, Stockholm*, **44**, 57-71.

HAKOVIRTA, H., Kaipia, A., Soder, O. and Parvinen, M. (1993). Effects of activin A, inhibin-A, and transforming growth factorB1 on stage-specific deoxyribonucleic acid synthesis during rat seminiferous epithelial cycle. *Endocrinology*, **133**, 1664-1668.

HALLIDAY, R. (1974). Animal Production, 19:301-308.

HANCOCK, J. L. (1959). The morphological characteristics of spermatozoa and fertility. *International Journal of Fertility*, **4**, 347-359.

HARRIS, G. W. (1955). Neural control of the pituitary gland. Arnold Publications, London.

HARRIS, G. W. (1964). Endocrinology, **75**: 627-648.

HARTE, J., Curran, S. and Vial, V. E., (1965). The production of beef from young bulls, I. *Irish Journal of Agricultural Research.*, **4**, 189-204.

HASEGAWA, Y., Miyamoto, K., Abe, Y., Nakamuro, T., Sugino, H., Eto, Y., Shibai, H. and Igarshi, M. (1988). Introduction of follicle-stimulating hormone receptor by erythroid differentiation factor on a rat granulosa calls. *Biochemical and Biophysical Research Communications*, **156**: 668-674

HASLER, J. F., McCauley, A. D., Schermerhorn, E. C. and Foote, R. H. (1983). Superovulatory responses of Holstein cows. *Theriogenology*, **19**: (1): 83-99.

HAUSER, M. A., Koob, M. D. and Roth, J. A. (1986) Variation of neutrophil function with age in calves. *American Journal of Veterinary Research*, **47**, 152.

HAWES, B. E., Waters, S. B., Janovick, J. A., Bleasdale, J. E. and Conn, P. M. (1992). Gonadotrophin-releasing hormone-stimulated intracellular Ca^{2+} fluctuations and luteinising hormone release can be uncoupled from inositol phosphate production. *Endocrinology*, **130**: 3475-3483.

HAY, F. C. and Thanavala, Y. M. (1984). Functional aspects of antibodies. In: Immunological Aspects of Reproduction in Mammals. Eds: D. B. Crighton. Chapter 3: 27-34

HAZUM, E. and Conn, P. M. (1988). Molecular mechanisms of gonadotrophin-releasing hormone (GnRH) action: I. The GnRH receptor. *Endocrine Reviews*, **9**, 379-385.

HENDERSON, K. M., Franchimont, P., Lecomte-Yerna, M. J., Hudson, N. and Ball, K. (1984). Increase in ovulation rate after active immunisation of sheep with inhibin partially purified from bovine follicular fluid. *Journal of Endocrinology*, **102**: 305-309.

HENDERSON, K. M., Ellen, R. L.., Weaver, A., Ball, K. and McNatty, K. P. (1989). Effect of active immunisation withfollicular fluid on ovulation rates in Romney ewes. *Proceedings of the New Zealand Society of Animal Production*, **49**, 97-101.

HERTAN, R., Farnworth, P. G., Fitzsimmons, K. L. and Robertson, D. M. (1999). Identification of high affinity binding sites for inhibin on ovine pituitary cells in culture. Endocrinology, 140: (1): 6-12.

HILEMAN, S. M. and Jackson, G. L. (1999). Regulation of gonadotrophin releasing hormone secretion by testosterone in male sheep. *Journal of Reproduction and Fertility, suppl.* **54**: 231-242

HILLARD, M. A., Wilkins, J. F., Cummins, L. J., Bindon, B. M., Tsonis, C. G., Findlay, J. K. and O'Shea, T. (1995). Immunological manipulation of ovulation rate for twinning cattle. *Journal of Reproduction and Fertility*, (suppl. 49): 351-364.

HILLIER, S. G. (1991). Regularory functions for inhibin and activin in human ovaries, *Journal of Endocrinology*, **131**: 171-175

HOCHEREAU-DE REVIERS, M. T. and Courot, M. (1978). *Annals of Biological Animal Biochemistry and Biophysics*, **18**, 573.

HOHL, M. C. K., Waidelich, S., Drochner, W. and Claus, R. (2002). Active immunisation of boars against GnRH at an early age: consequences on testicular function, boar taint accumulation and N-retention. *Livestock Reproduction Science*, **74**, 147-157.

HOKFELT, T., Johansson, O., Fux, K., Goldstein, M., Park, D., Ebstein, R., Fraser, H. M., Jeffcoate, S. L., Efendic, S., Luft, R and Arimura, A. (1976). Mapping the relationship of hypothalamic neurotransmitters and hypothalamic hormones. *Proceedings of the Sixth International Congress of Pharmacology*, **3**, 93.

HOLTZ, W. and Smidt, D. (1976). *Journal of Reproduction and Fertility*, **46**, 227-229.

HONARAMOOZ, A., Chandolia, R. K., Beard, A. P. and Rawlings, N. C. (1999). Effects of season of birth on the prepubertal pattern of gonadotrophin secretion and age at puberty in beef heifers. *Theriogenology*, **52**: 67-79.

HOOKER, C. W. (1970). The intertubular tissue of the testis. In: The Testis (volume I, Chapter 8). (Eds): A. D. Johnson, W. R. Gomes and N. L. Vandemark. Academic Press, New York and London pp: 483.

HOPPER, H. W., Silcox, R. W., Byerley, D. J. and Kiser, T. E. (1993). Follicular development in prepubertal heifers. *Animal Reproduction Science*, **31**: 7-12.

HORTSMAN, L.A., Callahan, C.J., Morter, R. L., and Amstutz, H. E. (1982). Ovariectomy as a means of abortion and control of estrus in feedlot heifers. *Theriogenology*, **17**: 273.

HOSKINSON, R. M., Scaramuzzi, R. J., Campbell, B. K., Downing, J. A., Welch, R. J. and Harrison, B. E. (1986). Effects of antibodies to steroid hormones on reproductive events of sheep and cattle. In: Immunological Approaches to Contraception and Promotion of Fertility, pp. 351-366 (Eds. G. P. Talawar) Plenum Press, New York.

HOSKINSON, R. M., Rigby, R. D. G., Mattner, P. E., Huynh, V. I., D'Occhio, M., Neish, A., Trigg, T. E., Moss, B. A., Lindsey, M. J., Coleman, G. D. and Schwartzkoff, C. L. (1990) Vaxstrate® : An anti-reproductive vaccine for cattle. *Australian Journal of Biotechnology*, **4**: (**3**): 166-170.

HSUEH, A. J. W. and Jones, P. B. C. (1981). Extrapituitary actions of gonadotrophin releasing hormone. *Endocrine Reviews*, **2**: 437-461.

HSUEH, A. J. W., Dahl, K. D., Vaughan, J., Tucker, E., Rivier, J. and Bardin, C. W. (1987). Heterodimers and homodimers of inhibin subunits have different paracrine action in the modulation of luteinizing hormone-stimulated androgen biosynthesis. *Proceedings National Academy of Sciences USA*, **84**: 5082-5086

HUCKLE, W. R. and Conn, P. M. (1988). The role of protein kinase C in pituitary gonadotrophin-releasing hormone action. In: Neural control of reproductive function. (Eds): J. M. Lakowski, J. R. Perez-Polo, D. K. Rassin. New York: A. Liss, pg: 441-446.

HUHTINEN , M., Raino, V., Aalto, J., Bredbacka, P. and Maki-Tanila, A. (1992). Increased ovarian responses in the absence of a dominant follicle in superovulated cows. *Theriogenology*, **37**: 475-463.

HURNIK, J. F., King, G. J. and Robertson, H. A. (1975). Oestrus and related behaviour in postpartum Holstein cows. *Applied Animal Ethiology*, **2**, 55-68.

HURNIK, J. F. and King, G. J. (1987). Oestrus behaviour in confined beef cattle. *Journal of Animal Science*, **65**, 431-438.

HUSBAND, A.J. and Lascelles, A.K. (1975). Antibody responses to neonatal immunisation in calves. Research in Veterinary Science, **18**:201-207.

HUTCHINSON, L. A., Findlay, J. K., deVos, F. L. and Robertson, D. M. (1987). Effects of bovine inhibin, transforming growth factor-β and bovine activin-A on granulosa cell differentiation. *Biochemical and Biophysical Research Communications*, **146**: 1405-1412

HUXSOLL, C. C., Price, E. O. and Adams, T. E. (1998). Testis function, carcass traits and aggressive behaviour of beef bulls actively immunized against gonadotrophin-releasing hormone. *Journal of Animal Science*, **76**: 1760-1766.

INWALLE, D. B. and Schillo, K. K. (2002). Castration increases pulsatile luteinizing release, but fails to diminish mounting behaviour in sexually experienced bulls. *Domestic Animal Endocrinology*, **22**: 223-235.

IRELAND, J. J. and Roche, J. F. (1982). Effect of progesterone on basal LH and episodic LH and FSH secretion in heifers. *Journal of Reproduction and Fertility*, **64**: 295-302.

IRELAND, J. J. and Roche, J. F. (1983). Development of non-ovulatory antral follicles in heifers: changes in steroids in follicular fluid and receptors for gonadotrophins. *Endocrinology*, **112**, 150-156.

IWASHITA, M, Evans, M. I. and Catt, K. J. (1986). Characterization of the gonadotrophin – releasing hormone receptor site in term placenta and chorionic villi. *Journal of Clinical Endocrinology and Metabolism*, **62**: 127- 133.

JACOBSON, J.D. (2000). Gonadotropin-releasing hormone and G proteins: potential role in autoimmunity. *Annals of the New York Academy of Science*, **917**:809-818.

JAGO, J. G., Lasenby, R. R., Trigg, T. E., Claxton, P. D., Matthews, L. R. and Bass, J. J. (1995). The effect of immunological castration on behaviour and growth of young bulls. *Proceedings of the New Zealand Society of Animal Production*, **55**: 190-192.

JAGO, J. G., Matthews, L., Bass, J. J., Knight T. W. (1996). A comparison of two methods of castration of post-pubertal beef cattle and their effect on behavior, growth and ultimate pH. *Proceedings of the New Zealand Society of Animal Production*, **56**: 394-397.

JAGO, J. G., Cox, N. R., Bass, J. J. and Matthews, L. R. (1997). The effect of pre-pubertal immunization against gonadotrophin-releasing hormone on the development of sexual and social behaviour of bulls. *Journal of Animal Science*, **75**: 2609-2619.

JAGO, J. G., Bass, J. J. and Matthews, L. R. (1997). Evaluation of a vaccine to control bull behaviour. *Proceedings of the New Zealand Society of Animal Production*, **57**: 91-95.

JAMESON, B.A. and Wolf, H. (1988). The antigenic index: a novel algorithm for predicting antigenic determinants. *Computer Applications in Bioscience, (CABIOS)*, **4**: 181-188.

JEFFCOATE, I. A., Lucas, J. M. S. and Crighton, D. B. (1982). Effects of active immunisation of ram lambs and bull calves against synthetic luteinizing releasing hormone. *Theriogenology*, **18**(1): 65-77.

JEFFERY, M., Loxton, I., Van der Mark, S., James, T., Shorthose, R., Bell, K. and D'Occhio, M. (1997). Liveweight gains, and carcass and meat characteristics of entire, surgically spayed or immunologically spayed beef heifers. *Australian Journal of Experimental Agriculture*, **37**: 719-726.

JIMENEZ-KRASSEL, F. Knight, P.G., Austin, E. J., Roche, J. F., Ireland, J. J. (2001). Inhibin suppresses oestradiol production by bovine granulosa cells isolated from first wave dominant follicles. *Biology of Reproduction*, **64**: (1), 110, abstract 31.

JIMENEZ-SEVERIANO, H., Mussard, M., Ehnis, L., Koch, J., Zanella, E., Lindsey, B., Enright, W., D'Occhio, M. and Kinder, J. (1998). Secretion of LH in bull calves treated with analogs of GnRH. *Journal of Reproduction and Fertility Supplement*, **54**.

JIMENEZ-SEVERIANO, H., D'Occhio, M. J., Lunstra, D.D., Mussard, M. L., Koch, J. W., Ehnis, L. R., Enright, W. J. and Kinder, J. E. (2003). Effect of chronic treatment with gonadotrophin-releasing hormone agonist azagly-nafarelin on basal concentrations of LH in prepubertal bulls. *Reproduction*, **125**: 225-232.

JOHNSON, S. K. and Smith, M. F. (1985). Effects of charcoal-extracted, bovine follicular fluid on gonadotropin concentrations, the onset of estrus and luteal function in heifers. *Journal of Animal Science*, **61**: 203-209

JOHNSON, H. E., DeAvila, D. M., Chang, C. F. and Reeves, J. J. (1988). Active immunization of heifers against luteinizing hormone-releasing hormone, human chorionic gonadotropin and bovine luteinizing hormone. *Journal of Animal Science*, **66**: 719-726.

JOHNSON, L. (1991). Spermatogenesis. Reproduction in Domestic Animals, Fourth Edition. Academic Press, Inc. Chapter 5, pg; 173-219.

JOSSO, N. J., Picard, Y., Dacheux, J. L. and Courot, M. (1979). *Journal of Reproduction and Fertility*, **57**, 379.

KAINER, R. A., (1993). Reproductive organs of the mare. In: McKinnon, A.O., Voss, J.L. (Eds.), Equine Reproduction. Lea & Febieger, Philadelphia, P.A, pp. 5-19.

KANEKO, H., Terada, T., Taya, K., Watanabe, G., Sasomoto, S., Hasegowa, Y. and Igarashi, M. (1991). Ovarian follicular dynamics and concentrations of oestradiol 17-beta, progesterone, luteinising hormone and follicle stimulating hormone during the peri-ovulatory phase of the oestrous cycle in the cow. *Reproduction, Fertility and Development*, **3**: 529-535.

KANEKO, H.,Yoshida, M., J., Hara, Y., Taya, K., Araki, K., Watanabe, G., Sasamoto, S. and Hasegawa, Y. (1993a). Involvement of inhibin in the regulation of FSH secretion in prepubertal bulls. *Journal of Endocrinology*, **137**, 15-19.

KANEKO, H., Nakanishi, Y., Taya, K., Kishi, H., Watanabe, G., Sasamoto, S. and Hasegawa, Y. (1993b). Evidence that inhibin is an important factor in the regulation of FSH secretion during the mid-luteal phase in cows. *Journal of Endocrinology*, **136**, 35-41.

KANEKO, H., Kishi, H., Watanabe, G. Taya, K., Sasamoto, S. and Hasegawa, Y. (1995). Changes in plasma concentrations in immunoreactive inhibin, oestradiol and FSH associated with follicular waves during the estrous cycle of the cow. *Journal of Reproduction and Development*, **41**, 311-320.

KAPPES, S. M., Bennett, G. L., Keele, J. W., Echternkamp, S. E., Gregory, K. E. and Thallman, R. M. (2000). Initial results of genomic scans for ovulation rate in cattle population selected for increased twinning rate. *Journal of Animal Science*, **78**, 3053-3059.

KATONGOLE, C. B., Naftolin, F. and Short, R. V. (1971). Relationship between blood levels of luteinising hormone and testosterone in bulls, and the effects of sexual stimulation. *Journal of Endocrinology*, **50**, 457-466.

KEELING, B. J. and Crighton, D. B. (1984). Reversibility of the effects of active immunisation against LH-RH. In: Immunological Aspects of Production in Mammals. (Ed): D. B. Crighton. Butterworths, London, pp, 379-398.

KEISLER, D. H., Daniel, J. A. and Morrison, C. D. (1999). The role of leptin in nutritional status and reproductive function. Journal of Reproduction and Fertility, Supplement, **54**: 425-435.

KENDRICK, J. W. and Franti, C. E. (1974) Bovine viral diarrhea: decay of colostrums-confirmed antibody in the calf. *American Journal of Veterinary Research*, **35**: 589-591.

KERR, J. B., Maddocks, S. and Sharpe, R. M. (1992). Testosterone and FSH have independent, synergistic and stage-dependent effect upon spermatogenesis in the rat testis. *Cell Tissue Research*, **268**, 179-189.

KESNER, J.S., Convey, E. M., Anderson, C. R. (1981) *Theriogenology*, **16**, 575.

KHODR, G. S. and Siler-Khodr, T. M. (1980). Placental luteinizing hormone- releasing factor and its synthesis. *Science*, **207**: 315-317.

KINDER, J. E., Day, M. L. and Kittok, R. J. (1987). Endocrine regulation of puberty in cows and ewes. *Journal of Reproduction and Fertility, Supplement*, **34**, 167-189.

KINDER, J. E., Bergfeld, E. G. M., Wehrman, M. E., Peters, K. E. and Kojima, F. N. (1995). Endocrine basis for puberty in heifers and ewes. *Journal of Reproduction and Fertility, Supplement*, **49**, 393-407.

KIRKPATRICK, J., Fulton, R. W., Burge, L. J., Dubois, W. R. and Payton, M. (2001) Passively transferred immunity in newborn calves, rate of antibody decay, and effects on subsequent vaccination with modified live virus vaccine. *Bovine Practioner*, **35**: 47-55.

KNIGHT, P. G., Beard, A. J., Wrathall, J. H. M. and Gastillo, R. J. (1989). Evidence that the bovine ovary secretes large amounts of monomeric inhibin α-subunit and its isolation from bovine follicular fluid. *Journal of Molecular Endocrinology*, **2**, 189-200.

KNIGHT, P.G., Groome, N. and Beard, A. J. (1991). Development of a two-site imunoradiometric assay for dimeric inhibin using antibodies against chemically-synthesized fragments of the α and β subunit. Journal of Endocrinology, **129**, R9-R12.

KNIGHT, P.G., Wrathall, J.H.M., Glencross, R.G. and McLeod, B.J. (1991). Effects of bovine follicular fluid on the secretion of LH and FSH in inhibin-immunized seasonally anoestrous ewes. *Journal of Endocrinology*, **128**, 403-410

KNIGHT, P. G. and Glister, C. (2001). Potential local regulatory functions of inhibins, activins and follistatin in the ovary. *Reproduction*, **121**: 503-512.

KNIGHT P.G and Glister, C. (2003). Local roles of TGF-β superfamily members in the control of ovarian follicle development. *Animal Reproduction Science*, **78**: 165-183.

KOVARIK, J. and Siegrist, C.A. (1997). Immunity in early life. *Immunology Today*, **19**:4:150-152.

KREIKEMEIER, K. K. and Unruh, J. A. (1993). Carcass traits and the occurrence of dark cutters in pregnant and nonpregnant feedlot heifers. *Journal of Animal Science*, **71**, 1699.

KUSINA, N. T., Meyer, R. L., Carlson, K. M. and Wheaton, J. E. (1994). Passive immunisation against an inhibin-peptide increases FSH, ovulation rate (OR) and lambs born in spring-mated ewes. *Abstracts*: *J. Ani. Sci*; **72** *suppl.* 1: *J. Dairy Sci*; **77** *suppl.* 1)

KUSINA, N. T., Meyer, R. L., Carlson, K. M. and Wheaton, J. E. (1995). Passive immunisation of ewes against an inhibin-like peptide increases follicle-stimulating hormone concentrations, ovulation rate, and prolificacy in spring-mated ewes. *Journal of Animal Science*, **73**: 1433-1439.

LADD, A., Tsong, Y-Y., Lok, J. and Thau, R. B. (1990) Effects of conjugation site and dose. *American Journal of Reproductive Immunology*, **22**, 56-63.

LAGERLOF, N. (1938). Infertility in domestic animals. *Veterinary Medicine*, **33**, 550-561.

LAMOTHE, C., Montiel, F., Fredriksson, G. and Galina, C. S. (1995). Reproductive performance of zebu cattle in mexico-influence of season and social interaction on the timing of expressed oestrus. *Tropical Agriculture*, **72**, (4): 319-323.

LAND, R.B., Baird, D.T. and Carr, W.R. (1981). Increased testicular growth of Tasmanian Merino ram lambs treated with antisera to oestrogens. *Journal of Reproduction and Fertility*, **62**:151-158.

LANDEFELD, T. D. and Kepa, J. (1979). The cell free synthesis of bovine lutotrophin B subunit. *Biochemical and Biophysical Research Communications*, **90**: 1111-1118.

LASTER, D. B., Glimp, H. A. and Gregory, K. E. (1972). Age and height at puberty and conception in different breeds and breed-crosses of beef heifers. Journal of Animal Science, 34, 1031-1036.

LAW, A. S., Baxter, G., Logue, D. N., O'Shea, T. and Webb, R. (1992). Evidence for the action of a bovine follicular fluid factor(s) other than inhibin in suppressing follicular development and delaying oestrus in heifers. *Journal of Reproduction and Fertility*, **96**, 603-616.

LEE, V. W. K., Cumming, I. A., de Kretser, D.M, Findlay, J. K., Hudson, B and Heogh, E. F. (1976). Regulation of gonadotrophin secretion in rams from birth to sexual maturity I. Plasma LH, FSH and testosterone levels. *Journal of Reproduction and Fertility*, **46**, 1-6.

LEGAN, S. J. and Karsch, F. J. (1980). Photoperiodic control of seasonal breeding in ewes: Modulation of the negative feedback action by estradiol. *Biology of Reproduction*, **23**: 1061.

LEHMAN, M. N., Robinson, J. E., Karsch, F. J. and Silverman, A. J. (1986). Immunocytochemical localization of luteinizing hormone-releasing hormone (LHRH) pathways in the sheep brain during anestrus and the mid-luteal phase of the estrous cycle. *The Journal of Comparative Neurology*, **244**: 19-35.

LESHIN, L.S., Rund, L.A., Crim, S.W. and Kiser, T.E. (1988). Immunocytochemical localisation of LHRH and pro-opiomelanocortin neurons within the pre-optic area and hypothalamus of the bovine brain. *Biology of Reproduction*, **39**: 963-975.

LEVERSHA, L. J., Robertson, D. M., de Vos, F. L., Morgan, F. J., Hearn, M. T. W., Wettenhall, R. E. H., Findlay, J. K., Burger, H. G. and de Kretser, D. M. (1987). Isolation of inhibin from ovine follicular fluid. *Journal of Endocrinology*, **113**, 213-221.

LEVINE, J. E. and Ramirez, V. D. (1982). Luteinizing hormone-releasing hormone release during the rat oestrous cycle and after ovariectomy as estimated with push-pull cannulae. *Endocrinology*, **111**: 1439-1448.

LEVINE, J. E., Pau, K-Y. F., Ramirez, V. D. and Jackson, G. L. (1982). Simultanious measurements of luteinising hormone-releasing hormone and luteinising hormone released in unanaesthetised, ovariectomized sheep. *Endocrinology*, **111**: 1449-1455.

LI, R., Philips, D.M. and Mather, J.P. (1995). Activin promotes ovarian follicular development *in vitro*. *Endocrinology*, **136**: 849-856.

LIEN, S., Karlsen, A., Klemetsdal, G., Vage, D. I., Olsaker, I., Klungland, H., Aasland, M., Heringstad, B., Ruane, J. And Gomez-Raya, L. (2000). A primary screen of the bovine genome for quantitative trait loci affacting twinning rate. *Mammalian Genome*, **11**: (**10**): 877-882.

LIN, S-Y., Morrison. J. R., Phillips, D.J., de Kretser, D.M. (2003). Regulation of ovarian function by the TGF-β superfamily and follistatin. *Reproduction*, **126**, 133-148.

LINCOLN, G. A. and Fraser, H. M. (1979). Blockage of episodic secretion of luteinizing hormone in the ram by the administration of antibodies to luteinizing hormone releasing hormone. Biology of Reproduction, 21: 1239-1245.

LINCOLN, G. A. and Fraser, H. M. (1987). Compensatory response of the luteinizing-hormone (LH)-releasing hormone (LHRH)/LH pulse generator after administration of a potent antagonist in the ram. *Endocrinology*, **120**: 2245-2250

LING, M., Ying, S-Y., Ueno, N., Esch, F. S., Denoroy, L. and Guillemin, R. (1985). Isolation and partial characterization of a 32,000 MW protein with inhibin activity from porcine follicular fluid. *Proceedings of National Acadaemic Science, USA*, **82**, 7217-7221.

LIU, X. and Spahr, S. L. (1993). Automated electronic activity measurement for detection of oestrus in dairy cattle. *Journal of Dairy Science*, **76**, 2906-2912.

LOBLEY, G. E., Connell, A., Morris, B., Anderson, R., Clayton, J. and Williams, P. E. V. (1992). The effect of active immunization against gonadotrophin- hormone-releasing-hormone on growth performance and sample joint composition of bulls. *Animal Production*, **55**: 193-202.

LOOPER, M.L.., Vizcarra, J.A., Wttemann, R.P., Malayer, J.R., Bradens, T.D., Geisert, R.D. and Morgan, G.L. (2003). Influence of estradiol, progesterone, and nutrition on concentrations of gonadotrophins and GnRH receptors, and abundance of mRNA for GnRH receptors and gonadotrophin subunits in pituitary glands of beef cows. *Journal of Animal Science*, **81**, 269-278.

LUCY, M. C., Collier, R. J., Kitchell, M. A., Dibner, J. J.,Hauser, S. D and Krivi, G. G. (1993). Immunohistochemical and nucleic acid analysis of somatotropin receptor populations in the bovine ovary. *Biology of Reproduction*, **48**: 1219-1227.

LUCY, M.C., Bilby, C. R., Kirby, C. J., Yuan, W. and Boyd, C. K. (1999). Role of growth hormone in development and maintenance of follicles and corpora lutea. *Journal of Reproduction and Fertility, suppl.*, **54**: 49-59.

LUCY, M.C. (2000). Regulation of ovarian follicular growth by somatotropins and insulin-like growth factors in cattle. *Journal of Dairy Science*, **83**: 1635-1647.

LUNDY, T., Smith, P., O'Connell, A., Hudson, N. L. and McNatty, K. P. (1999). Populations of granulosa cells in small follicles of the sheep ovary. *Journal of Reproduction and Fertility*, **115**: 251-262.

LUNSTRA, D. D., Ford, J. J. and Echternkamp, S. E. (1978). Puberty in beef bulls: hormone concentrations, growth, testicular development, sperm production and sexual aggressiveness in bulls of different breeds. *Journal of Animal Science*, **46**, (4): 1054-1062.

LUTZ, J. B., Rampack, G. B., Kraeling, R. R. and Pinkert, C. A. (1984). Serum luteinizing hormone and oestrogen profiles before puberty in the gilt. *Journal of Animal Science*, **58**: 686-691.

MAATJE, K. Loeffler, S. H. and Engel, B. (1997). Predicted optimal time of insemination in cows that show visual signs of oestrus by estimating onset of oestrus with pedometers. *Journal of Dairy Science*, **80**, (6): 1098-1105.

MACDONALD, R. D. and Page, R, D. (1986). Luteinising hormone response to pulsatile luteinising hormone releasing hormone in prepubertal heifers. Journal of Dairy Science, 69, 1922-1931.

MACDONALD, R. D., Deaver, D. R. and Schanbacher, B. D. (1991). Prepubertal changes in plasma FSH and inhibin in Holstein bull calves: responses to castration and (or) estradiol. *Journal of Animal Science*, **69**, 267-282.

MACMILLAN, K. L. and Hafs, H. D. (1968). Pituitary and hypothalamic endocryne changes associated with Holstein bulls. *Journal of Animal Science*, **27**, 1614-1620.

MACMILLAN, K. L. and Hafs, H. D. (1969). Reproductive tract of Holstein bulls from birth through to puberty. *Journal of Animal Science*, **28**, 233-239.

MADDOCKS, S. and Setchell, B. P. (1988). The physiology of the endocrine testis. *Oxford Reviews of Reproductive Biology*, **10**, 53-123.

MADDOCKS, S. and Sharpe, R. M. (1989). Dynamics of testosterone secretion by the rat testis: implications for measurement of the intratesticular levels of testosterone. *Journal of Endocrinology*, **122**, 323-329.

MADDOCKS, S., Kern, S. and Setchell, B. P. (1995). Investigating local regulation of the testes of ruminants. *Journal of Reproduction and Fertility*, **49**, 309-319.

MANDIKI, S. N. M., Noel, B., Bister, J. L., Peeters, R., Beerlandt, G., Decuypere, E., Visscher, A., Sueβ, R., Kaulfuβ, K. H. and Paquay, R. (2000). Pre-ovulatoory follicular characteristics and ovulation rates in different breed crosses, carriers or non-carriers of the Booroola or Cambridge fecundity gene. *Animal Reproducion Science*, **63**: 77-88.

MANN, T. (1964). The biochemistry of semen and of the male reproductive tract. John Wiley and Sons, Inc., New York, p. 316

MANN, T., Rowson, L. E. A., Short, R. V. and Skinner, J. D. (1967). The relationship between under nutrition and androgenic activity in pubescent twin calves and the effect of orchitis. *Journal of Endocrinology*, **38**, 455-468.

MANN, G. E., Campbell, B. K., McNeilly, A. S. and Baird, D. T. (1989). *Journal of Endocrinology*, **123**, 383.

MANN, G. E. (1990). The role of inhibin and oestradiol in the control of gonadotrophin secretion in the ewe. *PhD Thesis*.

MANN, G. E., Campbell, B. K., McNeilly, A. S. and Baird, D. T. (1990). Effects of passively immunising ewes against inhibin and oestradiol during the follicular phase of the oestrous cycle. *Journal of Endocrinology*, **125**, 417-424.

MANN, G. E., Campbell, B. K., McNeilly, A. S. and Baird, D. T. (1992). The role of inhibin and oestradio in the control of FSH secretion in the sheep. *Journal of Endocrinology*, **133**, 381-391.

MANN, G. E., Campbell, B. K., McNeilly, A. S. and Baird, D. T. (1993). Follicular development and ovarian hormone secretion following passive immunisation of ewes against inhibin or oestradiol. *Journal of Endocrinology*, **136**, 225-233.

MANN, G. E., Lamming, G. E. and Fray, M. D. (1995) Plasma oestradiol and progesterone during early pregnancy in the cow and the effects of treatment with buserelin. *Journal of Animal Reproductive Science*, **37**, 121-131.

MANTOVANI, R., Enright, W. J., Roche, J.F. and Boland, M. P. (1997). The effect of immunization of heifers against α 1-26 inhibin conjugate on the superovulatory response to pFSH. *Theriogenology*, **47**: 511-519.

MARION, G. B., Smith, V. R., Wiley, T. E. and Barrett, G. R. (1950). The effect of sterile copulation on the time of ovulation in dairy heifers. *Journal of Dairy Science*, **33**, 885-889.

MARSHALL-CLARKE, S., Reen, D., Tasker, L. and Hassan, J. (2000). Neonatal immunity: how well has it grown up? *Immunology Today*, **21**:1:35-41.

MARTIN, T. E., Henricks, D. R., Hill, J. R. and Rawlings, N. C (1978). Active immunisation of the cow against oestradiol-17 beta. *Journal of Reproduction and Fertility.*, **53**, 173-8.

MARTIN, T. L., Williams, G. L.., Lunstra, D.D. and Ireland, J. J. (1991). Immunoneutralisation of inhibin modifies hormone secretion and sperm production in bulls. *Biology of Reproduction*, **45**, 73-77.

MARTIN, J. T. (2000). Sexual dimorphism in immune function: the role of prenatal exposure to androgens and estrogens. *European Journal of Pharmacology*, 405: 251-261.

MARTENSZ, N. D. (1980). The biological effects of immunisation against steroids in female mammals. In: Oxford Reviews of Reproductive Biology, Vol.2, 232-62, (Eds. C. A. Finn) Clarendon Press, Oxford.

MARTINEZ, X., Brant, C., Saddallah, F., Tougne, C., Barrios, C., Wild, F., Dougan, G., Lambert, P. H. and Siegrist, C. A. (1997). DNA immunization circumvents deficient induction of T helper type 1 and cytotoxic T lymphocyte responces in neonates and during early life. *Proceedings of the National Academy of Science USA*, **94** (16): 8726-8731.

MASSAGUE, J. and Wotton, D. (2000). Transcriptional control by the TGF-beta/Smad signalling system. *Embo. J.*, **19**: 1745-1754

MATSUO, H., Arimura, A., Nair, R.M.C., and Schally, A.V. (1971). Structure of the porcine LH- and FSH-releasing hormone i. The proposed amino-acid sequence. *Biochemical and Biophysical Research Communications*, **43**, 1334-1339.

MATWIJIW, I. and Faiman, C. (1991). Control of gonadotropin secretion in the ovine fetus. IV. Male-specific entrainment of the hypothalamic control of LH secretion by testosterone in the female ovine fetus. *Endocrinology*, **129**:3: 1447-1451.

MATZUK, M. M., Kumar, T. R., Shou, W., Coerver, K. A., Lau, A. L., Behringer, R. R. and Finegold, M. J. (1996). Transgenic models to study the roles of inhibins and activins in reproduction, oncogenesis, and development. *Recent Progress in Hormone Research*, **51**: 123-154 (discussion 155-7).

MAZERBOURG, S., Zapf, J., Bar, S., Brigstock, D. R. and Monget, P. (2000). Insulin-like growth factor (IGF)-binding protein-4 proteolytic degradation in bovine, equine, and procine preovulatory follicles: regulation by IGF and heparin-binding domain-containing peptides. *Biology of Reproduction*, **63**: 390-400.

McANDREWS, J. M., Peters, J. L. and Deaver, D. R. (1994). Age related changes in the secretion of LH in vivo and in vitro in infantile and prepubertal Holstein bull calves. *Journal of Reproduction and Fertility*, **101**, 453-458.

McARDLE, C. A., Franklin, J., Green, L. and Hislop, J. N. (2002). Signalling, cycling and desensitisation of gonadotrophin-releasing hormone receptors. *Journal of Endocrinology*, **173**: 1-11.

McCANN, S. M. (1983). Present status of LHRH: Its physiology and pharmacology. In: Role of peptides and proteins in control of reproduction. Eds. S. M. McCann and D. S. Dhindsa. Elsevier, New York, pp: 3-26.

McCARTHY, M. S. and Swanson, L.V. (1976). Serum LH concentrations following castration, steroid hormone and gonaotrophin releasing hormone treatment in the male bovine. *Journal of Animal Science*, **43**: 151-158.

McCARTHY, M. S., Convey, E. M. and Hafs, H.D. (1979). Serum hormonal changes and testicular response to LH during puberty in bulls. *Biology of Reproduction*, **20**, 1221-1227.

McCARTHY, M.S., Hafs, H.D. and Convey, E. M. (1979) Serum hormone patterns associated with growth and sexual development in bulls. *Journal of Animal Science*, **49**: 1012

McCAULEY, I., Watt, M., Suster, D., Kerton, D. J., Oliver, W. T., Harrell, R. J. and Dunshea, F. R. (2003). A GnRF vaccine (Improvac®) and porcine somatotrophin (Reporcin®) have synergistic effects upon growth performance in both boars and gilts. *Australian Journal of Agricultural Research*, **54**: 11-20.

McCULLOGH, D. R. (1932). Dual endocrine activity of the testis. *Science*, **76**; 19-20.

McGUIRE, T.C., Pfeiffer, N.E., Weikel, J.M. and Bartsch, R.C. (1976). Failure of colostrual immunoglobulin transfer in calves dying from infectious disease. *Journal of the American Veterinary Medical Association*, **169**: 713-718.

McLEOD, B. J., Haresign, W., Peters, A. R. and Lamming, G. E. (1984). Plasma LH and FSH concentrations in prepubertal beef heifers before and in response to repeated injections of low doses of GnRH. *Journal of Reproduction and Fertility*, **70**, 137-144.

McLEOD, B. J., Peters, A. R., Haresign, W. and Lamming, G. E. (1985). Plasma LH and FSH responses and ovarian activity in prepubertal heifers treated with repeated injections of low doses of GnRH for 72 hours. *Journal of Reproduction and Fertility*, **72**, 589-596.

McLEOD, B. J., Hunter, M. G., Bleach, E. C. L., Glencross, R. G. and Wrathall, J. H. M. (1992). Preovulatory follicle development and luteal function in ewes immunised against a synthetic peptide sequence of the α-subunit of bovine inhibin. *Journal of Endocrinology*, **133**, 413-419.

McNATTY, K.P., Hudson, N. L., Gibb, M. and Collins, F. L. (1988). The plasma concentrations of FSH, LH, and progesterone in sheep immunised against a androstenedione-protein conjugate. *Journal of Reproduction and Fertility*, **82**, 63-69.

McNATTY, K.P., Smith, P., Hudson, N. L., Heath, D. A., Tisdall, D. J., O, W. –S. and Braw-Tal, R. (1995). Development of the sheep ovary during fetal and early neonatal life and the effect of fecundity genes. *Journal of Reproduction and Fertility, Supplement*, **49**: 123-135.

McNATTY, K. P., Juengel, J. L., Wilson, T., Galloway, S. M. and Davis, G. H. (2001). Genetic mutations influencing ovulation rate in sheep. Reproduction, Fertility and Development, 13: 549-555.

McNATTY, K.P., Juengel, J.L., Wilson, T., Golloway, S.M., Davis, G.H., Hudson, N.L., Moeller, C.L., Cranfield, M., Reader, K.L., Laitinen, M.P.E., Groome, N.P., Sawyer, H.R. and Ritvos, O. (2003). Oocyte derived growth factors and ovulation rate in sheep. *Reproduction Supplement*, **61**: 339-351.

McNEILLY, A. S., Jonassen, J. A. and Fraser, H. M. (1986). Suppression of follicle development after chronic LHRH immunoneutralisation in the ewe. *Journal of Reproduction and Fertility*, **76**, 481-490.

McNEILLY, A. S. (1988). The control of FSH secretion. *Acta Endocrinologica Supplementum*, **288**, 31-40.

McNEILLY, A. S., Crawford, J. L., Taragnat, C., Nicol, L. and McNeilly, J. R. (2003). The differential secretion of FSH and LH: regulation through genes, feedback and packaging. *Reproduction Supplement*, **61**, 463-476.

MECHOR, G. D., Virtala, A. M. K., Dobovi, E. J. and Grohin, Y. T. (2001). The half life for maternally derived immunoglobulin G anti-viral antibodies in data from an observational field study. *Bovine Practioner*, **35**: 131-136.

MEISEL, R. L. and Sachs, B. D. (1994). The physiology of male sexual behaviour. In: *The Physiology of Reproduction*, (Eds); E. Knobil & J. D. Neill. Second edition. New York: Raven Press.

MELOEN, R. H., Turkstra, J. A., Lankhof, H., Puijk, W. C., Schaaper, W. M. M., Dijkstra, G., Wensing, C. J. G. and Oonk, R. B. (1994). Efficient immunocastration of male piglets by immunoneutralization of GnRH using a new GnRH-like peptide. *Vaccine*, **12**: (8): 741-746.

MELOEN, R. H. (1995). Basic aspects of immunomodulation through active immunization. *Livestock Production Science*, **42**: 135-145.

MELVIN, E.J., Lindsey, B.R., Quintal-Franco, J., Zanella, E., Fike, K.E., Van Tassell, C.P. and Kinder, J.E. (1999). Circulating concentrations of oestradiol, LH and FSH during waves of ovarian follicular development in prepubertal cattle. *Biology of Reproduction*, **60**:405-412.

MEPHAM, T. B. and Forbes, J. M. (1995). Ethical aspects of the use of immunomodulation in farm animals. *Livestock Production Science*, **42**: 265-272.

MERCER, J. E., Clements, J. A., Funder, J. W and Clarke, I. J. (1989). Studies on the regulation of gonadotrophin of gene expression in the hypothalamo-pituitary intact and hypothalamo-pituitary disconnected ewe. In Glyco-protein Hormone pp 227-236. (Ed.WW Chin and I Boime). Serono Syposia USA, Norwell

MESIANO, S., Hart, C.S., Heyer, B.W., Kaplin, S.L. and Grumbach, M.M. (1991). Hormone ontogeny in the ovine fetus XXVI. A sex difference in the effect of castration on the hypothalamic-pituitary gonadotropinunit in the ovine fetus. *Endocrinology*, **129**:6:3073-3079.

METZ, C., Hohl, K., Waidelich, S., Drochner, W. and Claus, R. (2002). Active immunization of boars against GnRH at an early age: consequences for testicular function, boar taint accumulation and N-retention. *Livestock Production Science*, **74**: 147-157.

MEYER, D.G. (1987). 30,000 heifers prove success of ovarian-drop spaying technique. *Beef* **24**: (1A): 14.

MEYER, R. L., Carlson, K. M., Rivier, J. and Wheaton, J. E. (1991). Antiserum to an inhibin alpha-chain peptide neutralizes inhibin bioactivity and increases ovulation rate in sheep. *Journal of Animal Science*, **69**: 747-754.

MIHM, M., Good, T. E. M., Ireland, J. L. H, Ireland, J. J., Knight, P. G. and Roche, J. F., (1997). Decline in serum follicle-stimulating hormone concentrations alters key intrafollicular growth factors involved in selection of the dominant follicle in heifers. *Biology of Reproduction*, **49**: 1001-109

MIHM, M., Austin, E. J., Good, T. E. M., Ireland, J. L. H., Knight, P. and Roche, J. (2000). Indentification of potential intrafollicular factors involved in selection of dominant follicles in heifers. *Biology of Reproduction*, **63**: 811-819.

MILLER, D. W., Fraser, H. M. and Brooks, A. N. (1998). Suppression of fetal gonadotrophin concentrations by maternal passive immunisation to GnRH in sheep. *Journal of Reproduction and Fertility*, **113**: 69-73.

MIRO, F. and Hillier, S. G. (1996). Modulation of granulosa cell deoxyribonucleic acid synthesis and differentiation by activin. *Endocrinology*, **137**: 464-468.

MIYAMOTO, K., Hasegawa, Y., Fukuda, M. and Igarashi, M. (1985). Isolation of porcine follicular fluid inhibin of 32K daltons. *Biochemical and Biophysical Research Communications*, **136**, 1103-1109.

MIZUMACHI, M., Voglmayr, J. K., Washington, D. W., Chen, C-L. C. and Bardin, C. W. (1990). Superovulation of ewes immunised against the human recombinant inhibin subunit associated with increased pre- and post-ovulatory follicle-stimulating hormone levels. *Endocrinology*, **126**, 1058-1063.

MOBERG, G. P. (1987). A model for assessing the impact of behavioural stress on domestic animals. *Journal of Animal Science*, **65**: 1228-1235.

MOLENAAR, G. J., Lugard-Kok, C., Meloen, R. H., Oonk, R. B., de Koning, J. and Wesing, C. J. G. (1993). Lesions in the hypothalamus after active immunisation against GnRH in the pig. *Journal of Neuroimmunology*, **48**: 1-12.

MONGET, P. and Monniaux, D. (1995). Growth factors and the control of folliculogenesis. *Journal of Reproduction and Fertility*, **49**: 321-333.

MONGET, P., Fabre, S., Muslant, P., Lecerf, F., Elsen, J. M., Mazerbourg, S., Pisselet, C. and Monniaux, D. (2002). Regulation of ovarian folliculogenesis by IGF and BMP system in domestic animals. *Domestic Animal Endocrinology*, **23**: 139-154.

MONNIAUX, D., Mariana, J. C. and Gibson, W. R. (1984). Action of PMSG on follicular populations in the heifer. *Journal of Reproduction and Fertility*, **70**, 243-253.

MONNIAUX, D., Chupin, D. and Saumande, J. (1983). Superovulatory responses of cattle. *Theriogenology*, **19**, (1): 55-81.

MONNIAUX, D. and Pisselet, C. (1992). Control of proliferation and differentiation of ovine granulosa cells by insulin-like growth factor-1 and follicle-stimulating hormone in vitro. *Biology of Reproduction*, **46**: 109-119.

MONTGOMERY, G. W., Galloway, S. M., Davis, G. H. and McNatty, K. P. (2001). Genes controlling ovulation rate in sheep. *Reproduction*, **121**: 843-852.

MOR, G. (1998). Plasmid DNA: A new era in vaccinology. *Biochemical Pharmacology*, **55**:1151-1153.

MORAN, C., Prendiville, D. J., Quirke, J. F. and Roche, J. F. (1990). Effects of oestradiol, zeranol and trenbolone acetate implants on puberty, reproduction and fertility in heifers. *Journal of Reproduction and Fertility*, **89**: 527.

MOREIN, B., Abusugra, I. and Blomqvist, G. (2002). Immunity in neonates. *Veterinary Immunology and Immunopathology*, **87**:207-213.

MORRIS, D. G., McDermott, M. G. and Sreenan, J. M. (1991). Effect of immunizing prepubertal lambs of low and high ovulation rate genotypes with inhibin partially purified from bovine follicular fluid. *Theriogenology*, 35: (2): 339-350.

MORRIS, D. G., McDermott, M. G., Diskin, M. G., Morrison, C. A., Swift, P. J. and Sreenan, J. M. (1993). Effect of immunisation against synthetic peptide sequences of bovine inhibin α-subunit on ovulation rate and twin-calving rate in heifers. *Journal of Reproduction and Fertility*, **97**: 255-261.

MORRIS, D.G. and Grealy, M. (1993) Effect of immunisation against bovine inhibin peptides on FSH and LH in heifers/ Journal of Reproduction and Fertility Abstract Series, **11**: Abstract 83.

MORRIS, D. G., Grealy, M. and Sreenan, J. M. (1995a) Immunisation against inhibin: antibody titre, gonadotrophin concentrations, ovulation rate and fertility in cattle. In Proceedings of the Irish Grassland and Animal Production Association (21st Meeting), pp. 175-176.

MORRIS, D. G., Grealy, M. and Sreenan, J. M. (1995b) Effect of immunisation against synthetic peptide sequences of bovine inhibin alpha-subunit on gonadotrophin concentrations in heifers. *Animal Reproduction Science* **38**; 63-71.

MORRIS, D.G., McDermott, M.G., Grealy, M., Diskin, M.G., Morrison, C. A., Swift, P.J. and Screenan, J. M. (1995). Effect of immunisation against synthetic peptide sequences of the α_N-subunit of bovine inhibin on ovulation rate, gonadotrophin concentrations and fertility in heifers. *Journal of Reproduction and Fertility*, **103**, 285-291.

MOSMANN, T. R., Sad, S., Krishnan, L., Wegmann, T. G., Guilbert, L. J. and Belosevic, M. (1995). Differentiation of subsets of CD4+ and CD8+ T cells. *Ciba Found Symp*, **195**: 42-50.

MOSMANN, T. R., Li, L., Hengarter, H., Kagi, D., Fu, W. and Sad, S. (1997). Differentiation of functions of T cell subsets. *Ciba Found Symp*, **204**: 148-154.

MULSANT, P. Lecerf, F., Fabre, S., Schibler, L., Monget, P., Lanneluc, I., Pisselet, C., Riquet, J., Monniaux, D., Callebaut, I., Cribiu, E., Thimonier, J., Teyssier, J., Bodin, L., Cognie, Y., Chitour, N. and Elsen, J. -M. (2001). Mutation in bone morphogenetic receptor-1B is associated with increased ovulation rate in Booroola Merino ewes. *Procedings of National Academic Science, USA*, **98**, 5104-5109.

MURPHY, M. G., Boland, M. P. And Roche, J. F. (1998). The effects of dose and duration of administration of pFSH during the first follicular wave on the ovulation rate of beef heifers. *Theriogenology*, **49**: 557-569.

NAKADA, K., Ishikawa, Y., Nakao, T. and Sawamukai, Y. (2002). Changes in responses to GnRH on LH and FSH secretion in prepubertal heifers. *Journal of Reproduction and Development*, **48**, (6), 545-551.

NAMER, I. J. and Steibel, J. (2000). Antibody directed against mannan of the Mycobacterium tuberculosis cell envelope provokes blood-brain barrier breakdown. *Journal of Neuroimmunology*, **103**, (1): 63-68.

Naor, Z., Clayton, R. N. and Catt, K. J. (1980). Characterization of gonadotrophin-releasing hormone receptors in cultured rat pituitary cells. *Endocrinology*, **107**: 1144-1152

NICHOLAS, B. Scougall, R. K., Armstrong, D. G. and Webb, R. (2002). Changes in insulin-like growth factor binding protein (IGFBP) isoforms during bovine follicular development. *Reproduction*, **124**: 439-446.

NISHIKAWA, Y. and Horie, T. (1955). *National Institute of Agricultural Science, Japanese Bulletin Series G., Animal Husbandry*, **10**, 229.

NOEL, B., Bister, J. L. and Parquay R. (1993). Ovarian follicular dynamics in Suffolk ewes at different periods of the year. *Journal of Reproduction and Fertility*, **99**: 695.

NOLAN, R., O'Callaghan, D., Duby, R. T., Lonergan, P. and Boland, M. P. (1998). The influence of short-term nutrient changes on follicle growth and embryo production following superovulation in beef heifers. *Theriogenology*, **50**, 1263-1274.

NORWITZ, E.R., Xu, S., Jeong, K-H., Bedecarrats, G.Y., Winebrenner, L.D., Chin, W.W. and Kaiser, U.B. (2002). Activin A augments GnRH-mediated transcriptional activation of the mouse GnRH receptor gene. *Endocrinology*, **143**, (3): 985-997.

O, W. S., Robertson, D. M. and de Kester, D. M. (1989). Inhibin as an oocyte meiotic inhibitor. *Molecular and Cellular Endocrinology,* **62**: 307-311.

O'BRIEN, C.A., Bloss, R.E. and Nicks, E. F. (1968). Effect of melengestrol acetate on the growth and reproductive physiology of fattening heifers. *Journal of Animal Science*, **27**: 664.

O'CALLAGHAN, D. and Boland M. P. (1999). Nutritional effects on ovulation, embryo development and the establishment of pregnancy in ruminants. *Animal Science*, **68**: 299-314.

O'CALLAGHAN, D., Vaakub, H., Hyttel, P., Spicer, L. J. and Boland, M. P. (2000). Effect of nutrition and superovulation on oocyte morphology, follicular fluid composition and systemic hormone concentrations in ewes. *Journal of Reproduction and Fertility*, **118**: 303-313.

OFFIT, P.A. and Hackett, C.J. (2003). Multiple vaccines and the immune system. In: *Vaccines*. Eds: S.A. Plotkin, W.A. Orenstein and P.A. Offit. Pg: 1583-1589.

ORTH, J. M., Higginbotham, C.A. and Salisbury, R. L. (1984). *Biology of Reproduction*, **30**, 263.

O'SHEA, T., Cummins, L. J., Bindon. B. M. and Findlay, J. K. (1982). Increased ovulation rate in ewes vaccinated with an inhibin-enriched fraction from bovine follicular fluid. *Proceedings of Australian Journal of Reproductive Biology*, **14**, *Abstract*, 85.

O'SHEA, T., Al-Obaidi, S. A. R., Bindon, B. M., Cummins, L. J., Findlay, J. K. and Hillard, M. A. (1984). Increased ovulation rate in Merino ewes and advancement of puberty in Merino lambs immunised with a preparation enriched in inhibin. In: Reproduction in Sheep, pp. 335-337 (Eds. D. R. Lindsey and D. T. Pearce) Australian Academy of Science, Canberra.

O'SHEA, T., Bindon, B. M., Piper, L. A., Al-Obaidi, S. A. R. and Cox, R. I. (1987). Immunisation of Booroola ewes with Fecundin or an inhibin-enriched preparation. *Proceedings of the Australian Society of Reproductive Biology*, **19**, *Abstract*, 54.

O'SHEA, T., Anderson, S. T., Bindon, B. M., Hillard, M. A. and Munro, R. K. (1989a). Immunisation against a synthetic inhibin fragment advances onset of puberty in female Merino lambs. *Journal of Reproduction and Fertility, Abstract Series*, **4**, (8).

O'SHEA, T., Bindon, B. M., Hillard, M. A., Piper, L. A., Findlay, J. K. and Miyamoto, K. (1989b). Increase in ovulation rate in Merino ewes after active immunisation with inhibin preparations obtained by immuno-affinity chromatography. *Reproduction, Fertility and Development*, **1**, 347-355.

O'SHEA, T., Andrews, C. A., Bindon, B. M., Hillard, M. A. and Sinosich, M. J. (1989c). Differential effects on ovulation rate and plasma FSH following immunisation of Meriono ewes with various inhibin preparations. *Proceedings of Australian Society of Reproductive Biology*, **21**, *Abstract*, 133.

O'SHEA, T., Bindon, B. M., Forage, R. G. and Findlay, J. K. (1993). Active immunization of Merino ewe lambs with recombinant bovine alpha inhibin advances puberty and increases ovulation rate. *Reproduction, Fertility and Development*, **5**: 173-180.

O'SHEA, T., Hillard, M. A. Anderson, S.T., Bindon, B. M., Findlay, J. K., Tsonis, C. G. and Wilkins, J. F. (1994). Inhibin immunisation for increasing ovulation rate and superovulation. *Theriogenology* **41**; 3-17.

OTSUKA, F., Moore, R.K., Lemura, S., Ueno, N. and Shimasaki, S. (2001a). Follistatin inhibits the function of the oocyte-derived factor BMP-15. *Biochemical and Biophysical Research Communications*, **289**, 961-966.

OTSUKA, F., Yamamoto, S., Erikson, G.F. and Shimasaki, S (2001b). Bone morphogenetic protein-15 inhibits follicle-stimulating hormone (FSH) action by suppressing FSH receptor expression. *Journal of Biological Chemistry*, **276**, 11 387-11 392.

OUSSAID, B., Lonergan, P., Khatir, H., Guler, A., Monniaux, D., Touze, J. L., Beckers, J. F., Cognie, Y. and Mermillod, P. (2000). Effect of GnRH antagonist-induced prolonged follicular phase on follicular atresia and oocyte developmental competence in vitro in superovulated heifers. *Journal of Reproduction and Fertility,* **118**: 137-144.

PADMANABHAN, V., Kesner, J. S. and Convey, E. M. (1981). A primary effect of luteinising-releasing hormone on bovine pituitary cells in vitro. *Journal of Animal Science*, **52**, 1137-1142.

PADMANABHAN, V., McFadden, K., Mauger, D. T., Karsch, F. J. and Midgley, A. J. (1997). Neuroendocrine control of follicle stimulation hormone (FSH) secretion. I. Direct evidence of separate episodic and basal components of FSH secretion. *Endocrinology*, **138**, 424-432.

PADMANABHAN and McNeilly, (2001). Is there an FSH releasing factor? *Reproduction*, **121**, 21-30.

PADMANABHAN, V. and Sharma, T. P. (2001). Neuroendocrine versus paracrine control of FSH. *Archives of Medical Research*, **32**, 533-543.

PADMANABHAN, V., Karsch, F. J. and Lee, J. S. (2002). Hypothalamic, pituitary and gonadal regulation of FSH. Reproduction Supplement, 59, 67-82.

PARTHASARATHY, V., Price, E. O., Orihuela, A., Dally, M. R. and Adams, T. E. (2002). Passive immunization of rams (Ovis aries) against GnRH: effects on antibody titre, serum concentrations of testosterone, and sexual behaviour. *Animal Reproduction Science*, **71**: 203-215.

PELL, J. M. (1990). The nutritional regulation of growth hormone action. Nutrition Research Reviews, **3**: 163-192.

PELLETIER, J. (1970). Mode of action of testosterone proprionate on the secretion and release of luteinising hormone (LH) in the castrated ram. *Acta Endocrinologica (Copenhagen)*, **63**, 290.

PELLETIER, J., Carrez-Camous, S. and Thiery, J. C. (1981). Basic neuroendrocine events before puberty in cattle, sheep and pigs. *Journal of Reproduction and Fertility* Suppl. **30**: 91-102.

PERTMER, T.M., Oran, A.E., Madorin, C.A. and Robinson, H.L. (2001). Th1 genetic adjuvants modulate immune responses in neonates. *Vaccine*, 1764-1771.

PETERS, A.R. (1985). Hormonal control of the bovine oestrous cycle. I. The natural cycle. *British Veterinary Journal*, 141, 564 - 575.

PETTY, R.E. and Hunt, D.W.C. (1998). Neonatal dendritic cells. *Vaccine*, **16**:14/15: 1378-1382.

PFAU, K. O., Bartlett, J. W. and Shuart, C. E. (1948) A study of multiple births in a Holstein-Friesian herd. *Journal of Dairy Science* **31**: 241-254.

PHILIPON, P. and Draincourt, M. A. (1987). Potential of active immunisation against androstenedione to improve fecundity in sheep. *Animal Reproduction Science*, 15, 101-112.

PICTON, H. M. and McNeilly, A. S. (1991). Evidence to support a follicle stimulating hormone threshold theory for follicle selection in ewes chronically treated with gonadotrophin-releasing hormone agonist. *Journal of Reproduction and Fertility*, **93**, 43-51.

PICTON, H. M. and Hunter, M. G. (1995). Purification and characterisation of a porcine follicular fluid protein which inhibits aromatase activity in granulosa cells in vitro. *Journal of Endocrinology, Supplement*, **147**, *Abstract*, P96.

PIERSON, R. A. and Ginther, O. J. (1984). Ultrasonography of the bovine ovary. *Theriogenology*, **21**, 495-504.

PIERSON, R. A. and Ginther, O. J. (1988). Ultrasound imaging of the ovaries and uterus in cattle. *Theriogenology*, **29**, 21-27.

POLLOCK, J. M., Rowan, T.G., Dixon, J. B., Carter, S. D and Kelly, D. F. (1991). Estimation of immunity in the developing calf: Cellular and humoral responses to keyhole limpet haemocyanin. *Veterinary Immunology and Immunopathology*, **29**, 105.

POPKIN, R. M. and Fraser, H. M. (1985). Changes in pituitary and ovarian LHRH receptors after active immunisation of female rats against LH or LHRH. *Journal of Reproduction and Fertility*, **73**, 491-496.

PRENDIVILLE, D. J., Enright, W. J., Crowe, M. A., Finnerty, M., Hynes, N. and Roche, J. F. (1995a). Immunisation of heifers against gonadotropin-releasing hormone: antibody titres, ovarian function, body growth, and carcass characteristics. *Journal of Animal Science*, **73**: 2382-2389.

PRENDIVILLE, D. J., Enright, W. J., Crowe, M. A., Vaughan, L. and Roche, J. F. (1995b). Immunisation of prepubertal beef heifers against gonadotropin-releasing hormone: immune, estrus, ovarian and growth responses. *Journal of Animal Science*, **73**:3030-3037.

PRENDIVILLE, D. J., Enright, W. J., Crowe, M. A., Finnerty, M. and Roche, J. F. (1996). Normal and induced secretory patterns of luteinising hormone and follicle stimulating hormone in anoestrous gonadotrophin-releasing hormone-immunised and cyclic control heifers. *Animal Reproduction Science*, **45**: 177-190.

PRICE, E. O. and Wallach, S. J. R. (1991a). Development of sexual and aggressive behaviour in Hereford bulls. *Journal of Animal Science*, **69**: 1019-1027.

PRICE, E. O. and Wallach, S. J. R. (1991b). Sexual and aggressive behaviours of Hereford bulls: interrelationship of variables and problems with the use of composite scores to describe overall sexual performance. *Journal of Animal Science*, **69**: 1028-1033.

PRICE, E. O. and Wallach, S. J. R. (1991c). Effects of group size and the male-to-female ratio on the sexual performance and aggressive behaviour of bulls in serving capacity tests. *Journal of Animal Science*, **69**: 1034-1040.

PRICE, E. O. and Wallach, S. J. R. (1991d). Ability to predict the adult sexual performance of bulls by prepubertal sexual behaviours. *Journal of Animal Science*, **69**: 1041-1046.

PRICE, T. D. and Wiltbank, J. N. (1978a) Dystocia in cattle. A review and implications. *Theriogenology* **9**: 195-219.

PRICE, T. D. and Wiltbank, J. N. (1978b) Predicting dystocia in heifers. *Theriogenology* **9**: 221- 249.

PRICE, M. A. and Tennesson, T. (1981). Pre-slaughter management and dark cutting in the carcasses of young bulls. *Canadian Journal of Animal Science*, **61**: 205-208.

PRICE, C. A., Morris, B. A., O'Shea, T. and Webb, R. (1987). Active immunization of cattle against partly purified follicular fluid from sheep. *Journal of Reproduction and Fertility*, **81**: 161-168.

RALPH, J. H. (1996). Factors affecting follicle and oocyte development in cattle. Ph.D. thesis, University of Edinburgh.

RAMIREZ, D. V. and McCann, S. M. (1963). Comparison of the regulation of luteinising hormone (LH) secretion in immature and adult rats. *Endocrinology*, **72**, 452-464.

RAWLINGS, N. C., Hafs, H. D. and Swanson, L. V. (1972). Testicular and blood plasma androgens in Holstein bulls from birth through puberty. Journal of Animal Science, **34**, 435-440.

RAWLINGS, N.C., Fletcher, P. W., Hendricks, D. M. and Hill, J. R. (1978). Plasma luteinising hormone (LH) and testosterone levels during sexual maturation in beef bull calves. *Biology of Reproduction*, **19**, 1108-1112.

RAWLINGS, N. C. and Churchill, I. J. (1990). Effects of naloxone on gonadotrophin secretion at various stages of development in the ewe lamb. *Journal of Reproduction and Fertility*, **89**: 503-509.

RAWLINGS, N. C. and Evans, A. C. O. (1995). Androgen negative feedback during the early rise in luteinising hormone secretion in bull calves. *Journal of Endocrinology*, **145**, 243-249.

RAWLINGS, N. C., Evans, A. C. O., Honaramooz, A. and Bartlewski, P.M. (2003). Antral follicle growth and endocrine changes in prepubertal cattle, sheep and goats. *Animal Reproduction Science*, **78**: 259-270.

REEVES, J. J., Chang, C. F., deAvila, D. M., Greiger, D. M., Johnson, H. E. and Roberts, A. J. (1989). Vaccines against endogenous hormones: A possible future tool in animal production. *Journal of Dairy Science*, **72**: 3363-3371.

REIBIGER, I. and Spanel-Borowski, K. (2000). Difference in localization of eosinophils and mast cells in the bovine ovary. *Journal of Reproduction and Fertility*, **118**: 243-249.

REICHERT, L. E. Jr. (1962). Preparation of purified bovine luteinizing hormone. *Endocrinology*, **71**: 729-733.

REICHERT, L. E. Jr. and Jiang, N. S. (1965). Studies on bovine pituitary follicular stimulating hormone. *Endocrinology*, **77**: 124-127.

RIDGE, J. P., Fuchs, F. and Matzinger, M. (1996). Neonatal tolerance revisited: turning on newborn T cells with dendritic cells. *Science*, **271**: 1723-1726.

RIVERA, G. M., Chandrasekher, Y. A., Evans, A. C. O., Giudice, L. C. and Fortune, J. E. (2001). A potential role for insulin-like growth factor binding protein-4 proteolysis in the establishment of ovarian follicular dominance in cattle. *Biology of Reproduction*, **65**: 102-111.

ROBERTS, V.J., Meunier, H., Vaughan, J., Rivier, J., Rivier, J., Vale, C., Sawchenko, P. (1989). Production and regulation of inhibin subunits in pituitary gonadotrophs. *Endocrinology*, **124**: 552-554.

ROBERTS, A.J. and Reeves, J.J. (1989). Reproductive and endocrine changes in ewes actively immunised against luteinizing hormone. *Journal of Reproductive Immunology*, **16**, 302.

ROBERTS, A.J. and Reeves, J.J. (1990). Reproductive and endocrine changes in ewes actively immunised against luteinizing hormone. *Journal of Reproductive Immunology*, **69**, 38.

ROBERTS, A.J. and Reeves, J.J. (1990). Reproductive and endocrine changes in ewes actively immunised against luteinizing hormone. *Journal of Reproductive Immunology*, **69**, 38.

ROBERTS, A.J., DeAvila, D.M., Gerber, J.D. and Reeves, J.J. (1990). Active immunsation of beef heifers against LH, I: Evaluation of protein carriers and adjuvants on antigenicity of LH. *Journal of Animal Science*, **68**: 3742.

ROBERTS, V. J., Barth, S., El-Roeiy, A. and Yen, S. S. C. (1993). Expression of inhibin/activin subunits and follistin messenger ribonucleic acids and proteins in ovarian follicles and the corpus luteum during the human menstrual cycle. *Journal of Clinical Endocrinology and Metabolism* **77**: 1402-1410.

ROBERTSON, D. M., Foulds, L. M., Leversha, L., Morgan, F. J., Hearn, M. T. W., Burger, H. G., Wettenhall., R. E. H. and de Kretser, D. M. (1985). Isolation of inhibin from bovine follicular fluid. *Biochemical and Biophysical Research Communications*, **126**, 220-226.

ROBERTSON, I. S., Wilson, J. C and Fraser, H. M. (1979). Immunological castration in male cattle. *The Veterinary Record*, **105**: 556-557.

ROBERTSON, I. S., Wilson, J. C., Rowland, A. C. and Fraser, H. M. (1981). Further studies on immunological castration in male cattle. *The Veterinary Record*, **108**: 381-382.

ROBERTSON, I. S., Fraser, H. M., Innes, G. M. and Jones, A.S. (1982). Effect of immunological castration on sexual and production characteristics in male cattle. *The Veterinary Record*, **111**: 529-531.

ROBERTSON, I. S., Wilson, J. C., Fraser, H. M., Innes, G. M. and Jones, A. S. (1984). Immunological castration of young bulls for beef production. In: Manipulation of growth in farm animals. (Eds.) Roche, J. F. and O'Callaghan, D. Martinus Nijhoff, The Hague, pp. 137-145.

ROBERTSON, D.M., Giacometti, M. F., Lahnstein, L.M., Goss, N.H., Hearn, M.T.W. and de Kretser, D.M. (1989). Isolation of inhibin α-subunit precursor proteins from bovine follicular fluid. *Endocrinology*, **125**: 2141-2149.

ROBERTSON, D. M., Hertan, R. and Farnworth, P. G. (2000). Is the action of inhibin mediated via a unique receptor? *Reviews of Reproduction*, **5**, 131-135.

ROBINSON, T. J. and Shelton, J. N. (1991). Reproduction in cattle. In: Reproduction in domestic animals, (4th edition). Eds: P. T. Cupps. New York and London, Academic Press, pp; 445-470.

ROBINSON, J. J. (1990). Nutrition in the reproduction of farm animals. *Nutrition Research Reviews*, **3**, 253-276.

ROBINSON, J. E., Birch, R. A., Grindrod, J. A. E., Taylor, J. A and Unsworth, W.P (2003). Sexually differentiated regulation of GnRH release by gonadal steroid hormones in sheep. *Reproduction Supplement*, **61**, 299-310.

ROCHA, A., Carpenter, B. B., Hawkins, H. E., Sprou, L. R. and Forrest, D. W. (1994). Relationship between seminal vesicle size and copulatory activity in bulls. *Theriogenology*, **42**, 1177-1181.

ROCHE, J. F. and Crowe, M. A. (2004). Suppression of sexual behaviour in farm animals by GnRH immunization and the implications for productivity and welfare. *Proceedings of the British Society of Animal Science (abstract)* pg 260.

RODEN, L.D., Smith, B.P., Spier, S.J. and Dilling, G. W. (1992). Effect of calf age and Salmonella bacterin type on ability to produce immunoglobulins directed against salmonella whole cells or lipopolysaccharide. *American Journal of Veterinary Research*, **53**, 1895.

RODRIGUEZ, R. E. and Wise, M. E. (1989). Ontogeny of pulsatile secretion of gonadotrophin-releasing hormone in the bull calf during infantile and pubertal development. *Endocrinology*, **124**, 248-256.

RODTIAN, P., King, G., Subrod, S. and Pongpiachan, P. (1996). Oestrus behaviour of Holstein cows during cooler and hotter tropical conditions. *Animal Reproduction Science*, **45**, 47-58.

ROUILLIER, P., Matton, P., Sirard, M.-A. and Guilbault, L. A. (1998). Effects of bovine follicular fluid from healthy and atretic follicles on follicle-stimulating hormone-induced production of estradiol be bovine granulosa cells cultured in vitro. *Journal of Animal Science*, **76**: 1172-1177

ROUSSEAU, L., Dupont, A., Labrie, F. and Courture, M. (1998). Sexuality changes in prostate cancer patient receiving antihormonal therapy combining the antiandrogen flutamide with medical (LHRH agonist) or surgical castration. *Archives of Sexual Behaviour*, **17**, 87-98.

ROWE, J., Poolman, J.T., Sly, P.D., Loh, R. and Holt, P.G. (2004). Enhancement of vaccine-specific cellular immunity in infants by passively aquired maternal antibody. *Vaccine, in press* : www.sciencedirect.com

ROY, S. K. and Greenwald, G. S. (1989). Hormonal requirements for the growth and differentiation of hamster preantral follicles in long-term culture. *Journal of Reproduction and Fertility*, **87**, 103-114.

RUDDER, T.H. and Corlis, P.L. (1974). Spaying permits planned sales. *Queensland Agricultural Journal*, **100**: 619.

KUKAVINA, D. and Podack, E. R. (2000). Abundant perforin expression at the maternal-fetal interface: guarding the semiallogenic transplant?. *Immunology Today*, **21** (4): 160-163.

RUTLEDGE, J. J. (1975) Twinning in cattle. *Journal of Animal Science* **40**: 803-815.

SACHS, B. D. and Meisel, R. L. (1988). The physiology of male sexual behaviour. In: *The Physiology of Reproduction*, II. (Eds): E. Knobil and J. D. Neill. New York, Raven Press. Pg: 1393-1482.

SAUMANDE, J. (1990). Culture of bovine granulosa cells in a chemically defined serum free medium. The effect of insulin and fibronectin on the response to FSH. *Journal of Steroid Biochemistry*, **38**: 189-196

SAVIO, J. D., Keenan, L.., Boland, M. P. and Roche, J. F. (1988). Pattern of growth of dominant follicles during the oestrous cycle in heifers. Journal of *Reproduction and Fertility*, **83**, 663-671.

SCANLON, A. R., Sunderland, S. J., Martin, T. L., Goulding, D., O'Callaghan, D., Williams, D. H., Headon, D. R., Boland, M. P., Ireland, J. J. And Roche, J. F. (1993). Active immunisation of heifers against a synthetic fragment of bovine inhibin. *Journal of Reproduction and Fertility*, **97**: 213-222.

SCARAMUZZI, R. J. and Baird, D. T., (1976). The oestrous cycle of the ewe after active immunisation against prostaglandin F-2α. *Journal of Reproduction and Fertility*, 46, 39-47.

SCARAMUZZI, R. J., Davidson, W. G. and Van Look P. F. A. (1977). Increaing ovulation rate in sheep by active immunization against an ovarian steroid, androstenedione. *Nature*, **269**: 817-818.

SCARAMUZZI, R. J., Martinez, N. D. and Van Look, P. F. A. (1980). Ovarian morphology and the concentrations of steroids, and of gonadotrophins during the breeding season in ewes actively immunised against oestradiol-17β or oestrone. *Journal of Reproduction and Fertility*, **59**, 303-310.

SCARAMUZZI, R. J., Baird, D. T., Clarke, I. J., Martinez, N. D. and Van Look, P. F. A. (1980). Ovarian morphology and the concentration of steroids during the oestrous cycle of sheep actively immunised against androstenedione. *Journal of Reproduction and Fertility*, **58**, 27-35.

SCARAMUZZI, R. J. and Hoskinson, R. M. (1984). Active immunisation against steroid hormones for increasing fecundity. In *Immunological Aspects of Reproduction in Mammals*, pp: 445-474. Ed. D. B. Crighton. Butterworths, London.

SCARAMUZZI, R. J., Adams, N. R., Baird, D. T., Campbell, B. K., Downing, J. A., Findlay, J. K., Henderson, K. M., Martin, G. B., McNatty, K. P., McNeilly, A. S. and Tsonis, C. G. (1993). A model for follicle selection and the determination of ovulation rate in the ewe. *Reproduction, Fertility and Development*, **5**, 459-478.

SCARAMUZZI, R. J. (1994) The role of metabolic hormones in mediating nutritional influences on ovarian function. *Journal of Reproduction and Fertility*, Abstract Series **No. 14**, p.2.

SCHALLENBERGER, E. and Peterson, A. J. (1982). Effect of ovariectomy on tonic gonadotrophin secretion in cyclic and post-partum dairy cows. *Journal of Reproduction and Fertility*, **64**, 47-52.

SCHALLY, A.V., Arimura, A., Kastin, A.J., Matsuo, H., Baba, Y., Redding, T.W., Nair, R.M.G. and Debeljuk, L. (1971). The gonadotrophin-releasing hormone: One peptide regulates the secretion of luteinising and follicle stimulating hormones. Science. **173**, 1036-1038.

SCHALLY, A. V., Arimura, A and Kastin, A. J. (1973) Hypothalamic regulating hormones. *Science*, **179**: 341.

SCHAMS, D. and Karg, H. (1969). *Acta endocrinologica, Copenhagen* 61, 69.

SCHAMS, D. and Schallenberger, E. (1976). *Acta endocrinologica, Copenhagen*, **81**, 461.

SCHAMS, D., Schallenberger, E., Hoffman, B. and Karg, H. (1977). The oestrous cycle of the cow: hormonal parameters and time relationships concerning oestrus, ovulation and electrical resistance of the vaginal mucus. *Acta Endocrinologica*, **86**: 180-192.

SCHAMS, D., Gombe, S., Schallenberger, E., Reinhardt, V. and Claus, R. (1978). *Journal of Reproduction and Fertility*, **54**, 145.

SCHAMS, D., Schallenberger, E., Gombe, S. and Karg, H. (1981). Endocrine patterns associated with puberty in male and female cattle. *Journal of Reproduction and Fertility, Supplement.* **30**, 103-110.

SCHANBACHER, B. D. and Ford, J. J. (1977). Gonadotrophin secretion in cryptorchid and castrate male rams and the acute effects of exogenous steroid treatment. *Endocrinology*, **100**, 387.

SCHANBACHER, B. D. (1979). Relationship of in vitro gonadotrophin binding to bovine testes and the onset of spermatogenesis. *Journal of Animal Science*, **48**: 591.

SCHANBACHER, B. D. (1980). Testosterone regulation of luteinising hormone and follicle stimulating hormone secretion in young male lambs. *Journal of Animal Science*, **51**, 679.

SCHANBACHER, B. D. (1981). Importance of the episodic nature of luteinizing hormone secreation for normal development of the bovine testis during puberty: Interference with oestradiol-17 beta. *Journal of Endocrinology*, **88**: 393

SCHANBACHER, B. D. (1982). Responses of ram lambs to active immunisation against testosterone and luteinising hormone releasing hormone. *Americam Journal of Physiology*, **242**, E201.

SCHANBACHER, B. D., D'Occhio, M. J. and Gettys, T. W. (1983). Pulsatile luteinising secretion in the castrate male bovine: effects of testosterone or oestradiol replacement therapy. *Journal of Animal Science*, **56**, 132-138.

SCHANBACHER, B. D. (1984). Active immunisation against LHRH in the male. In: Crighton, D. B. (Ed.), Immunological aspects of reproduction in mammals. Butterworths, London, pp. 345-362.

SCHANBACHER, B. D. (1985). Effects of active immunisation of the ram and bull against luteinising hormone. *Theriogenology*, **24**, 59.

SCHANBACHER, B. D. (1988). Increased ovulatory response of Suffolk sheep vaccinated against a synthetic fragment of porcine inhibin. *Biology of Reproduction*, **38**: *(Suppl 1.)* : 40.

SCHANBACHER, B. D. (1989). Ovulatory response and pituitary FSH charge microheterogeneity in beef heifers vaccinated against synthetic porcine inhibin alpha. *Journal of Animal Science*, **67**: *(Suppl. 1)*: *Abstract* 876.

SCHANBACHER, B. D. (1991). Pituitary and testicular responses of beef bulls to active immunisation against inhibin alpha. *Journal of Animal Science*, **69**, 252-257.

SCHANBACHER, B. D., Schemm, S. R. and Rhind, S. M. (1991). Gonadotrophin concentrations and ovulation rates in Suffolk ewes actively of passively immunised against inhibin alpha. *Journal of Reproduction and Fertility*, **93**, 133-139.

SCHILLO, K. K., Dierschke, D. J. and Hauser, E. R. (1982). Influences of month of birth and age on patterns of luteinizing hormone secretion in prepubertal heifers. Theriogenology, **18**: (**5**): 593-598.

SCHWALL, R. H., Mason. A. J., Wilcox, J. N., Basset, S. G. and Zeleznik, A. J. (1990). Localization of inhibin/activin subunit mRNAs within the primate ovary. *Molecular Endocrinology,* **4**: 75-79

SEEBURG, P.H. and Adelman, J.P. (1984). Characterisation of cDNA for precursor of human luteinising hormone. *Nature*, **311**, 666-668.

SEEBURG, P. H., Mason, A. J., Stewart, T. A. and Nikolics, K. (1987). The mammalian GnRH gene and its pivotal role in reproduction. Recent Progress in Hormone Research, 43, 69-97.

SEJRSEN, K., Enright, W. J. and Prendiville, D. J. (1994). Prevention of puberty by GnRH immunization of prepubertal heifers: plasma hormones and mammary growth. *Journal of Animal Science*, **72**: (1): *Abstract*, 1098; pg; 285. *Journal of Dairy Science*, **77**: (1) *Abstract*, 1098; pg; 285.

SENGER, P. L. (1997) Pathways to Pregnancy and Parturition (ed. P. L. Senger) Current Conceptions, inc. Washington.

SETCHELL, B. P. and Jacks, F. (1974). Inhibin-like activity in rete testis fluid. *Journal of Endocrinology*, **62**, 675-676.

SHACKELL, G. H., Hudson, N. L., Heath, D. A., Lun, S., Shaw, L., Condell, L., Blay, L. R. and McNatty, K. P. (1993). Plasma gonadotrophin concentrations and ovarian characteristics in Inverdale ewes that are heterozygous for a major gene ($FecX^{I}$) on the X chromosome that influences ovulation rate. *Biology of Reproduction*, **48**, 1150-1156.

SHARPE, P.J. and Fraser, H. M. (1978). Control of reproduction. In: The Endocrine Hypothalamus. (Eds), S. L. Jeffcoate and J. S. M. Hutchinson). Acadaemic Press. London. Pp: 271-332.

SHARPE, R. M., Donachie, K. and Cooper, I. (1988). Re-evaluation of the intratesticular levels of testosterone required for quantitative maintenance of spermatoogenesis in the rat. *Journal of Endocrinology*, **117**, 19-26.

SHARPE, R. M. (1990). Intratesticular control of steroidogenesis (review). *Clinical Endocrinology*, **33**, 787-807.

SHIMASAKI, S., Moore, R. K., Erickson, G. F. and Otsuka, F. (2003). The role of bone morphogenetic proteins in ovarian function. *Reproduction Supplement*, **61**: 323-337

SHORT, R. V. (1974). *Sexual Endocrinology of the perinatal period*, pg 121-142. Ed by Forest, M. G. and Bertrand, J. INSERM, Paris.

SHORT, R. V., Flint, A. P. F. and Renfree, M. B. (1985). Influence of passive immunisation against GnRH on pregnancy and parturition in the tammar wallaby, Macropus eugenii. *Journal of Reproduction and Fertility*, **75**: 567- 575.

SIEGRIST, C. A., Saddallah, F., Tougne, C., Martinez, X., Kovarik, J. and Lambert, P. H. (1998). Introduction of neonatal TH1 and CTL responses by live viral vaccines: a role for replication patterns within antigen presenting cells ?. *Vaccine*, **16**: 1473-1478.

SILVA, C. C. and Knight, P. G. (1998) Modulatory actions of activin-A and follistatin on the developmental competence of in vitro-matured bovine oncycles, *Biology of reproduction*, **58**: 558-565

SILVA, C. C., Groome, N. P. and Knight, P. G. (1999). Demonstration of a suppressive effect of inhibin α-subunit on the developmental competence of in vitro matured bovine oocytes. *Journal of Reproduction and Fertility*, **115**: 381-388.

SILVA, C. C., Groome, N. P. and Knight, P. G. Immunohistochemical localization of inhibin/activin α, βA and βB subunits and follistatin in bovine oocytes during in vitro maturation and fertilization (2003). *Reproduction*, **125**: 33-42.

SILVA, J. C. e., da Costa, L. L.. and Silva, J. R. (2002). Embryo yield and plasma progesterone profiles in superovulated dairy cows and heifers. *Animal Reproduction Science*, **69**, 1-8.

SILVERMAN, A. J. (1987). The gonadotrophin releasing hormone (GnRH) releasing neural systems: Immunocytochemistry. In: The physiology of reproduction. (Eds.E. Knobil, J. Neill, L. L. Ewing, G. S. Greenway, C. L. Markert and D. W. Pfaff). Raven Press, New York. pp1283-1305.

SILVERSIDES, D.W., Allen, A. F. Misra, V., Qualtiere, L., Mapletoft, R. J, and Murphy, B. D. (1988) A synthetic luteinizing hormone releasing hormone vaccine. I. Conjugation and specificity trials in BALB/c mice. *Journal of Reproductive Immunology*, **13**, 249-261.

SINGH, R. R., Hahn, B. H. and Sercarz, E. E. (1996). Neonatal peptide exposure can prime T cells and, upon subsequent immunization, induce their immune deviation: implications for antibody vs. T cell-medicated autoimmunity. *Journal of Experimental Medicine*, **183**: 1613-1621

SIROIS, J. and Fortune, J. E. (1988). Ovarian follicular dynamics during the oestrous cycle in heifers monitored by real-time ultrasonography. *Biology of Reproduction*, **39**: 308-317.

SIROIS, J. and Fortune, J. E. (1990). Lengthening the bovine oestrous cycle with low levels of exogenous progesterone. *Endocrinology*, **127**, 916-925.

SKAGGS, C. L., Able, B. V. and Stevenson, J. S. (1986). Pulsatile or continuous infusion of luteinising hormone-releasing hormone and hormonal concentrations in prepubertal beef heifers. *Journal of Animal Science*, **62**, 1034-1048.

SMITH, J. F., Jagusch, K. T. and Farquhar, P. A. (1983). The effects and timing of flushing on the ovulation rate of ewes. *Procceedings of the New Zealand Society Animal Production*, **43**: 13-16.

SMITH, J. F. (1985). Steroid immunization of ewes to increase lambing. *Proceedings of the New Zealand Society of Animal Production*, **45**: 171-198.

SMITH, F. J. (1988). Nutrition and ovulation rate in the ewe. *Australian Journal of Biological Science*, **41**: 27-36.

SMITH, P., Hudson, O, W-S., Hudson, N. L., Shaw, L., Heath, D. A., Condell, L., Philips, D. J. and McNatty, K. P. (1993). Effects of Booroola (Fec B) on body weight, ovarian development and hormone concentrations during fetal life. *Journal of Reproduction and Fertility*, **98**: 41-54.

SMITH, M. J. and Jennes, L. (2001). Neural signals that regulate GnRH neurones directly during the oestrous cycle. *Reproduction*, **122**: 1-10.

SOSA, J. M., Zhang, Y., de Avila, D. M., Bertrand, K. P. and Reeves, J. J. (2000). Technical note: Recombinant LHRH fusion protein suppresses estrus in heifers. *Journal of Animal Science*, **78**: 1310-1312.

SOUZA, C. J. H., MacDougall, C., Campbell, B. K., McNeilly, A. S. and Baird, D. T. (2001). The booroola (FecB) phenotype is associated with a mutation in the bone morphogenetic receptor type 1 B (BMPR1B) gene. *Journal of Endocrinology*, **169**: R1-R6

SOUZA, C. J. H., Campbell, B. K., McNeilly, A. S. and Baird, D. T. (2002). Effect of bone morphogenetic protein 2 (BMP2) on oestradiol and inhibin A production by sheep granulosa cells, and localization of BMP receptors in the ovary by immunohistochemistry. *Reproduction*, **123**, 363-369.

SOUZA, C. J. H., Campbell, B. K., McNeilly, A. S. and Baird, D. T. (2003). Bonemorphogenetic proteins and folliculogenesis: lessons from the Booroola mutation. *Reproduction Supplement*, **61**: 361-370.

SPICER, L. J., Alpizar, E. and Echternkamp, S. E. (1993). Effects of insulin-like growth factor I, and gonadotropins on bovine granulosa cell proliferation, progesterone production, estradiol production and (or) insulin-like growth factor I production in vitro. *Journal of Animal Science*, **71**: 1232-1241

SREENAN, J. M. (1984). Steroid immunization in cows: potential for increasing ovulation and twinning rates. *10th International Congress on Animal Reproduction and Artificial Insemination (Urbana-Champaign)* **4**:22-27.

STAIGMILLER, R. B. and England, B. G. (1982) Folliculogenesis in the bovine. *Theriogenology*, **17**: 43-52.

STEINBERGER, A. (1981). Regulation of inhibin secretion in the testis. In *Intragonadal regulation of reproduction*, pg: 283-298 (Eds) P. Franchimont and C. P. Channing. Acadaemic Press, London.

STEINBERGER and Steinberger, (1976). *Endocrinology*, **99**, 918.

STEVENS, V. (1993). Vaccine delivery systems: potential methods for use in antifertility vaccines. *American Journal or Reproductive Immunology*, **29**, 176-188.

STOCK, A. E. and Fortune, J. E. (1993). Ovarian follicular dominance in cattle: relationship between prolonged growth of the ovulatory follicle and endocrine parameters. Endocrinology, 132, 1108-1114.

STRAIGMILLER, R. B., England, B. G., Webb, R., Short, R. E. and Bellows, R. A. (1982). Estrogen secretion and gonadotropin binding by individual bovine follicles during estrus. *Journal of Animal Science*, **55**: (6): 1473-1482.

STUMPF, T. T., Wolfe, M. W., Robertson, M. S., Kittok, R. J and Kinder, J. E. (1993). Season of the year influences concentration and pattern of gonadotrophins and testosterone in circulation of the bovine male. *Biology of Reproduction*, **49**, 1089-1095.

SUEN, Y., Lee, S., Qian, J., Van de Ven, C. and Cairo, M.S. (1998). Dysregulation of lymphokine production in the neonate and its impact on neonatal cell mediated immunity. *Vaccine*, **16**:14/15:1369-1377.

SUJARIT, S. and Pholpramool, C. (1985). *Journal of Reproduction and Fertility*, **74**, 497-502.

SUNDBY, A. and Velle, W. (1980). Plasma concentrations of testosterone in young bulls in relation to age, rate of mass gain and stimulation with human chorionic gonadotrophin. *Journal of Endocrinology*, **86**, 465-469.

SUNDERLAND, S.J., Goulding, D., Kane, M., Headon, D. R., Boland, M. P. and Roche, J. F. (1991). Ovarian responces in beef heifers following immunization against the α1-26 bovine inhibin fragment. *Journal of Reproduction and Fertility*, Abstract Series **7**: 27

SUNDERLAND, S. J., Crowe, M. A., Boland, M. P. Roche, J. F. and Ireland, J. J. (1994). Selection, dominance and atresia of follicles during the oestrous cycle of heifers. *Journal of Reproduction and Fertility*, **101**: 547-555.

SWANSON, L.V., Wetteman, R. P., Rawlings, N. C., Hafs, H. D and Magee, W. T. (1971). Pubertal relationships of some endocrine and reproductive criteria in Hereford bulls. *Journal of Animal Science*, **33**, 823-828.

SYRSTAD, O. (1984). Inheritance of multiple births in cattle. Livestock Production Science, **11**, 373-380.

TAKEDOMI, T., Aoyagi, Y., Konishi, M., Kishi, H., Taya, K., Watanabe, G. and Sasamoto, S. (1995). Superovulation of Holstein heifers by a single subcutaneous injection of FSH dissolved in polyvinylpyrrolidone. *Theriogenology*, **43**, 1259-1268.

TAKEDOMI, T., Kaneko, H., Aoyagi, Y., Konishi, M., Kishi, H., Watanabe, G. And Taya, K. (1997). Effect of passive immunisation against inhibin on ovulation rate and embryo recovery in holstein heifers. *Theriogenology*, **47**: 1507-1518.

TANABE, T. Y. and Salisbury, G. W. (1946). The influence of age on breeding efficiency of dairy cattle in artificial insemination. *Journal of Dairy Science*, **29**: 337.

TANNETTA, D., Fray, M. D., Wrathall, J. H. M., Bleach, E. C. L., Glencross, R. G. and Knight, P.G. (1997). Effects of supplementary treatment with bovine growth hormone on hormonal and ovulatory responses to inhibin immunisation in the ewe. *Journal of Reproduction and Fertility*, 110, 255-262.

TANNETTA, D., Feist, S. A., Bleach, E. C. L., Groome, N. P., Evans, L. W. and Knight, P.G. (1998). Effect of active immunisation of sheep against an amino terminal peptide of the inhibin α_C –subunit on intrafollicular levels of activin A, inhibin A and follistatin. *Journal of Endocrinology*, **157**, 157-168.

TANRIVERDI, F., Silveira, L.F., MacColl, G.S. and Bouloux, P.M. (2003). The hypothalamic-pituitary-gonadal axis: immune function and autoimmunity. *Journal of Endocrinology*. **176**:3:293-304.

TEAGUE, G. L., Boyd, G. W., Smith, G. C., Finnerty, M., Rollin, B. E., Field, T. G., Grandin, T., Kniffen, D. M., Taylor, R. E. and Enright, W. J. (1992). Bovine carcass characteristics and overall lean meat production efficiency of early castrates (EC), late castrates (LC), immunocastrates (IC) and intact males (IM). *Journal of Animal Science, suppl, Abstract,* **70**: (**1**): 103.

TERQUI, M., Wrathall, J. H. M., Draincourt, M. A. and Knight, P. G. (1995). Modulation of ovarian function by steroid and inhibin immunisation. *Livestock Production Science*, 42: 181-192.

THIBIER, M. (1976). Diurnal testosterone and 17α-hydroxyprogesterone in peripheral plasma of young post-pubertal bulls. *Acta Endocrinologica*, **81**, 623-634.

THOMAS, G. B., McNeilly, A.S., Gibson, F and Brooks, A. N. (1994). Effects of pituitary-gonadal suppression with a gonadotrophin-releasing hormone agonist on steroid-secretion in the sheep. *Journal of Endocrinology*, **141**, 317-324.

THOMAS, G. B., McNeilly, A.S. and Brooks, A. N. (1995). Effect of suppression of pituitary-testicular function during fetal life with a GnRH agonist on reproductive development in ram lambs during the first 28 weeks of life. *Journal of Reproduction and Fertility* (*Suppl.*), **49**: 533-537.

THOMPSON Jr., D. L. (2000). Immunization against GnRH in male species (comparative aspects). *Animal Reproduction Science*, **60-61**: 459-469.

THUN, R., Jaros, P., Claus, R. and Hennessy, D. P. (2003). Immunocastration as an alternative to surgical castration of male piglets in Switzerland. *Reproduction in Domestic Animals*, **38**, (4). *Abstract*, W4.1, pp; 321.

THUNDATHIL, J., Palasz, A. T., Mapletoft, R. J. and Barth, A. D. (1999). An investigation of the fertilizing characteristics of pyriform-shaped bovine spermatozoa. *Animal Reproduction Science*, **57**, 35-50.

TILBROOK, A.J., De Kretser, J.T., Cummins, J.T. and Clarke, I.J. (1991). The negative feedback effects of testicular steroids are predominantly at the hypothalamus in the ram. *Endocrinology*, **129** (6): 3080-3092.

TILDERS, F. J. H., van Oers, J. W. A. M., White, A., Menzaghi, F. and Burlet, A. (1990). Antibodies to neuropeptides: biological effects and mechanisms of action. In: *Circulating Regulatory and Neuroendocrine Function*. (Eds): J. C. Porter and D. Jezova. Plenum Press, New York, NY, PP, 135-146.

TISDALL, D.J., Watanabe, K., Hudson, N.L., Smith, P. and McNatty, K.P. (1995). Fsh receptor gene expression during follicle development in sheep. *Journal of Molecular Endocrinology*, **15**: 273-281.

TIZZARD, I. (1977). Immunity in the fetus and newborn animal. In: An Introduction to Veterinary Immunology, **10**:155-168. Saunders, Philadelphia.

TIZZARD, I. (1982). Antigens and antigenicity. In: An Introduction to Veterinary Immunology (second edition). W. B Saunders Company, London, pp, 34-37.

TODD, M. G., and Cowell, M. D. (1981). Within sale price variation of cattle and carcase auctions. *Australian Journal of Agricultural Economy*, **25**, 30-47.

TORTONESE, D. J. and Inskeep, E. K. (1992). Effects of melatonin treatment on the attainment of puberty in the heifer. *Journal of Animal Science*, **70**, 2822-2827.

TRIGG, T. E. (1994). Vaccines in Agriculture-Reproduction Vaxstrate-the commercial perspective.

TRIMBERGER, G. W. (1948). Breeding efficiency in dairy cattle for artificial insemination at various intervals before and after ovulation. *Nebraska Agricultural Experimental Station Research Bulletin*, **153**.

TSAI, H-W. and Legan, S. J. (2002). Loss of luteinising hormone surges induced by chronic estrdiol is associated with decreased activation of gonadotrophin-releasing hormone neurons. *Biology of Reproduction*, **66**, 1104-1110.

TSONIS, C. G., Pearson, M., Hungerford, J., Borchers, C. E., Greenwood, P. E., Forage, R. G., Doughton, B. and Findlay, J. K. (1989). Immunising sheep with recombinant inhibin subunit increases the number of lambs born. *Proceedings of Australian Society of Reproductive Biology*, **21**, *Abstract*, 99.

TUCHSCHERER, M., Kanitz, E., Otten, W. and Tuchscherer, A. (2002). Effects of prenatal stress on cellular and humoral immune responses in neonatal pigs. *Veterinary Immunology and Immunopathology*, **86**: 195-203.

TURIN, E. M., Nagle, C. A., Lahoz, M., Torres, M., Turin, M., Mendizabel, A. F. and Escofet, M. B. (1997). Effects of a copper bearing intrauterine device on ovarian function, body weight gain and pregnancy rate of nulliparous heifers. *Theriogenology*, **47**, 1327-1336.

TURZILLO, A. and Fortune, J. (1990). Suppression of secondary FSH surge with bovine follicular fluid is associated with delayed ovarian follicular development in heifers. *Journal of Reproduction and Fertility*, **89**: 643-653.

ULIBARRI, C. and Yahr, P. (1996). Effects of androgens and oestrogens on sexual differentiation of sex behaviour, scent marking, and the sexually dimorphic area of the gerbil hypothalamus. *Hormones and Behaviour*, **30**:107-130.

VAITUKAITIS, J. L., Ross, G. T., Braunstein, G. D. and Rayford, P. L. (1976). Gonadotrophins and their subunits: basic and clinical studies. *Recent Progress in Hormone Research*, **32**: 289-331.

VANDEMARK, N. L. (1956). "Quantitative aspects of semen production in bulls. 3rd International Congress on Animal Reproduction, Cambridge, England. Pg: 80.

VAN DISSEL-EMILIANI, F. M., Grootenhuis, A. J. de Jong, F. H. and de Rooij, D. G. (1989). Inhibin reduces spermatogonial numbers in testes of adult mice and Chinese hamsters. *Endocrinology*, **125**, 1899-1903.

VAN VLECK, L. D., Gregory, K. E. and Echternkamp, S. E. (1991) Ovulation rate and twinning rate in cattle: Heritabilities and genetic correlation. *Journal of Animal Science* **69**: 3213-3219.

VAN VLECK, L. D. and Gregory, K. E., (1996) Genetic trend and environmental effects in a population of cattle selected for twinning. *Journal of Animal Science* **74**: 522-528

VERHOEVEN, G. and Cailleau, J. (1988). Follicle-stimulating hormone and androgens increase the concentration of the androgen receptor in Sertoli cells. *Endocrinology*, **122**, (4): 1541.

VILCHEZ-MARTINEZ, J. A. and Marin De Lopez, G. (1980). *Federation Proceedings*, **39**: 822 (abstract 2935).

VIZCARRA, J. A. and Wettemann, R. P. (1994). Immunization of beef heifers against GnRH prevents luteal activity and pregnancy. *Journal of Animal Science, Abstract*: **72**: (**1**): pg; 285: *Journal of Dairy Science, Abstract*: **77**: (1): 1099.

VOGEL, F.R. and Hem, S.L. (2003). Immunological adjuvants. In: *Vaccines*. Eds: S.A. Plotkin, W.A. Orenstein and P.A. Offit. Pg: 69.

WALKER, W. L., Nebel, R. L. and McGilliard, M. L. (1996). Time of ovulation in relation to mounting activity in dairy cattle. *Journal of Dairy Science*, **79**, 1555-1561.

WALTERS, D. L., Short, R. E., Convey, E. M., Staigmiller, R. B., Dunn, T. G. and Kaltenbach, C. C. (1982). Pituitary and ovarian function in postpartum beef cows. III. Induction of oestrus, ovulation and luteal function with intermittent small-dose injections of GnRH. *Biology of Reproduction*, **26**: 655-662.

WALTERS, D. L., Schams, D. and Schallenberger, E. (1984). Pulsatile secretion of gonadotrophins, ovarian steroids and ovarian oxytocin during luteal phase of the oestrous cycle in the cow. *Journal of Reproduction and Fertility*, **71**: 479-491.

WALTON, J. S. (1985). Effect of active immunization against androstenedione on ovarian function in dairy cows and prepubertal gilts. *Animal Reproduction Science*, **8**: 349-364.

WALTON, J. S., Veenhuizen, L. P. and King, G. J. (1987). Relationship between time of day, oestrus behaviour and the preovulatory luteinising hormone surge in Holstein cows after treatment with cloprostenol. *Journal of Dairy Science*, **70**, 1652-1663.

WANDJI, S. A., Pelletier, G. and Sirard, M. A. (1992). Ontogeny and cellular localization of ^{125}I-labelled insulin-like growth factor-1, ^{125}I-labelled follicle-stimulating hormone, and ^{125}I-labelled human chorionic gonadotropin binding sites in ovaries from bovine fetuses and neonatal calves. *Biology of Reproduction*, **47**: 814-822

WANG, H., Wu, M., Patt, D., Murphy, B. D. and Mapletoft, R. J. (1988). Superovulation in beef heifers with PMSG: effect of dose and monoclonal antibodies to PMSG. *Theriogenology*, **29**, (1): 323.

WANG, X-N and Greenwald, G.S. (1993). Hypophysectomy of the cyclic mouse. 1. Effects on folliculogenesis, oocyte growth and follicle-stimulating hormone and human chorionic gonedotophin receptors. *Biology of Reproduction*, **48**: 585-594.

WEBB, R., Lamming, G. E., Haynes, N. B. and Foxcroft, G. R. (1980). Plasma progesterone and gonadotrophin concentrations and ovarian activity in postpartum dairy cows. *Journal of Reproduction and Fertility*, **50**: 203-210.

WEBB, R. and England, B. G. (1982). Relationship between LH receptor concentrations in thecal and granulosa cells and in-vivo and in-vitro steroid secretion by ovine follicles during the preovulatory period. *Journal of Reproduction and Fertility*, **66**: 169-180.

WEBB, R., Baxter, G., McBride, D., Nordblom, C. D. and Shaw, M. P. K. (1985). The measurement of testosterone and oestradiol- 17β using iodinated tracers and incorporating an affinity chromatography extraction proceedure. *Journal of Steroid Biochemistry*, **23**: 1043-1051.

WEBB, R. and Morris, B. A. (1988). The effect of steroid and inhibin immunity on FSH and ovulation. *Proceedings of the 11th International Congress on Animal Reproduction and A.I.*, **3**, 183-191.

WEBB, R., Gauld, I. K. and Draincourt, M. A. (1989). Morphological and functional characterization of large antral follicles in three breeds of sheep with different ovulation rates. *Journal of Reproduction and Fertility*, **87**: 243-255.

WEBB, R., Gong, J. G., Law, A. S. and Rusbridge, S. M. (1992a). Control of ovarian function in cattle. *Journal of Reproduction and Fertility (suppl.)* **45**: 141-156.

WEBB, R., Baxter, G., McBride, D., Ritchie, M. and Springbett, A. J. (1992b). Mechanism controlling ovulation rate in ewes in relation to seasonal anoestrous. *Journal of Reproduction and Fertility*, **94**: 143-151.

WEBB, R., Campbell, B. and Draincourt, M. A. (1995). Follicular cells from sheep carrying the FecB gene are more sensitive to gonadotrophins *in vitro*. Abstracts series

WEBB, R., Gosden, R. G., Telfer, E. E. and Moor, R. M. (1999a). Factors affecting folliculogenesis in ruminants. *Animal Science*, **68**: 257-284.

WEBB, R., Campbell, B. K., Garverick, H. A., Gong, J. G., Gutierrez, C. G. and Armstrong, D. G. (1999b). Molecular mechanisms regulating follicular recruitment and selection. *Journal of Reproduction and Fertility*, Suppl., **54**: 33-48.

WEBB, R., Garnsworthy, P. C., Gong, J. G., Robinson, R. S. and Wathes, D. C. (1999c). Consequences for reproductive function of metabolic adaptation to load. *Metabolic Stress in Dairy Cows, Occaisional Publication*, Number **24**. *British Society of Animal Science*, pp, 99-112.

WEBB, R., Nicholas, B., Gong, J. G., Campbell, B. K., Gutierrez, C. G., Gaverick, H. A. and Armstrong, D. G. (2003a). Mechanisms regulating follicular development and selection of the dominant follicle. *Reproduction Supplement*, **61**, 71-90.

WEBB, R., Garnsworthy, Gong, J. G. and Armstrong, D. G. (2003b). Control of follicular growth: local interactions and nutritional influences. (*In press*).

WETTEMANN, R. P. and Castree, J. W. (1988). Ovarian luteal activity of heifers immunised against LHRH. Journal of Animal Science, 66: (1): Abstract 62.

WETTEMANN, R. P. and Castree, J. W. (1994). Immunisation of heifers against gonadotropin releasing hormone delays puberty and causes the cessation of oestrous cycles. *Animal Reproduction Science*, **36**, 49.

WHEATON, J. E., Carlson, K. M. and Kusina, N. T. (1992). Active and passive immunoneutralization of inhibin increases follicle-stimulating hormone levels and ovulation rate in ewes.*Biology of Reproduction*, **47**: 361-367.

WIGZELL, H. (1987). Regulation of immunity by anti-idiotype antibodies. *Veterinary Immunology and Immunopathology*. **17**:1-4:161-172.

WILBER, J. F., Montoya, E., Plotnikoff, N. P., White, W. F., Gendrich, R., Renaud, L. and Martin, J. B. (1976). Gonadotrophin releasing hormone and thyrotrophin-releasing hormone: distribution and effects in the central nervous system. *Recent Progress in Hormone Research*, **32**: 117-159.

WILLETS, E. L. and Ohm's, J. I. (1957). *Journal of Dairy Science*, **40**, 1559.

WILLIAMS, W.W. and Savage, A. (1925). Observations on the seminal micropathology of bulls. *Cornell Veterinary Journal*, **15**, 353-375.

WILLIAMS, B., Brooks, A. N., Aldridge, T. C., Pennie, W. D., Stephenson, R. and McArdle, C. A. (2000). Oestradiol is a potent mitogen and modulator of GnRH signalling in αT3-1 cells: are these effects causally related? *Journal of Endocrinology*, **164**: 31-43.

WILLIAMSON, N. B., Morris, R. S., Blood, D. C., Cannon, C. M. and Wright, P. J. (1972). A study of oestrus behaviour and oestrus detection methods on a large commercial dairy herd II. Oestrus signs and oestrus patterns. *Veterinary Record*, **91**, 58-62.

WILLIE, R. (1944) Early maturity and its significance in cattle breeding (translated title). *Animal Breeding Abstract*, **12**: 196.

WILSON, T., Wu, Xi-Yang., Juengel, J. L., Ross, I. K., Lumsden, J. M., Lord, E. A., Dodds, K. G., Walling, G. A., McEwan, J. C., O'Connell, A. R., McNatty, K. P. and Montgomery, G. W. (2001). Highly prolific Booroola sheep have a mutation in the intracellular kinase domain of bone morphogenetic protein IB receptor (ALK-6) that is expressed in both oocytes and granulosa cells. *Biology of Reproduction*, **64**: (4): 1225-1235.

WILTBANK, M. C., Fricke, P. M., Sangsritavong, S., Sartori, R. and Ginther, O. J. (2000). Sex and the single follicle: mechanisms that prevent and produce double ovulations in dairy cattle. *Journal of Dairy Science*, **83**, 1-10.

WISE, T. H. and Schanbacher, B. D. (1983). Reproductive effects of immunizing heifers against androstenedione and oestradiol 17β. *Journal of Reproduction and Fertility*, **69**: 605-612.

WISE, T., and Ferrell, C. (1984). Effects of immunisation of heifers against estradiol on growth, reproductive traits, and carcass characteristics. *Proceedings of theSociety of Experimental Biology and Medicine*, **176**, 243.

WISE, M. E., Nieman, D., Stewart, J. and Nett, T. M. (1984). Effect of number of receptors for gonadotropin-releasing hormone on the release of luteinizing hormone. *Biology of Reproduction*, **31**: 1007-1013.

WISE, M.E., Rodriguez, R.E. and Kelly, C.M. (1987). Gonadal regulation of LH secretion in prepubertal bull calves. *Domestic Animal Endocrinology*, **4**:3:175-181.

WISHORT, D.F. (1972). Observations on the oestrous cycle of the Friesian heifer. *Veterinary Record*, **90**: 595-597.

WOLF, F. R., Almquist, J. O. and Hale, E. B. (1965). Prepubertal behaviour and pubertal characteristics of beef bulls on high nutrient allowance. *Journal of Animal Science*, **24**, 761-765.

WOOD, S. C., Glencross, R. G., Bleach, E. C. L., Lovell, R., Beard, A. J. and Knight, P. G. (1993). The ability of steroid-free bovine follicular fluid to suppress FSH secretion and delay ovulation persists in heifers actively immunised against inhibin. *Journal of Endocrinology*, **136**, 137-148.

WOODLAND, D.L. (2004). Jump-starting the immune system:prime-boosting comes of age. *Trends in Immunology*, **25**:2: 98-104.

WORLD HEALTH ORGANISATION. (1999). Laboratory manual for the examination of human semen and sperm cervical mucus interaction. Fourth edition. Cambridge University Press.

WRATHALL, J. H. M., McLeod, B. J., Glencross, R.G., Beard, A. J. and Knight, P. G. (1990). Inhibin immunoneutralization by antibodies raised against synthetic peptide sequences of inhibin α subunit: effects on gonadotrophin concentrations and ovulation rate in sheep. *Journal of Endocrinology*, **124**: 167-176.

WRATHALL, J. H. M., McLeod, B. J., Glencross, R. G., Beard, A. J. and Knight, P. G. (1992). Effects of active immunisation against a synthetic peptide sequence of the inhibin α-subunit on plasma gonadotrophin concentrations, ovulation rate and lambing rate in ewes. *Journal of Reproduction and Fertility*, **95**, 175-182.

WRATHALL, J. H. M. and Knight, P. G. (1993). Production of immunactive inhibin and activin by bovine granulosa cells in serum-free culture: effects of exogenous steroids and FSH. *Domestic Animal Endocrinology*, **10**: 289-304.

WRATHALL, J. H. M. and Knight, P. G. (1995). Effects of inhibin-related peptides and oestradiol on androstenedione and progesterone secretion by bovine theca cells in vitro. *Journal of Endocrinology*, **145**: 491-500.

WU, M., Wang, H., Murphy, B. D. and Mapletoft, R. J. (1988). Superovulation of beef cows with Follitropin: A dose trial. *Theriogenology*, **29**, (1): 332.

XU, Z. Z., Garverick, H. A., Smith, G. W>, Hamilton, S. A and Youngquist, R. S. (1995). Expression of messenger RNA encoding cytochrome P450 17α-hydroxylase and cytochrome P450 aromatase in bovine follicles during the first follicular wave. *Endocrinology*, **136**, 981-989.

YADAV, M. C., Watson, J. S. and Leslie, K. E. (1986) Ovarian function in Holstein cows immunized against pregnant male serum gonadotrophin. *Theriogenology*, **26**: 541-549

YAMOTO, M., Minami, S., Nakano, R. and Kobayashi M. (1992). Immunohistochemical localization of inhibin/activin subunits in human ovarian follicles during the menstrual cycle. *Journal of Clinical Endocrinology and Metabolism*, **74**: 989-993

YOUNG, A. W., Cundiff, L. V and Bradley, N. W. (1969). Effects of an oral progestogen on feedlot heifers. *Journal of Animal Science*, **28**, 224.

ZEITOUN, M. M., Yassen, A. M., HassaN, a. a., Fathelbab, A. Z., Wise, T. H. and Maurer, R. R. (1988). Superovulation using PMSG and anti-PMSG in beef cows. *Theriogenology*, **29**, (19): 339.

ZENG, X. Y., Turkstra, J. A., Meloen, R. H., Liu, X. Y., Chen, F. Q., Schaaper, W. M. M., Oonk, R. B., Guo, D. Z. and van de Wiel, D. F. M. (2002). Active immunization against gonadotrophin-releasing hormone in Chinese male pigs: effects of dose on antibody titre, hormone levels and sexual development. *Animal Reproduction Science*, **2234**: 1-11.

ZHAO, J., Taverne, M. A., van der Weijden, G. C., Bevers, M. M. and van der Hurk, R. (2001). Effects of activin A on in vetro development of rat pre-antral follicles a nd localization of activin A and activin receptor II. *Biology of Reproduction*, **65**: 967-977

ZOBELL, D. R., Goonewardene, L. A. and Ziegler, K. (1993). Evaluation of the bloodless castration procedure for feedlot bulls. *Canadian Journal of Animal Science*, **73**, 967-970.

APPENDIX I

Assay Calculations

Composite standard curve:

Use % binding (B/Bo) throughout.

Calculate mean and standard error of the means (s.e.m.) for each point of the standard curve across all assays. Plot these as a standard curve. This is a composite standard curve for the set of assays.

Minimum detectable dose (lower sensitivity):

Calculate TB/mean TB (within assay) x 100 for each TB. Calculate the standard deviation of all TB across all assays.

Then: 100-(SDx2)=% binding. This percentage binding is then read from the composite standard curve to give a minimum detectable dose in ng $tube^{-1}$. If the standards are per tube, the calculation must be corrected for amount assayed, by multiplying by 1000/assayed µl.

If the assay is extracted, the data must be adjusted for the extraction efficiency.

Inter-assay variation:

Calculate the standard deviation of the QCs across all the assays, and average them. Mean the values of all QCs also.

Coefficient of variation =

$$\frac{100 \times SD}{X}$$

where X = mean of QC values

SD = mean of SD of QCs

Intra-assay variation:

$$S = \text{square root of } \frac{\text{sum of } d^2}{2n}$$

where d = difference in QC/sample pairs

n= number of pairs used.

For the intra assay variation one can also just mean all the sample coefficients of variation (covs) for each assay.

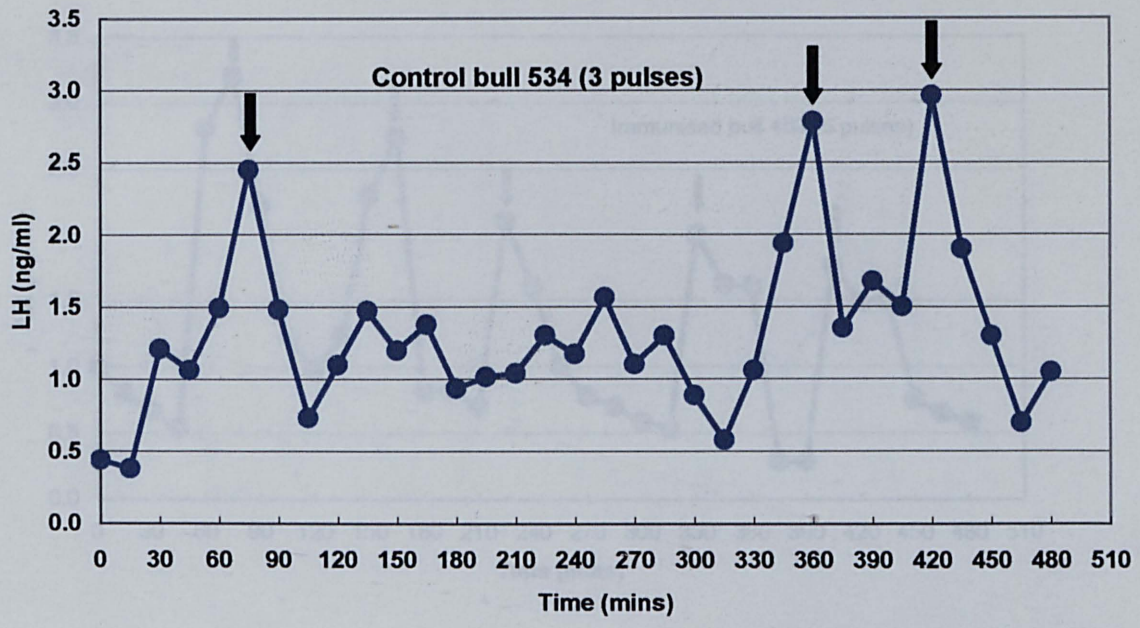
APPENDIX II.

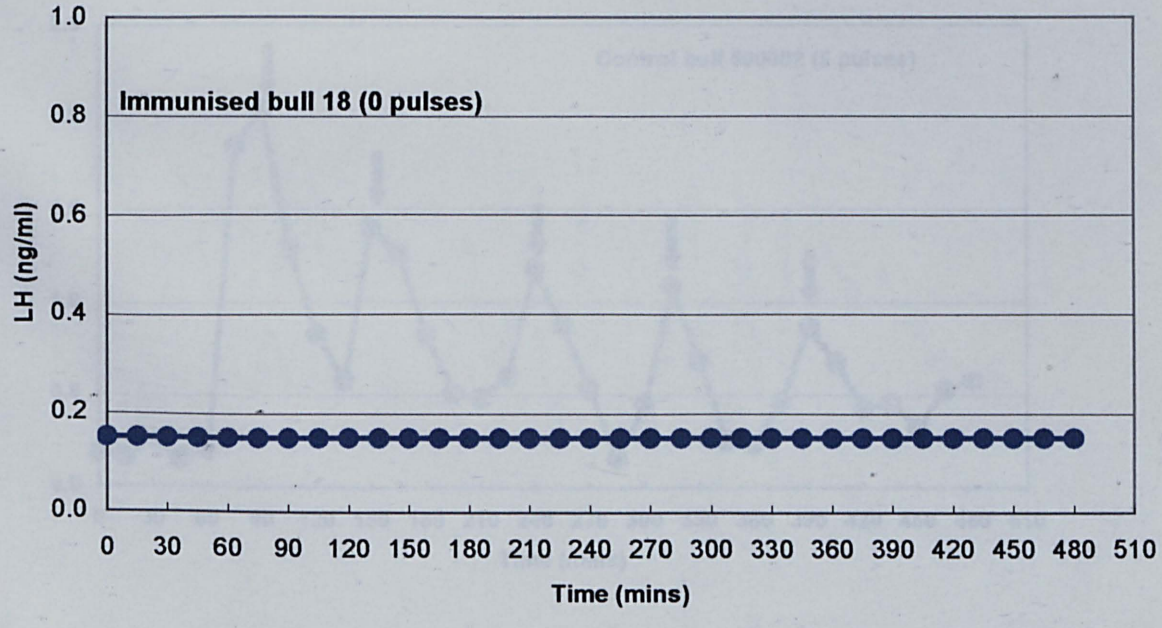
MUNRO Settings

Algorithm:	**MUNRO**	
Smoothing window:	**60 mins**	
Nadir window:	**35 mins**	
Minimum pulse interval:	**30 mins**	
Rise threshold:	**2 standard deviations (s.d.)**	
Baxter parameters:	**B1:**	**0.02920**
	B2:	**0.02970**
	B3:	**0.00089**
G parameters (s.d. units):	**G1:**	**3.979**
	G2:	**2.399**
	G3:	**1.679**
	G4:	**1.239**
	G5:	**0.930**

APPENDIX III

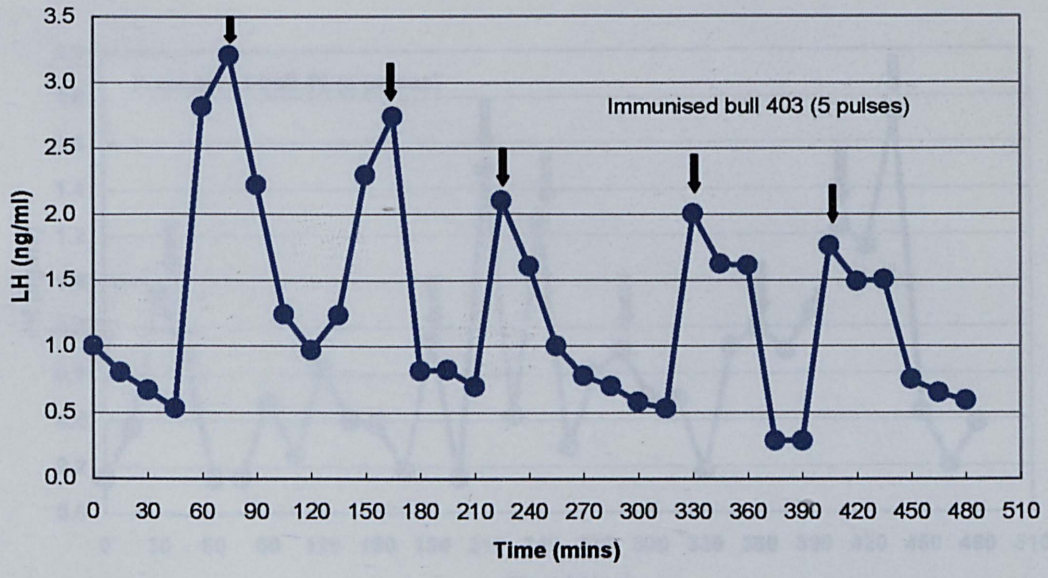
Examples of LH pulse profiles from GnRH immunised and control bulls during a serial bleed at 16 weeks of age. Black arrows indicate the Author's estimation of an LH pulse occurrence.

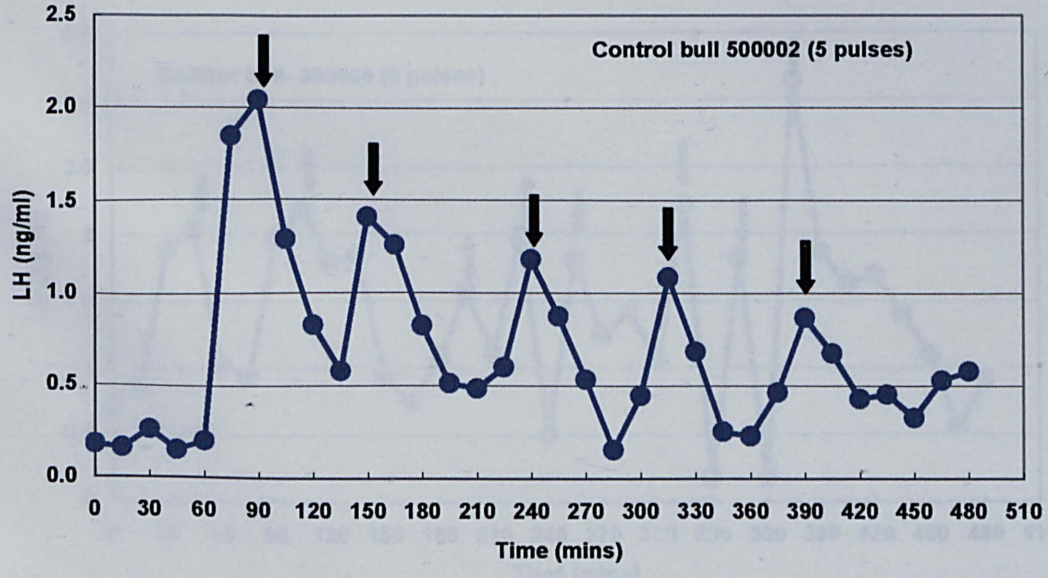




APPENDIX IV

Examples of LH pulse profiles from GnRH immunised and control bulls during a serial bleed at 16 weeks of age. Black arrows indicate the Author's estimation of an LH pulse occurrence.





APPENDIX V

Examples of LH pulse profiles from GnRH immunised and control bulls during a serial bleed at 16 weeks of age. Black arrows indicate the Author's estimation of an LH pulse occurrence.