Chapter 5 Model for the Study of Ovarian Cysts

Ovarian cyst formation is a major cause of infertility in cattle, but little is known of the aetiology of this ovarian pathology (Vanholder *et al.,* 2006). Implantation of osmotic mini-pumps containing the GnRH agonist, buserelin, over a period of 48 days, resulted in at least 60% of heifers developing ovarian cysts after termination of treatment (Gong *et al.,* 1996). The aim of this study was to evaluate this experimental model for studying the endocrine and physiological changes associated with ovarian cyst development.

5.1 Materials and methods

5.1.1 Hyper-stimulation protocol (performed by Professor Bruce Campbell in order to collect a large quantity of oocytes for unrelated research)

Previously, twelve beef × dairy heifers were selected for this trial all of which were weighed, body condition scored, and split into two even groups. Group 1 had an average weight of 439kg (±12.6 SEM) and BCS of between 3.5 and 4.0, while Group 2 had an average weight of 437kg (±13.4 SEM) and BCS of between 3.5 and 4.0. An osmotic mini-pump (2ML4: Alzet, CA, USA) containing 2ml of buserelin (Hoechst Animal Health, Hounslow, UK, release rate: 25μg/hour), was implanted subcutaneously in the upper shoulder area of each heifer. Four weeks (28 days) later the osmotic mini-pumps were changed to replenish the buserelin and heifers were exposed for a further 21 days. Two weeks after the mini-pumps were changed, group 1 were cannulated and an infusion of ovine FSH (NIDDK-oFSH-16, National Institute of Diabetes and Digestive and Kidney Disease (NIDDK), Bethesda, MD, USA) was started to

stimulate follicle recruitment until the lead follicle reached a diameter of 10mm. Approximately 2-3 days after the FSH infusion began, FSH was stepped down (75% for 6h, 50% for 6h, and 25% for 6h) and substituted by LH (NIDDK-oLH-26, National Institute of Diabetes and Digestive and Kidney Disease (NIDDK), Bethesda, MD, USA) (25µg pulses of oLH-S26 every 2h for 24h followed by 12.5µg pulses for a further 24-36h) to emulate the shift from FSH to LH dependency that occurs in the follicular phase. Heifers were injected (im) with a single dose (1500IU) of hCG (Chorulon; MSD Animal Health, Milton Keynes, UK) 120h after the FSH infusion began to promote the later stages of follicular growth. Sixteen hours post hCG, oocytes were collected from the heifers by transvaginal ultrasound guided follicular aspiration as outlined by Pieterse *et al.,* (1991), also known as, ovum pick up (OPU), and the mini-pumps were removed. Heifers in group 2 underwent the same treatment regime but 2 days later than group 1 to facilitate treatment administration. An overview of the treatment protocol is illustrated in Figure 5.1.

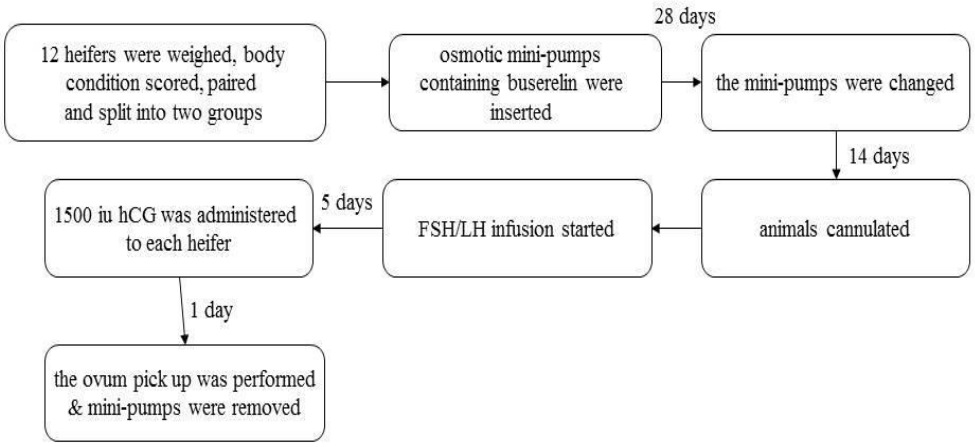

Figure 5.1: Summary view of the hyper-stimulation model.

hCG = human chorionic gonadotropin; FSH = follicle stimulating hormone.

5.1.2 Post-buserelin model for ovarian cysts

Seven days after OPU, the ovaries of all heifers were examined using a 7.5 MHz linear array rectal probe (Easi-scan; BCF Technology, Livingston, UK) to determine ovarian physiology. At this time heifers were reorganised into two different groups that displayed similar ovarian physiologies. Group A (n=6) was treated 2 days ahead of group B (n=6) as heifers in this group had larger, more developed follicles (average diameter = 18.7mm) than those in group B (average diameter = 12.2mm). Treating group A ahead of group B allowed follicles in heifers of group B to develop to a similar size as those in group A before treatment began. Eight days after OPU, all heifers had a CL and received a luteolytic dose of the prostaglandin $F2_{\alpha}$ analogue (PG), cloprostenol (Estrumate, Schering-Plough Animal Health, Welwyn Garden City, UK) and a blood sample from each heifer was withdrawn from the jugular cannulae into lithium heparin spray coated vacutainers (Becton,

Dickson Ltd., Oxford, UK) and centrifuged immediately at 3000**g** for 15mins. Plasma was removed from the samples, frozen and stored at -20˚C until analysis. Ovarian physiology was monitored by daily transrectal ultrasonography and endocrine status by 8 hourly blood sampling for 120h following PG. Over a period of 4h on one day of the trial (Group A PG +96 hours, Group B PG +48 hours), blood was withdrawn from the jugular cannula every 15 minutes in order to study pulsatile release of LH.

The majority of heifers were expected to come into oestrus 72-96h post PG, with ovulation occurring 24h later. All heifers were sacrificed 120h after administration of PG. In the abattoir, the hypothalamus (removed in order to successfully obtain the pituitary gland), the pituitary and the reproductive tract of each heifer was collected. A summary of the protocol can be found in Table 5.1.

Table 5.1: A summary of the treatment protocol for groups A and B for the post buserelin experimental protocol. Shaded day (Group A PG +96h and Group B PG +48h) depicts the day on which a 4 hour window of pulse bleeds were taken.

hCG = human chorionic gonadotropin; PG = prostaglandin; bleed = withdrawal of blood for analysis; OPU = ovum pick up.

Date	Hour of Day (24h)				
		hCG ↓ 1 day **OPU** ↓ 2 days **Predicted ovulation** ↓ 5 days			
19/06/08		TRANSRECTAL ULTRASONOGRAPHY (scan)			
		GROUP A		GROUP B	
		Hours since PG	Action	Hours since PG	Action
20/06/08	0800	PG	Scan & 0h bleed		
	1600		8h bleed		
	2400		16h bleed		
21/06/08	0800	PG+24h	Scan & 24h bleed		
	1600		32h bleed		
	2400		40h bleed		
22/06/08	0800	PG+48h	Scan & 48h bleed	PG	Scan & 0h bleed
	1600		56h bleed		8h bleed
	2400		64h bleed		16h bleed
23/06/08	0800	PG+72h	Scan & 72h bleed	PG+24h	Scan & 24h bleed
	1600		80h bleed		32h bleed
	2400		88h bleed		40h bleed
24/06/08	0800	PG+96h	Scan & 96h bleed	PG+48h	Scan & 48h bleed
	1600		104h bleed		56h bleed
	2400		112h bleed		64h bleed
25/06/08	0800	PG+120h	120h bleed & sacrifice	PG+72h	Scan & 72h bleed
	1600				80h bleed
	2400				88h bleed
26/06/08	0800			PG+96h	Scan & 96h bleed
	1600				104h bleed
	2400				112h bleed
27/06/08	0800			PG+120h	120h bleed & sacrifice

5.1.3 Laboratory protocols

Hormone concentrations were measured in plasma and follicular fluid by immunoassay and protein expression levels of CYP19A1, CYP11A1 and CYP17A1 were determined by immunohistochemistry.

5.1.3.1 Follicle dissection

Follicular fluid from follicles >8mm was aspirated and snap frozen in liquid nitrogen. The follicle itself was then quartered with each quarter encompassing both granulosa and theca layers. One quarter was fixed in Bouins solution (Sigma-Aldrich, Dorset, UK) for 24 hours before being transferred into 70% ethanol for long term storage. All other quarters were snap frozen and archived (at -80˚C). Of the 24 ovaries collected, 3 representing a diverse selection of follicles (primary to dominant), were fixed whole in Bouins solution (Sigma-Aldrich, Dorset, UK), to serve as control tissue, and transferred after 24 hours to a 70% ethanol solution.

5.1.3.2 Pituitary dissection

Using the pituitary stalk for orientation, transverse cross sections were taken which included both the anterior and posterior pituitary. The pituitary gland was dissected into quarters with one quarter fixed in Bouins solution (Sigma-Aldrich, Dorset, UK) for 24 hours and then transferred to 70% ethanol. The other 3 quarters were snap frozen and stored at -80˚C.

5.1.3.3 Tissue processing

Sample preparation

Fixed tissue samples were trimmed and placed into cassettes for processing (Leica Microsystems, Newcastle-upon-Tyne, UK). A cross section from each of the 3 whole ovaries was removed and placed into cassettes while the remaining tissue was returned to storage pots with fresh 70% ethanol solution.

Tissue processing

A Leica TP 1020 (Leica Microsystems, Newcastle-upon-Tyne, UK) was used to dehydrate the tissue samples overnight in an ascending series of alcohols and then histoclear (70% ethanol for 1 hour, 80% ethanol for 1.5 hours, 95% ethanol for 1.5 hours, 100% ethanol for 1 hour, 100% ethanol for 1 hour, 100% ethanol for 1.5 hours, 100% ethanol for 1.5 hours), followed by histoclear (histoclear for 1 hour, histoclear for 1.5 hours, histoclear for 0.5 hours), and molten paraffin wax (1 x 2 hours; 1 x 3 hours) before immediate transfer into the paraffin wax bath of the embedder. Tissue embedding was done on a Leica EG 1160 (Leica Microsystems, Newcastle-upon-Tyne, UK) and all tissue types were embedded in a similar orientation to each other. Sections of follicle (5μm) or pituitary tissue (7μm) were microtomed and mounted onto Menzel Polysine slides (Fisher Scientific UK Ltd., Loughborough, Leicestershire, UK).

5.1.3.4 Measurement of hormones

5.1.3.4.1 Radioimmunoassay (RIA)

Peripheral luteinising hormone (LH), follicle stimulating hormone (FSH) and oestradiol concentrations as well as follicular fluid concentrations of androstenedione were determined using direct radioimmunoassays.

General methodology - RIA

The standard curve, NSBs, TCs, and QCs as well as the unknown samples were pipetted out according to Table 5.2. Assay buffer (0.01M PBS with 0.01% bovine serum albumin (BSA) and 0.1% sodium azide), primary antibody, and tracer were added (Table 5.2) before all tubes were mixed with a vortex mixer and incubated for 24 hours (overnight) at room temperature (24-25˚C) (LH and FSH), or at 4˚C (androstenedione and oestradiol). The secondary antibody was added to all samples except TCs and tubes were mixed with a vortex mixer, covered and incubated for one further hour (LH at 4˚C, and oestradiol at 24-25˚C), or for 24 hours overnight (FSH and androstenedione at 4˚C). Specific volumes of each element are outlined in Table 5.2.

Luteinising hormone radioimmunoassay

Peripheral LH concentrations were determined using a direct iodinated radiomimmunoassay described in Mann & Lamming (2000). The primary LH antibody (NIDDK-anti-oLH) was supplied by the National Institute of Diabetes and Digestive and Kidney Disease (NIDDK), Bethesda, MD, USA.

The secondary antibody consisted of donkey anti-rabbit serum (S022-220, Scottish Antibody Production Unit [SAPU]) used at a 1:160 dilution, and normal rabbit serum (R-4505, Sigma Aldrich, Dorset, UK) at a 1:800 dilution. Three quality controls were included in this RIA; Low - 0.5ng/ml, Medium - 5-6ng/ml, and High - 10-12ng/ml. A standard curve was prepared using 40, 20, 10, 5, 2.5, 1.25, 0.625, 0.31 and 0.156ng/ml. Assay sensitivity was 0.2ng/ml, inter-assay % CV were as follows; Low QC 13.9%, medium QC 11.4% and high QC 3.9% and intra-assay % CV was calculated at <18.1%. **NB:** It is unlikely that there was any cross reactivity with the previously administrated hCG as the half-life of hCG does not normally extend beyond 38h (De Rensis *et al.*, 2010) post injection. Although hCG half-life can be slightly extended following im injection (De Rensis *et al.*, 2010), there was 192h between the injection of hCG and the commencement of this trial.

Follicle stimulating hormone radioimmunoassay

Peripheral concentrations of FSH were measured using a direct iodinated radioimmunoassay described by Campbell *et al.*, (1994). The primary FSH antibody (teri.oFSH.Ab1) was obtained from the Tucker Endocrine Research Institute, Stone Mountain, GA, USA. The secondary antibody was a combination of donkey anti-rabbit serum, used at 1:160 dilution (S022-220, Scottish Antibody Production Unit, UK), and normal rabbit serum, used at a 1:800 dilution (R-4505, Sigma-Aldrich, Dorset, UK). Three quality controls were included in this RIA; Low – unspiked plasma, medium – plasma spiked with 4ng/ml FSH, and high – plasma spiked with 8ng/ml FSH. A set of standards were prepared by diluting 500µg/ml standard solution (NIDDK-

oFSH-SIAFP-RP-2, AFP4117A) 1:1000 in assay buffer (0.01M PBS with 0.1% BSA and 0.1% sodium azide), diluting further 1:30 to give 16.67ng/ml and then double diluting to 0.13ng/ml as well as including a '0' standard of assay buffer. Assay sensitivity was 0.16ng/ml, inter-assay % CV were as follows; Low QC 17.0%, medium QC 11.1%, and high QC 11.2% and intra-assay % CV was calculated at <23.3%.

Androstenedione radioimmunoassay

Androstenedione concentrations were measured in the follicular fluid by direct radioimmunoassay, described by Campbell *et al.*, (1990). The primary antibody, Rabbit C (supplied by Brian Cook, Glasgow Royal Infirmary, Glasgow UK), was used at a 1:300 dilution. The secondary antibody comprised of both donkey anti-rabbit serum (S022-220, Scottish Antibody Production Unit, UK) used at 1:160 dilution as well as normal rabbit serum (R-4505, Sigma-Aldrich, Dorset, UK) used at a 1:800 dilution. Four quality controls were included in this RIA; 1) 0ng/ml; 2) 0.5ng/ml; 3) 2.5ng/ml and 4) 4ng/ml. No extraction step is needed when utilising this assay to determine androstenedione concentrations in follicular fluid, however follicular fluid was diluted 1:10 with assay buffer and results were multiplied up accordingly at the end. Assay sensitivity was 0.04ng/ml; inter-assay % CV were as follows; QC 2) 4.9%, QC 3) 6.2% and, QC 4) 1.6% and intra-assay % CV were calculated at <20.4%.

Table 5.2: Quantity of reagents, radioactive tracer, antibodies and wash solution in non-specific binding (NSB), total count (TC), total bound (TB), standard curve, quality controls (QCs) and samples for luteinising hormone (LH), follicle stimulating hormone (FSH), androstenedione (A4) and oestradiol (E2) radioimmunoassay.

RIA		No. of Replicates	SMP/ STD/ QC	Assay Buffer (µl)	Tracer (µl)	1˚Ab (µl)	2˚Ab (µl)	Wash Buffer (µl)
LH	NSB	3	-	400	100	-	200	500
	TC	3	-	-	100	-	-	-
	Standard	3	100	400	100	100	200	500
	QC/Sample	2	100	400	100	100	200	500
FSH	NSB	3	-	200	50	-	100	500
	TC	3	-	-	50	-	-	-
	Standard	3	200	200	50	50	100	500
	QC/Sample	2	200	200	50	50	100	500
A4	NSB	4	-	200	100	-	250	250
	TC	4	-	-	100	-	-	-
	TB	4	-	100	100	100	250	250
	Standard	4	100	-	100	100	250	250
	QC/Sample	2	100	-	100	100	250	250
E2	NSB	3	-	300	50	-	100	1000
	TC	3	-	-	50	-	-	-
	TB	3	-	250	50	50	100	1000
	Standard	3	50	200	50	50	100	1000
	QC/Sample	2	*	250	50	50	100	1000

*Please refer to chapter 2 for full details of QC and sample extraction.

5.1.3.4.2 Enzyme linked immuno-sorbent assays (ELISAs)

General methodology - ELISA

Stage 1: Capture antibody (100µl) was diluted in coating buffer (0.05M carbonate buffer) and applied to a 96 well plate (Fisher Scientific UK Ltd, Loughborough, Leicestershire, UK). The plate was covered and left overnight to incubate at 4˚C. The following day the plate was washed 3 times with wash buffer (0.05% tween 20 in 10x PBS). A blocking solution (100µl) (3% BSA in

PBS) was added to each well and left to incubate at room temperature for 2 hours, after which the plate was washed 3 times in wash buffer as before.

Stage 2: Standards were set up in quadruplicate on one plate. QCs (quadruplicate) and unknown samples (duplicate) were set up on a separate plate. A horseradish peroxidase (HRP) conjugate (Fisher Scientific UK Ltd, Loughborough, Leicestershire, UK) was diluted in assay buffer (0.1% BSA in PBS) and applied to each well and left to incubate for 2 hours at room temperature, after which the plate was washed 3 times in wash buffer, as before. The visualising reagent, tetramethylbenzidine (TMB) (50µl) (T0440-Sigma-Aldrich, Dorset, UK) was applied to all wells and allowed to incubate at room temperate, before 50µl of a stop solution (1N H_2SO_4) was added to all wells and the absorbance was read at 450nm. Table 5.3 indicates the differences between the progesterone and the oestradiol ELISA protocols.

Progesterone ELISA

The progesterone antibody (SAPU R7044X) (Scottish Antibody Production Unit) was used at a working dilution of 1:32000 in assay buffer (0.1% BSA in PBS). A set of standards were prepared by double diluting the top standard 20ng/ml down to 0.078ng/ml, and the QCs included QC 1) 0ng/ml, QC 2) 1ng/ml, QC 3) 5ng/ml and, QC 4) 8 ng/ml. The follicular fluid was diluted 1:10 in assay buffer (0.1% BSA in PBS). Assay sensitivity was 0.078ng/ml, inter-assay % CV were calculated as follows; QC 2) 16.6%, QC 3) 12.3% and, QC 4) 9.2%, and intra-assay % CV was calculated to be <17.9%.

Oestradiol ELISA

The oestradiol antibody (ab1024) (ABCAM, Cambridge, UK) was stored at 1:100 dilution, and diluted further to a working dilution of 1:64000 in assay buffer (0.1% BSA in PBS). A set of standards were prepared by double diluting the top standard 10ng/ml down to 0.039ng/ml and the QCs included QC 1) 0ng/ml, QC 2) 0.5ng/ml, QC 3) 2.5ng/ml and, QC 4) 4ng/ml. The follicular fluid was diluted 1:20 in assay buffer (0.1% BSA in PBS). Assay sensitivity was 0.039ng/ml, inter-assay % CV were measured as follows; QC 2) 6.8%, QC 3) 5.8%, QC 4) 8.6% and intra-assay % CV was calculated at <19.5%.

Table 5.3: Summary of differences in methodology between the progesterone and oestradiol enzyme linked immuno-sorbent assays (ELISA).

HRP = horseradish peroxidase; TMB = tetramethylbenzidine

ELISA	Dilution of Capture Antibody	HRP (µl)	Dilution of HRP	Incubation with TMB (minutes)
Progesterone	1:32,000	50	1:32,000	15
Oestradiol	1:64,000	100	1:40,000	120

5.1.3.5 Immunohistochemistry (IHC)

Immunohistochemistry was used to detect the presence or absence of the steroidogenic enzymes CYP19A1, and CYP17A1 in follicular tissue and LH and FSH in pituitary tissue. Positive tissue controls and negative IgG controls were also included in the immunohistochemistry.

CYP19A1 and CYP17A1 in follicular tissue

Presence of CYP19A1 and CYP17A1 was determined in follicular tissue using a Bond-max autostainer (Leica Microsystems, Newcastle-upon-Tyne, UK) and Bond Refine Polymer Detection Kit (Leica Microsystems, Newcastle-upon-Tyne, UK). No antigen retrieval was required and DAB substrate was used.

Antibodies were as follows;

- Mouse anti-human CYP19A1 (MCA2077S, Serotec, Kidlington, UK)
- Rabbit anti-human CYP17A1 (kindly provided by Ian Mason from the University of Edinburgh)

The CYP19A1 antibody was stored neat and diluted to a working dilution of 1:100. Antibody for CYP17A1 was kept at a 1:1000 dilution and diluted to a working dilution of 1:800, making the overall dilution 1:800,000.

Luteinising hormone/follicle stimulating hormone IHC in pituitary tissue

The immunostaining protocol for pituitary LH and FSH was based on Mukherjee *et al.*, (2001) and Stubbs *et al.*, (2005, 2007). Primary antibodies were 1) anti-LH antibody (AFP-192279) or, 2) anti-FSH (AFP-C528813), both raised in rabbit against sheep. Both primary antibodies were diluted 1:400 and rabbit control serum was also used at a 1:400 dilution. Immunostaining was visualised using a Vectastatin ABC Elite universal kit (Vector Laboratories Ltd., Peterborough, UK) and DAB substrate.

Luteinising hormone/follicle stimulating hormone IHC protocol

To de-wax and dehydrate the tissue, slides were soaked in xylene (2 x 5 mins), 100% ethanol (2 mins), 95% ethanol (2 mins), 70% ethanol (2 mins) and washed using PBS. To make the 10mM citrate buffer for epitope retrieval, two solutions were required. Solution A (citric acid buffer) consisted of 4.2g of citric acid (Fisher Scientific UK Ltd, Loughborough, Leicestershire, UK) in 200ml of distilled water. Solution B (citrate buffer) consisted of 14.7g sodium citrate (Sigma-Aldrich, Dorset, UK) in 500ml distilled water. To make the 10mM citrate buffer, 18ml of solution A was mixed with 82ml of solution B as well as 900ml of distilled water, pH to 6.0. To facilitate epitope retrieval, 10mM citrate buffer was boiled using a microwave prior to adding slides (5 min at 900W), slides were then added and boiled for 1 min at 900W and 15 mins at 750W (checked every 5 min and buffer topped up if required). Slides were left to cool in the buffer for approximately 20 min after which they were washed in PBS. Slides were transferred into a humidity chamber to commence staining, for the peroxidase blocking step, fresh 3% H_2O_2 was prepared in PBS, and 100µl was added to each slide for 10 min. All slides were washed in PBS. Normal horse serum (20%) was added to all slides and left to incubate for 30 min. Excess horse serum was blotted from all slides except control slides once incubation was completed. Primary antibody (100µl) was added to all sections except controls and left to incubate for 1 hour at room temperature. All slides were washed in 0.1% triton-X-100 in PBS. At this point the avitin-biotin (AB) complex was prepared, and allowed to stand for 30 min prior to use to allow the avitin and biotin to bind. The secondary horse anti-rabbit antibody (100ul) diluted 1:200, was added to all slides and allowed to incubate at room

temperature for 30 min. All slides were washed in 0.1% triton-X-100 in PBS followed by PBS. The AB complex (100µl) was added to all slides and allowed to incubate for 30 min. All slides were washed in PBS. The DAB substrate was prepared and added to slides. Colour change was closely observed and allowed to develop between 2-4 min. All slides were rinsed in running water and counterstained as follows; haematoxylin (1 min), washed until clear under running water, ammoniated water (1%) (1-2 min), running tap water, 70% ethanol (2 min), 95% ethanol (2 min), 100% ethanol (2 min), xylene (2 x 5 min). Coverslips were mounted using DPX (Fisher Scientific UK Ltd., Loughborough, Leicestershire, UK) and slides were left to dry overnight in a fume hood.

5.1.4 Statistical analysis

In order to further characterise LH pulses during the 4 hour intense 'pulse bleed' a pulse analysis programme MUNRO© (Zaristow Software, Haddington, East Lothian, UK) was used to determine frequency, amplitude, mean, and basal concentrations of LH using Baxter and 'G' parameters. As described in Collet *et al.*, (1999) Baxter parameters are those determined by Baxter (1980), calculated by studying the duplicate values of all human LH standards used in 54 standard curves. G parameters were determined by Merriam & Wachter (1982), to obtain less than 5% of false positive pulses. Baxter parameters used were B1- 0.0292, B2- 0.0297, and B3- 0.00089 and G parameters were G1- 3.979, G2- 2.399, G3- 1.679, G4- 1.237, and G5- 0.930. In addition to these, a smoothing window of 60 minutes, a nadir window of 35 minutes, a minimum pulse interval of 15 minutes and a rise threshold of 2

standard deviations were also applied. Resulting data were compared using a one way ANOVA (Genstat 12, VSN International, Hemstead, UK) and a Fishers Exact test (Graphpad Software Inc., CA, USA). Peripheral hormone concentrations were examined using ANOVA for repeated measurements. The main effects tested included treatment, time, and interaction of treatment x time (Genstat 12, VSN International, Hemstead, UK). Mean hormone concentrations in the follicular fluid and follicle sizes were compared using a one way ANOVA (Genstat 12, VSN International, Hemstead, UK). All residuals were tested for normality and no transformations were required. Probability values $P<0.05$ or lower were considered to be significant, while values $P<0.1$were considered to show a tendency towards difference.

5.2 Results

5.2.1 Dynamics of ovarian follicle growth and development

Large aberrant follicular structures (>10mm) formed in all 12 heifers following treatment with the GnRH agonist, buserelin, with 8 heifers having multiple follicles >8-9mm developed. Follicle appearance ranged from healthy to haemorrhagic and full of bloody follicular fluid with varying degrees of luteinisation observed. CL were observed in one of the twelve heifers. Table 5.4 illustrates that a range of follicles and CL were observed and that no distinct pattern could be determined.

Table 5.4: Number of follicles present at dissection, following euthanasia at 120 hours post prostaglandin. *886 had 5 CLS across both ovaries; **Largest follicle present on the ovaries of cow 870 (11mm) was dark in colour and full of bloody follicular fluid.

Follicle appearance code as follows; A) Healthy; B) Healthy with patches of luteinised tissue; C) Healthy but with some degree of luteinisation; D) Healthy with a thickened follicle wall (50% luteinised).

Group	Cow ID	Number of Follicles Present ≥8mm diameter		Number of Follicles Present 2-8mm diameter	Follicle Appearance
		n	Diameter (mm)	n	
A	874	2	22, 20	>20	B, A
A	877	1	10	9	D
A	881	3	15, 14, 12	2	A, C, A
A	886	3	20,16, 11	0	D, C, A *
A	887	1	22	0	A
A	890	2	23, 15	0	A, A
B	868	2	21, 10	0	C, A
B	870	2	11, 10	19	**, C
B	873	1	17	19	C
B	876	3	15, 15,14	7	D, A, A
B	878	1	15	1	D
B	889	3	13, 10, 9	32	D, A, A

5.2.2 Peripheral hormone concentrations

Measurements of LH concentrations demonstrated that within the group of 12 heifers there were two separate cohorts. One cohort (n=6; Group B) maintained basal LH concentrations until they exhibited an LH surge at 104 hours post PG (defined as heifers exceeding LH concentrations of 5ng/ml) before returning to basal concentrations by 120 hours post PG. The other cohort (n=6; Group A) maintained basal LH concentrations throughout the observation period. To facilitate discussion of these results, those heifers that did not exhibit an LH surge will be referred to as the 'no-surge' (NS) heifers and heifers that did exhibit an LH surge will be referred to as the 'surge' (S) heifers.

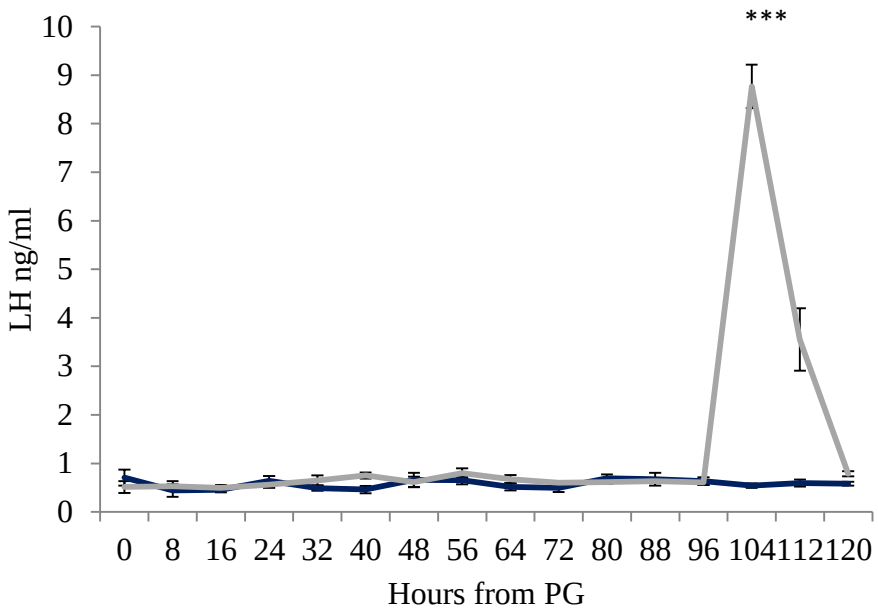

Figure 5.2: Peripheral concentrations of luteinising hormone (LH) (ng/ml) (±SEM) in heifers that did (grey) and did not (blue) exhibit an LH surge across the 120 hours following administration of the prostaglandin (PG) analogue, cloprostenol. Over the period from 104 to 120 hours post PG, LH concentrations were significantly elevated ($P<0.001$) in heifers that did exhibit an LH surge compared to heifers that did not.

Peripheral FSH concentrations were significantly higher (P<0.05) in the first 24 hours post PG in NS heifers compared to S heifers. Conversely, in the final 24 hours, FSH concentrations were significantly lower (P<0.01) in NS heifers compared to S heifers (Figure 5.3). The pattern of FSH release in NS heifers fluctuates around a constant value of 1.5ng/ml whereas FSH concentrations in S heifers appeared to steadily increase over time, although this was not significant, and there was no significant time x treatment interaction.

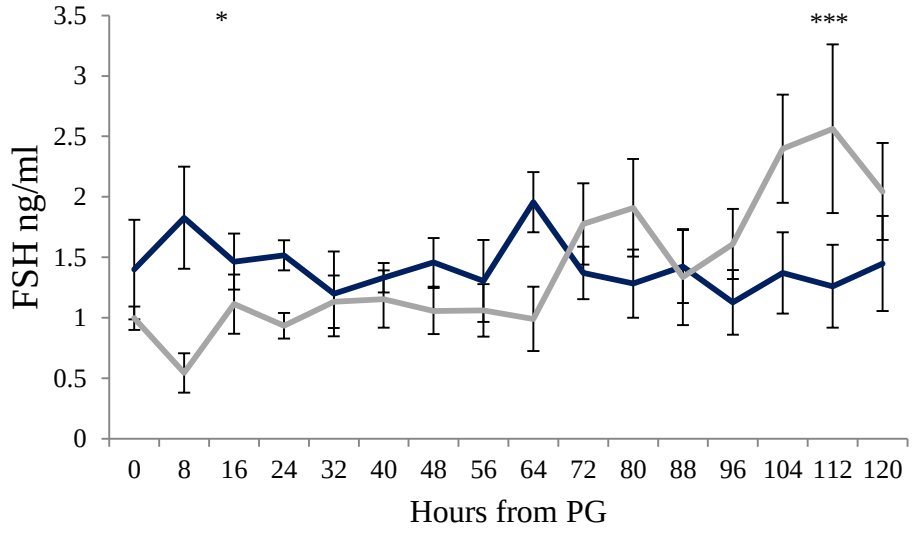

Figure 5.3: Peripheral concentrations of follicle stimulating hormone (FSH) (ng/ml) (±SEM) in heifers that did (grey) and did not (blue) exhibit an LH surge across 120 hours following administrations of the prostaglandin (PG) analogue, cloprostenol. Between 8 and 24 hours post PG, FSH concentrations were significantly (P<0.05) higher in heifers that did not exhibit an LH surge compared to heifers that did. Between 104 and 120 hours post PG, FSH concentrations in heifers that did not exhibit an LH surge were significantly lower (P<0.001) that heifers that did.

5.2.3 Follicle size and intra-follicular steroid hormone concentrations.

Concentrations of oestradiol, progesterone, and androstenedione in follicular fluid for NS heifers are described in Table 5.5, while those from the S heifers are described in Table 5.6.

Overall, follicles were larger in the NS heifers compared to the S heifers ($P<0.1$), and Figure 5.4 illustrates that, although not significant, there was a greater variety in follicle size in NS heifers. Furthermore, there was also a greater variety in intra-follicular concentrations of oestradiol and progesterone, but not androstenedione in the NS heifers, compared to the S heifers. Mean intra-follicular oestradiol, progesterone and androstenedione concentrations as well as the oestradiol:progesterone were numerically greater in the NS heifers compared to the S heifers, however, these differences were not significant (Table 5.7).

Table 5.5: Steroid hormone concentrations in the follicular fluid of follicles ≥8mm diameter in heifers that did not exhibit an LH surge.

E2 = oestradiol and P4 = progesterone.

Cow ID	Follicle size mm	Oestradiol ng/ml	Progesterone ng/ml	E2:P4	Androstenedione ng/ml
874	22	4.3	241.9	0.02	6.5
874	20	4.4	242.8	0.02	14.3
877	10	4.3	*	*	27.4
881	15	8.2	17.8	0.46	67.7
881	14	8.6	305.3	0.03	6
881	12		897.6	*	5.6
886	20	5.2	985.7	0.01	8.9
886	16	3.3	990.3	0.003	6.6
886	11	8.6	101.1	0.08	5.2
887	22	4.2	638.1	0.01	6.3
890	23	5.2	182.0	0.03	13.8
890	15	*	18.9	*	*
MEAN	16.7	5.6	420.1	0.07	15.3
MEDIAN	15.5	4.8	242.8	0.02	6.6
RANGE	10-23	3.3-8.6	17.8-990.3	0.03-0.46	5.2-67.7
SEM	1.3	0.6	115.9	0.049	5.6

*Concentrations/value not determined due to low sample volume

Table 5.6: Steroid hormone concentrations in the follicular fluid of follicles ≥8mm diameter in heifers that exhibited an LH surge.

E2 = oestradiol and P4 = progesterone.

Cow ID	Follicle Size mm	Oestradiol ng/ml	Progesterone ng/ml	E2:P4	Androstenedione ng/ml
868	21	4.5	134.0	0.03	4.5
868	10	4.2	438.2	0.01	6.7
870	11	5.2	477.4	0.01	40.7
870	10	4.1	462.1	0.01	3.2
873	17	4.8	124.8	0.04	0.5
876	15	4.8	339.3	0.01	0.7
876	15	4.9	389.2	0.01	15.3
876	14	5.4	305.9	0.02	27.3
878	15	8.7	834.1	0.01	3.3
889	13	4.5	318.1	0.01	4.59
889	10	5.4	*	*	41.4
889	9	7.8	448.3	0.02	*
MEAN	13.3	5.4	388.3	0.02	13.5
MEDIAN	13.5	4.9	389.2	0.01	4.6
RANGE	9-21	41.-8.7	124.8-834.1	0.01-0.04	0.5-41.4
SEM	1.0	0.4	57.7	0.003	4.7

*Concentrations/value not determined due to low sample volume

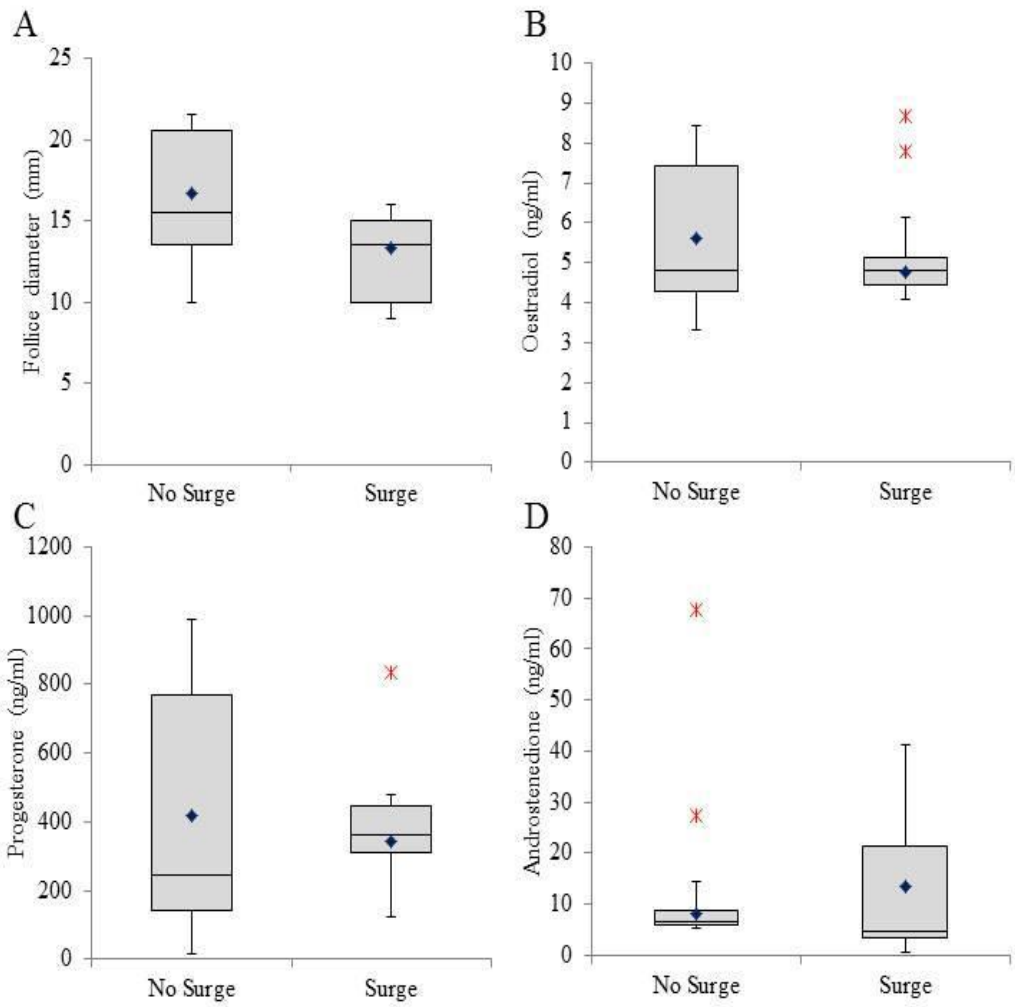

Figure 5.4: Comparison of the range of follicle size (A) and intra-follicular steroid hormone concentrations, oestradiol (B), progesterone (C) and androstenedione (D), between the no-surge and surge heifers. The top and bottom of the box represent the upper and lower quartiles respectively, the line within the box represents the median value and the whiskers represent the minimum and maximum of the data set. The blue diamonds indicate mean values and red stars indicate outliers.

Table 5.7: Summary of mean follicle size (mm), follicular fluid hormone concentrations and oestradiol:progesterone ratios, with standard error of the mean (SEM) between the surge and no-surge heifers on average and within the dominant structure for each group. There were no significant differences between groups.

Progesterone (P4) (ng/ml), oestradiol (E2) (ng/ml) and androstenedione (A4) (ng/ml).

Average of All Follicles >8mm							
	No-surge			Surge			P
		SEM	n		SEM	n	
Size (mm)	16.7	± 1.3	12	13.3	± 1.0	12	<0.1
P4 (ng/ml)	420	± 115.9	11	388	± 57.7	11	NS
E2 (ng/ml)	5.6	± 0.6	10	5.4	± 0.4	12	NS
E2:P4	0.07	± 0.049	10	0.017	± 0.003	11	NS
A4 (ng/ml)	15.3	± 5.6	12	13.5	± 4.7	11	NS
Largest Follicle							
	No-surge			Surge			
		SEM	n		SEM	n	
Size (mm)	17.3	± 2.0	6	15.3	± 1.4	6	NS
P4 (ng/ml)	413.1	± 175.7	5	371.3	± 107.5	6	NS
E2 (ng/ml)	5.22	± 0.6	6	5.42	± 0.7	6	NS
E2:P4	0.103	± 0.089	5	0.02	± 0.005	3	NS
A4 (ng/ml)	21.8	± 9.7	6	9.1	± 6.4	6	NS

5.2.4 Immunohistochemistry

FSH and LH were localised in gonadotropes of the anterior pituitary by IHC in 11/12 heifers, (6 NS + 5 S). Positive immunostaining for LH (Figure 5.5, A and C) was detected in 10/12 heifers (6 NS [cows 874, 877, 881, 886, 887, 890] *vs.* 4 S [cows 868, 873, 876, 889]). Positive immunostaining for FSH (Figure 5.5, B and D) was detected in 8/12 heifers, (3 NS [cows 881, 886, 890] *vs.* 5 S [cows 868, 873, 876, 878, 889]).

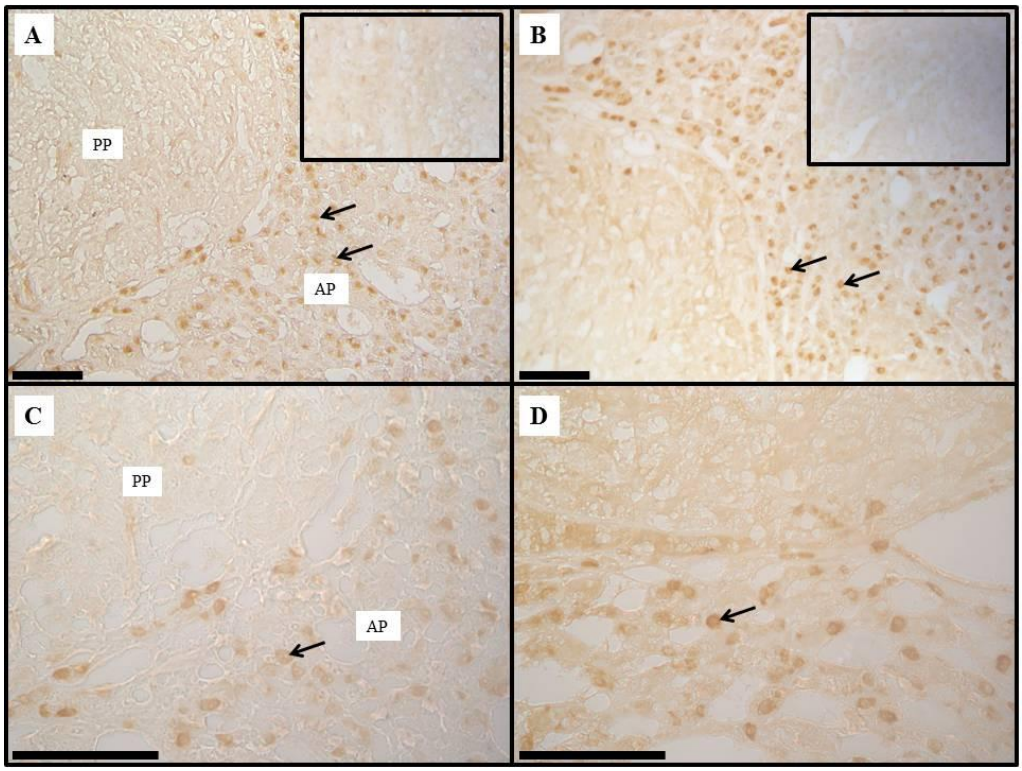

Figure 5.5: Immunolocalisation of the expression of LH (A and C) and FSH (B and D) in pituitary tissue. Inset images represent IgG controls and arrows indicate positive staining. NB: Haematoxylin staining was omitted. Scale bar is equal to 50µm.

AP = anterior pituitary; PP = posterior pituitary.

The steroidogenic enzymes CYP19A1 (Figure 5.6, A and C) and CYP17A1 (Figure 5.6, B and D) were detected in the granulosa and theca cells, respectively, of 3/24 (1 NS [cow 881] *vs.* 2 S [cows 878, 889]) follicles ≥8mm diameter. Follicles demonstrating positive immunostaining for CYP19A1 and CYP17A1 also had the highest concentrations of oestradiol in their follicular fluid.

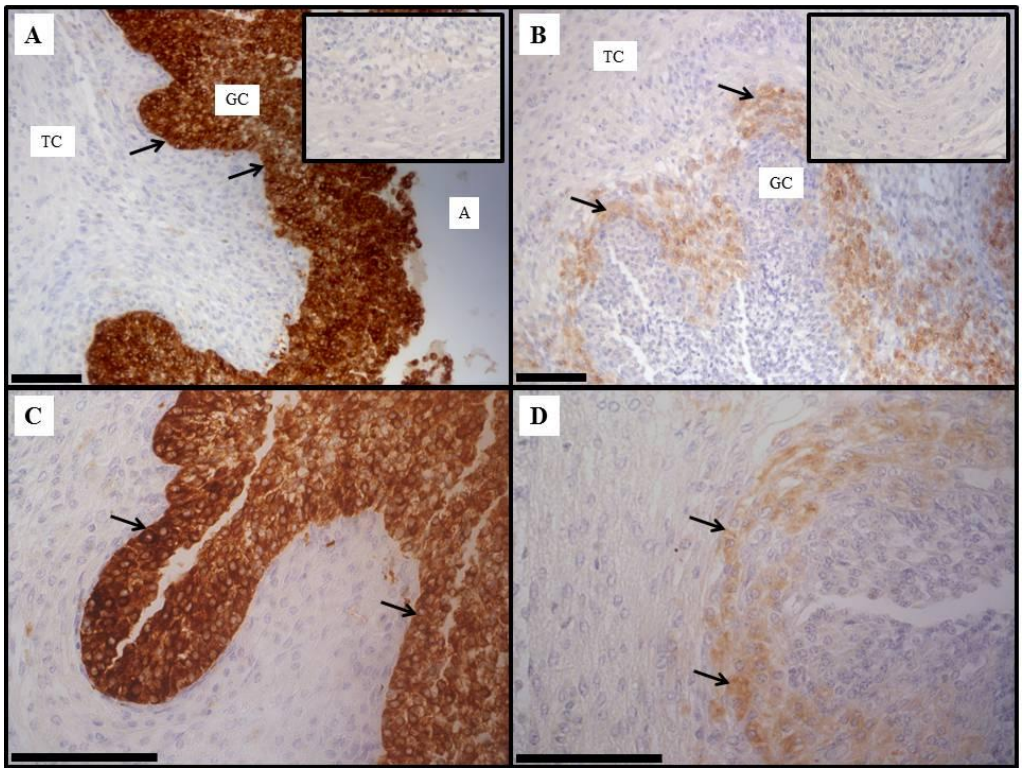

Figure 5.6: Immunolocalisation of the expression of CYP19A1 (A and C) and CYP17A1 (B and D) in follicular tissue. Inset images represent IgG controls and arrows indicate positive staining. Scale bar is equal to 50μm.

GC = granulosa cells; TC = theca cells; A = antrum.

5.2.5 LH pulse data

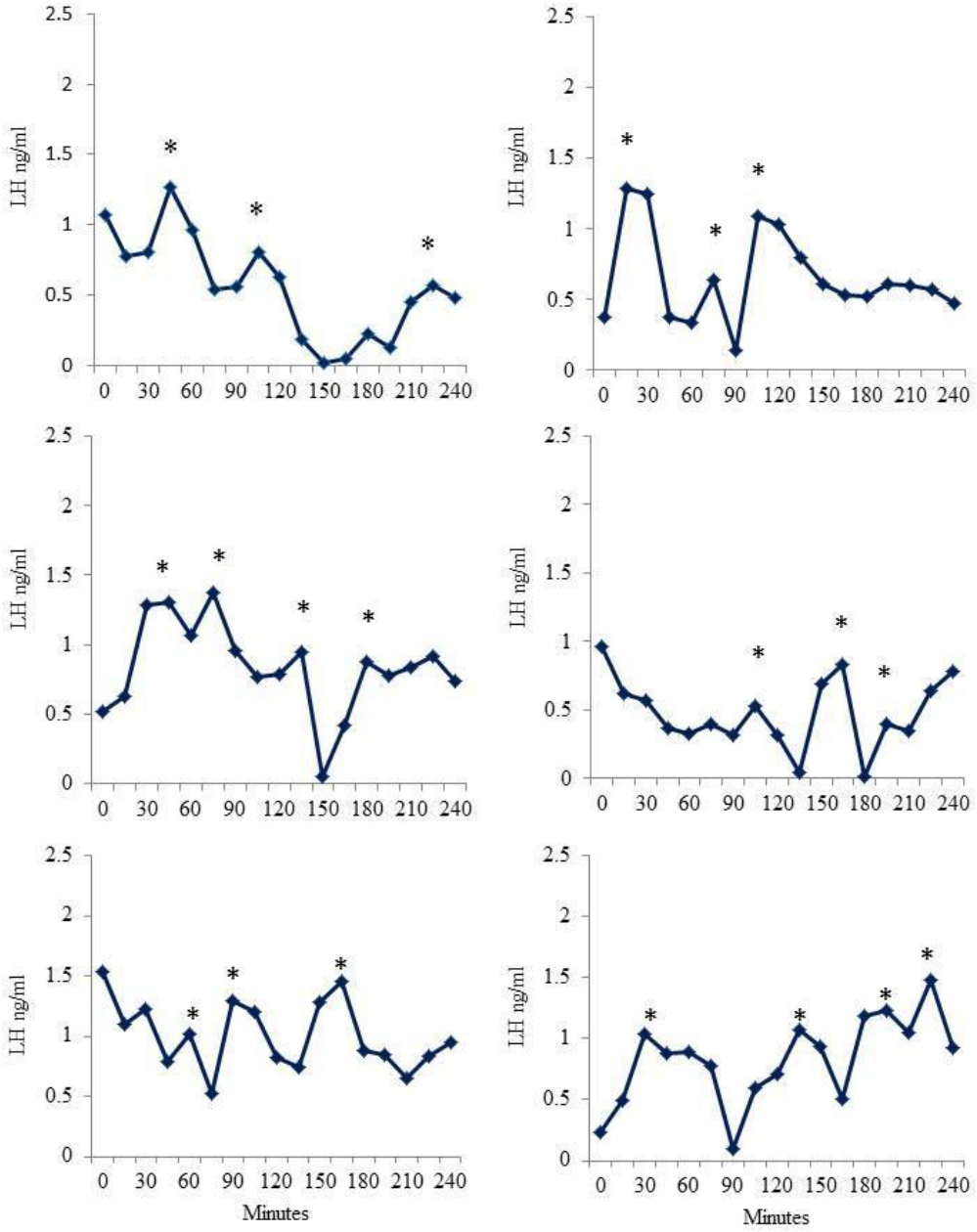

Figure 5.7: Pulsatile release of luteinising hormone (LH) (ng/ml) during the four hour pulse bleed in heifers that did not exhibit an LH surge. Samples were taken at 15 minute intervals 96 hours after administration of the prostaglandin analogue, cloprostenol. Stars indicate true LH peaks as determined using MUNRO© software.

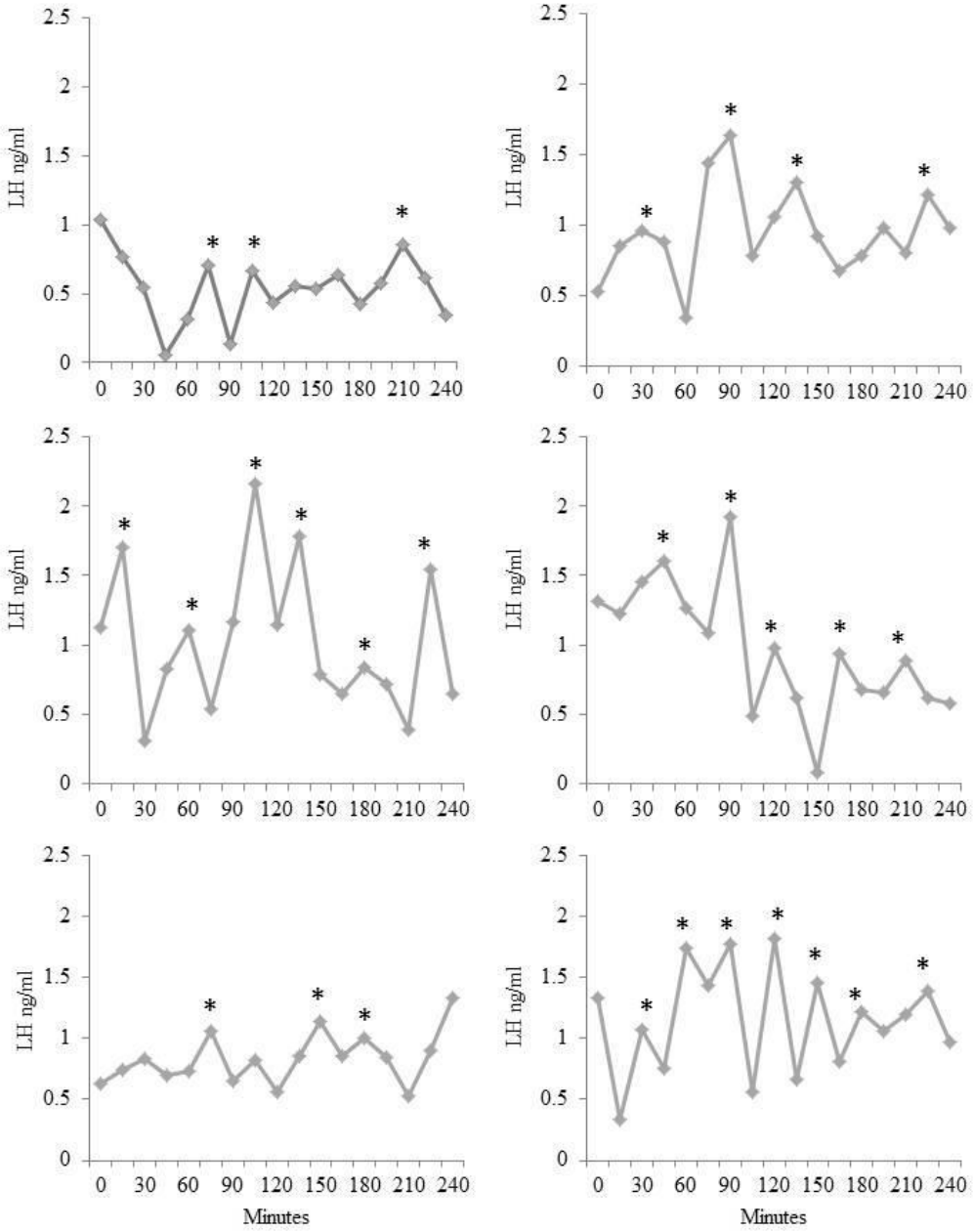

Figure 5.8: Pulsatile release of luteinising hormone (LH) (ng/ml) during the four hour pulse bleed in heifers that did exhibit an LH surge. Samples were taken at 15 minute intervals 48 hours after administration of the prostaglandin analogue, cloprostenol. Stars indicate true LH peaks as determined using MUNRO© software.

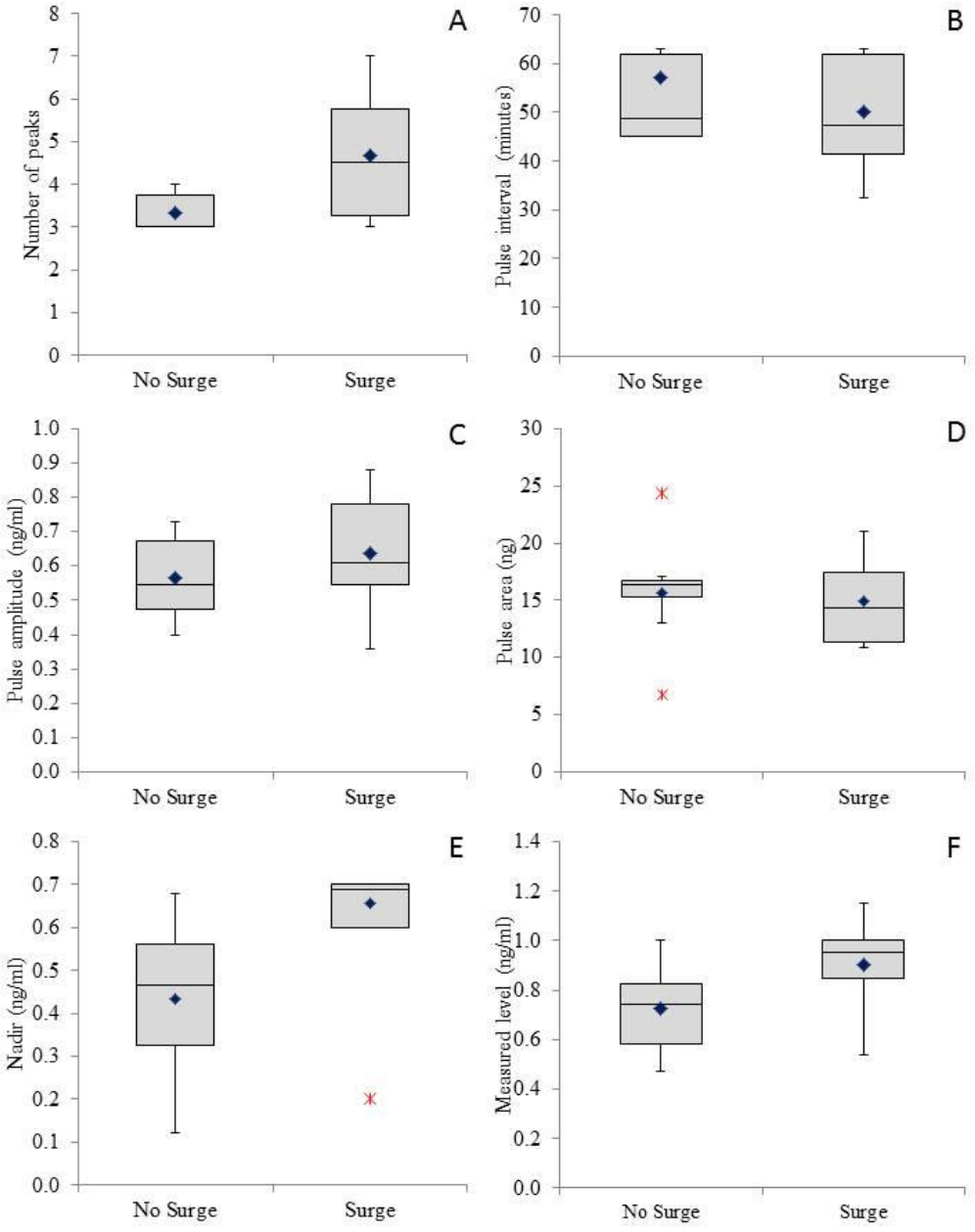

Figure 5.9: LH pulse characteristics in heifers with and without an LH surge. Box and whisker plots illustrate (A) number of peaks; (B) pulse interval; (C) pulse amplitude; (D) pulse area; (E) nadir and, (F) measured level, of LH during the 4 hour pulse bleed at prostaglandin +48 hours, and prostaglandin +96 hours for the surge and no surge heifers, respectively. The top and bottom of the box represent the upper and lower quartiles respectively, the line within the box represents the median value and the whiskers represent the minimum and maximum of the data set. The blue diamonds indicate mean values and red stars indicate outliers. Surge heifers exhibited a significantly higher LH pulse frequency ($P<0.01$; ANOVA) than no surge heifers, and 4/6 Surge heifers exhibited higher amplitude LH peaks than all 6 no surge heifers ($P<0.05$; Fishers Exact test). In (A) the median =3.

Table 5.8: LH pulse characteristics in heifers with and without an LH surge. Mean number of peaks, pulse interval, pulse amplitude, pulse area, nadir and measured levels for the no-surge (prostaglandin +96 hours) and surge (prostaglandin +48 hours) heifers, (±SEM). Mean number of peaks was higher, but not significantly, in heifers that exhibited an LH surge (P<0.1).

	No-surge	SEM	Surge	SEM	P
Number of Peaks (n)	3.3	± 0.3	4.7	± 0.5	P<0.1
Pulse Interval (minutes)	57.1	± 1.7	50.1	± 1.4	NS
Pulse Amplitude (ng/ml)	0.6	± 0.3	0.6	± 0.2	NS
Pulse Area (ng)	15.7	± 1.0	14.9	± 0.8	NS
Nadir (ng/ml)	0.4	± 0.2	0.6	± 0.1	NS
Measured level (ng/ml)	0.7	± 0.2	0.9	± 0.1	NS

Four hour pulse bleeds for LH analysis were taken from NS (Figure 5.7) and S (Figure 5.8) heifers. Examination of the profiles revealed that at 48 hours following PG administration, the S heifers had a higher LH pulse frequency (P<0.1, ANOVA) than the NS heifers had at 96 hours post PG, although this difference failed to reach significance. In addition, 4/6 S heifers exhibited higher amplitude LH peaks than all 6 NS heifers (P<0.05; Fishers Exact test).

5.3 Discussion

Six heifers exhibited an LH surge within 104 hours of luteal regression while six heifers did not, however no heifers had evidence of ovulation at slaughter 16h later. Mean FSH concentrations in the S but not the NS heifers, showed a divergence comparable with an LH surge. Follicle development was variable: all heifers had at least one follicle 2-8mm and 5/12 had at least one follicle >20 mm. Follicle appearance ranged from healthy, well vascularised to heavily luteinised with 63% of follicles showing some degree of luteinisation, and protein expression of CYP17A1 and CYP19A1 was only detected in 12.5% of follicles.

NS heifers appeared to have higher basal concentrations of FSH than the S heifers, around 64 hours post PG, FSH levels in the S heifers begin to increase while those of the NS heifers remain constant. It is likely that there was a GnRH surge in the S heifers which would explain the slight increase in FSH around the time of the LH surge (104h). Despite an LH surge in S heifers, the interval (104h) between luteolysis and the LH surge was longer than would have been expected in cows since during a natural cycle, cows exhibit an LH surge within 62 hours of luteolysis on average (Starbuck *et al.,* 2006).

Despite the presence of large follicles, steroidogenesis is evidently aberrant as concentrations of oestradiol within the follicular fluid were lower than the expected 1.1μg/ml seen in dominant follicles of typically cyclic cows (Henderson *et al.,* 1982; Fortune & Hansel, 1985; Wise, 1987). Mean androstenedione concentrations in the follicular fluid of the largest follicles (irrespective of surge) were also consistently lower than expected values (200ng/ml; Henderson *et al.,* 1982; Wise, 1987; Singh *et al.,* 1998). This

combination of both low oestradiol and androstenedione concentration has been previously identified in the follicular fluid of confirmed bovine ovarian cysts (Short, 1962). The lack of CYP19A1 detected in the largest follicles recovered from these heifers, may explain why oestradiol concentrations were so low, but if this were the case androstenedione concentrations would be high. Androstenedione concentrations may also have been low due to a lack of sensitivity to LH (Campbell *et al.,* 1998), but most likely in this case were low due to the absence of CYP17A1 detected in most follicles, as CYP17A1 is an essential steroidogenic enzyme in the production of androgens and oestrogens. Furthermore, a lack of LHr in the theca cells of follicles from the NS heifers, may also have been the reason for low androstenedione concentrations (Ginther *et al.,* 2001a) Moreover, expression of LHr mRNA in granulosa cells of bovine follicles has been shown to be dependent on the synchronous expression of mRNAs for CYP11A1 and CYP19A1 (Bao *et al.,* 1997). Follicles demonstrated a distinct lack of CYP19A1 in this study, which may also have been a contributing factor to the absence of an LH surge; however, without identifying LHr in the tissue, no definitive conclusions can be drawn and this warrants further investigation. Progesterone concentrations in the follicular fluid of the largest follicles were greater than those expected (16-24ng/ml; Mihm *et al.,* 1999). This was most likely due to the high proportion of follicles (63%) that showed some degree of luteinisation. Elevated intra-follicular progesterone concentrations are likely to suppress oestradiol synthesis from the largest follicles (Fortune *et al.,* 2004), thus indirectly decreasing LH pulse frequency and removing the high oestradiol trigger required for an LH surge (via GnRH). However, identification of LH pulses in

all heifers, despite low oestradiol concentrations in the follicular fluid, indicates that all 12 heifers had the potential to exhibit an LH surge. Hypothetically, if large oestrogenic cysts developed soon after the completion of the hyper-stimulation protocol, prior to this experiment, the hypothalamus may have become hyper- or hypo-sensitive to oestradiol.

The erratic pattern of follicle growth observed suggests that some of the follicular structures were perhaps follicles haemorrhaged during OPU that have refilled with blood instead of being newly emerged follicles. These refilled follicles may have misrepresented the pattern of follicle growth after the hyper-stimulation protocol had ended. It is possible that refilled follicles may not have been distinguishable from newly developed follicles when heifers were scanned by transrectal ultrasonography seven days after OPU as blood and follicular fluid are both anechoic (Battino, 1992; Ginther *et al.*, 1997).

5.4 Limitations

No plasma oestradiol measurements were available due to technical difficulties. It was therefore not possible to determine if oestradiol concentrations were higher in the S heifers as would be predicted. Also difficulties in optimising the CYP11A1 immunohistochemistry also lead to inconclusive results which have also been omitted from this thesis.

If cost and time had permitted, determination of IGF-I concentrations in the follicular fluid of cyst-like structures would have been beneficial in creating a fuller picture of the mechanisms involved in cyst formation. As discussed in the literature review, IGF-I is important in follicle development up

to post deviation size, stimulating granulosa cell proliferation and synergising with FSH to increase oestradiol synthesis (Spicer *et al.,* 1993). Concentrations of IGF-I, as well as insulin, in the follicular fluid of ovarian cysts have been reported to be lower than that of pre-ovulatory follicles (Braw-Tal *et al.,* 2009). Furthermore, increased concentrations of IGFBPs have also been identified in the follicular fluid of ovarian cysts; an increase in IGFBPs indicates a decrease in the bioavailability of IGFs. A lack of IGF-I within the follicle will limit the capacity of the follicle to synthesise and secrete oestradiol, eliminating positive feedback effects of oestradiol on the hypothalamus, failing to induce a GnRH and subsequently an LH surge and therefore potentially resulting in no ovulation and subsequent cyst formation. This idea also supports the hypothesis of Spicer *et al.,* (2004) that bioavailability rather than total concentration of IGFs is important during follicle development.

Inhibin is secreted by the granulosa cells in response to FSH, NS heifers had higher circulating FSH concentrations immediately post PG, when compared to those S heifers. Determination of circulating inhibin concentrations, specifically inhibin A, would have been interesting as inhibin has been reported to be high during cystic follicle development (Todoroki *et al.,* 2004).

5.5 Conclusion

In conclusion, follicles developing in the recovery period immediately post treatment were abnormal and in a wide range of physiological states, confirming that, aberrantly steroidogenic ovarian cyst-like structures develop at a high frequency following prolonged exposure to a GnRH agonist. As cyst development occurred in heifers regardless of whether they exhibited an LH surge or not, the obvious conclusion is that more than one mechanism must be involved. Data presented in this chapter indicate that an endocrine imbalance may exist and this supports previous published studies on ovarian cyst development. Although these studies have shown that prolonged exposure to buserelin is a good model for ovarian cyst development, further mechanistic studies are necessary in order to fully elucidate the physiological processes which underlie this ovarian pathology.